CHEMICAL REVIEWS

Review

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Functionalizing Nanoparticles with Biological Molecules: Developing Chemistries that Facilitate Nanotechnology

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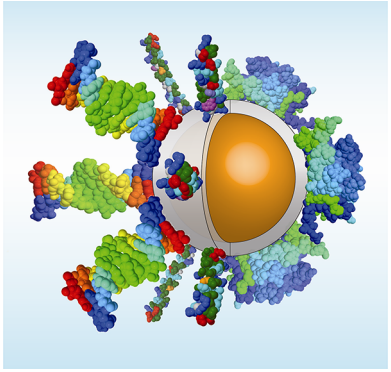
Supporting Information



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1. INTRODUCTION

Bionanotechnology has now become firmly established in its own right as one of the principle and focused subdisciplines within nanotechnology.[1−10] Bionanotechnology can be defined as a field representing all facets of research at the intersection of biology and nanomaterials (NMs) and is generally characterized as having two somewhat opposite functional goals. The first goal focuses on utilizing the inherent structural, specific recognition, or catalytic properties of biomolecules to assemble composite nanoscale materials or devices with unique or novel properties. Perhaps the most visible example of this is utilizing nucleic acid structures to control the placement and ordering of nanoparticles (NPs) in pursuit of creating single molecule or molecular electronic devices at the sublithographic scale.[2] The second goal focuses on utilizing the unique properties available to NMs within a biological setting. For example, using NPs to mediate targeted drug delivery or *in vivo* imaging, with the intent of overcoming many of the problems associated with systemic administration of drugs and contrast agents.[10−13] The number of different NP bioconjugates currently being developed is already beyond counting, as highlighted in Table 1, where just a superficial listing of some NP biocomposites and their intended utility amply reflects the breadth of intended applications and the vibrancy underlying this field. NM biocomposites are typically considered "value-added" in that they are jointly capable of more than the sum of each component individually. Regardless of the exact utility or research goal, interfacing biologicals with NMs or NPs into some type of *de novo* designer functional entity is at the heart of this endeavor. Here, we focus on NP biomolecular linkage chemistry as the common and crucial element that will ultimately determine the success of almost all these composites within their intended application.[4,14−18]

For the purposes of this review, we define biological molecules or *biologicals* to include all forms of proteins, peptides, nucleic acids (i.e., DNA/RNA/PNA/LNA as genes, oligomers, aptamers, and ribozymes/DNAzymes), lipids, fatty acids, carbohydrates, etc., whether they are monomeric building blocks (i.e., amino acids, nucleotides, monosaccharides) or the fully formed, functional "polymers" such as proteins, plasmids, and cellulose. The biologicals may be functionally active, offering binding, catalytic, or therapeutic activity (e.g., antibodies or enzymes) or, alternatively, may be passively utilized as an inert coating or scaffolding material. We further expand our definition to include enzymatic cofactors and all forms of drugs and other biologically active small molecules such as reporters and contrast agents (e.g., fluorescent dyes, MRI probes, radiolabels). A variety of other biocompatible molecules such as poly(ethylene glycol) (PEG) or metal complexes are further included in some special circumstances.

The definition of what exactly constitutes a NM is not as easily addressed. The initially accepted working definition was any material, either organic or inorganic (but not purely chemical or a naturally occurring biomolecule such as a sugar or lipid), that was <100 nm in at least one of its dimensions. More importantly, these materials were "intentionally produced" on this scale "to have a specific property or composition".[48] Recently, some national and international standards organizations have proposed somewhat more-acceptable and modified nomenclature and terminology for use when describing nanoscale materials (see American Society for Testing and Materials International E2456

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Table 1. Applications of Representative Nanoparticle-Bioconjugates[a]

nanoparticle material	biological	application	reference
gold	DNA	templated assembly/ diagnostics	19, 20
gold	proteins/ enzymes	energy harvesting/ bioelectronics	19
gold	carbohydrate/ peptide	cellular labeling	20
gold	lipid/DNA	gene therapy/DNA delivery to cells	21
platinum	enzyme	sensing	22
palladium	protein	biocatalysis	23
silver	antiamyloid ligand	Alzheimer's detection	24
silver	antibodies	cell surface detection	24
iron oxide	proteins/ peptides	MRI imaging/sample concentration/therapy	25−27
iron oxide	DNA/siRNA	gene therapy	25
silica	DNA/drug	drug and gene delivery	28
silica	antibody/DNA	bioanalysis/bioprobes	29
semiconductor QD	DNA/proteins/ peptide	sensors/probes/cellular delivery	30
semiconductor QD	antibody	*in vivo* imaging/ diagnostics/PDT	31, 32
semiconductor QD	virus	bioelectronics/molecular memory	33, 34
titanium dioxide	DNA	subcellular organelle targeting	35
nickel oxide	protein	biocatalysis	36
copper sulfide	DNA	bioanalytical probe	37
carbon nanotube	DNA/proteins	bioelectronics and bioanalysis	38, 39
carbon nanotube	protein/ enzyme	drug delivery	40
zirconium oxide	enzyme	bioanalysis and sample capture	41
Composite Nanoparticles			
semiconductor QD/ iron oxide	antibodies	multimodal MRI/ fluorescent imaging	42
manganese-doped iron oxide	adenovirus/ DNA	gene delivery	43
iron oxide coated silica	antibodies	multimodal MRI/ fluorescent imaging	44
semiconductor QD/ Gd-DTPA	chitosan	multimodal MRI/ fluorescent imaging	45
semiconductor QD/ polymer bead	DNA	optically coded hybridization probe	46
carbon nanotube/ QD	drug	cancer imaging and drug delivery	47

[a]Abbreviations: QD, quantum dot; PDT, photodynamic therapy; Gd-DTPA, gadolinium(III)-diethylenetriaminepentaacetate.

and International Organization for Standardization technical specification documents 27687 and 80004). These definitions still describe an upper size limit of ∼100 nm in at least one of the dimensions. However, there is currently limited scientific evidence to strictly support this upper limit for all NMs. Such rigid definitions are not a trivial undertaking, because they have important regulatory and legal implications, see refs 48−50 and references therein.

For the purposes of this review, we define NMs as any material at the submicrometer level, be it biotic or abiotic, that can be interfaced with a "biological" in pursuit of creating a novel "value-added" entity. Further, this material must be *intentionally produced* on this scale, *have discrete functional or structural parts arrayed on its surface or internally*, and display a *unique property or composition* that may not be available to the same material in the bulk scale.[48,51] This allows us to include all types of NPs as synthesized from metals, noble metals, oxides, and semiconductors, along with a variety of nontraditional and nonmetal NPs. The latter include carbon allotropes, polymeric, dendrimer, and chemical NPs, protein, and virus-derived NPs, along with lipid, carbohydrate, liquid crystals, and other assorted materials. Within the discussion below, the acronyms NP and NM are sometimes used interchangeably. For a particular NP material, limited discussion of the differences in their size, shape, density, and other physicochemical or opto-electronic properties, as determined by their composition, is also provided where appropriate.

The interest in using NMs, and especially NPs, as part of biomolecular composites arises from the unique size-dependent physical, optical, electronic, and chemical properties that they can contribute to the resulting conjugate. These may include quantum confined properties as typified by the size-tunable photoluminescence (PL) of nanocrystalline semiconductor quantum dots (QDs), the plasmon resonances of gold NPs, the electrical properties of carbon allotrope NMs, and the paramagnetism and catalytic properties available to certain metal alloy and metal oxide NPs.[4,20,52−55] NPs are also characterized by having very high surface-to-volume (S/V) ratios (i.e., at <2 nm, the S/V atomic ratio is >50%) and nontrivial surface areas.[3,16] This allows for the display of multiple (possibly different) biologicals on their surfaces, which can contribute to increased avidity and multifunctionality.[14,16] In the case of dendrimeric NPs, multiple and sometimes different reactive sites or chemical handles can be isolated and presented within a very small volume.[56,57] NPs can act as carriers for insoluble materials (e.g., drugs, radioactive isotopes) while also providing long-term chemical stability and protection against photo- or chemical degradation. Alternatively, the converse may be desired, and NPs can be engineered to degrade *in vivo* over time.[58] When considered cumulatively, these properties are also the main motivation behind developing NP-based theranostic agents, that is, designer NP bioconjugates capable of multiple, simultaneous activities such as tumor-targeting, active sensing, diagnostics, and the delivery of drugs or imaging contrast agents.[4,10−12,59−61] Moreover, it is anticipated that many new NP materials will be developed in the near future, and as their unique properties are understood, this may stimulate interest in subsequent bioconjugation for new or improved applications.

It is likely that humanity has had almost continuous interactions with NM−bioconjugate composites, albeit unknowingly. Heterogeneous, carbonaceous NMs in many different forms (e.g., nanotubes and fullerenes) and coupled to various proteins, carbohydrates, lipids, and their degradation products have probably been present in some form or other in foods cooked over an open flame.[62−64] The rise of alchemy resulted in the intentional addition of various metals and their salts (e.g., Au, Ag, As, Hg) to food and plant extracts, which were then cooked into medicinal potions (or poisons), potentially yielding complex mixtures of NM biocomposites.[65] Colloidal Au, in particular, has a long history of medicinal use from ancient times up to the present day.[20,66] Perhaps the first scientific reference to a "designed" NP bioconjugate can be traced to the physician H.H. Helcher. In his tome on colloidal Au published in 1718, he remarked on the use of boiled starch in "drinkable" Au preparations (colloids) as having the effect of enhancing stability.[67] We can surmise that the different sugars present in these preparations were likely interacting with the Au colloids and providing a surface-stabilizing ligand-type function to them.

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The report of dyeing silk with colloidal Au toward the end of the same century may represent the creation of another designer NM bioconjugate.[20] It is only in the last ~20 years that the synthesis, characterization, and application of specific designer NM bioconjugates has become a dedicated field of research. This was spurred primarily by the development of robust Au, magnetic, and semiconductor NP bioconjugates.[20,30,68,69] Before reviewing how NPs have been bioconjugated, we first examine the important criteria that need to be considered when performing these types of modifications, along with providing an overview on many of the relevant NP biological interaction or attachment chemistries.

2. BACKGROUND: NANOPARTICLES, BIOCONJUGATES, AND CHEMISTRY

2.1. The Challenge of Working with Nanoparticles

The many challenges associated with preparing NP bioconjugates are not trivial. To begin with, NPs have wide variability in their structure. Some NPs are uniform in that they consist of only one type of material, for example, Au NPs and similar NPs, including FePt, which while uniform in composition, is a binary alloy and lacks a shell structure. In other cases, NPs have core/shell or core/$(shell)_n$ structures, where the outer shells protect and insulate the inner layers, which are sometimes needed within biological applications. As synthesized, many metal, semiconductor, and carbon NPs are hydrophobic and need to be made hydrophilic and biocompatible by chemical modification of their surfaces. This usually involves attaching or replacing the hydrophobic ligands that stabilize the NP during or after synthesis with hydrophilic ligands or other coatings capable of mediating aqueous dispersion. The chemistry of hydrophilic ligands is both complex and diverse and can range from small charged molecules to dendrimers or amphiphilic block copolymers that completely encapsulate the NP.[70] The ligands may also provide additional chemical "handles" that can act as sites for subsequent bioconjugation. Ligand chemistry and ligand–NP interactions also have important implications for the colloidal stability of the NP, especially within intracellular environments.[71] It is also worth noting that ligand–NP coverage will usually be highly heterogeneous and that most values that try to describe this metric are almost invariably an ensemble average and may not be sufficient for the rigorous characterization required for regulatory clearance.[3,72]

Biomolecules can be conjugated directly to the surface of some NPs and to surface-bound stabilizing ligands or coatings, either directly or using small cross-linking molecules and other intermediaries. Bioconjugation usually aids in specifically targeting the NP in a biological environment through biorecognition motifs (e.g., antibodies, aptamers) or imbues the composite with a bioderived activity such as catalysis (e.g., enzymes, DNAzymes). NP–bioconjugate structure can quickly become complex as many different permutations and iterations of the core/shell/ligand/biomolecule multilayered structure can occur.[3] Figure 1 highlights this point by schematically depicting the structure of eight different NP–biological assemblies. In the simplest format, the biological interacts directly with the NP core. In more complex formats, the NP surrounds the biological or, alternatively, the biological is encapsulated within the NP.[3]

The diversity of NP materials is wide and their dimensionality can vary by 2 orders of magnitude (1–100 nm). Several properties are important to consider: NPs will usually be larger than most biomolecules, except perhaps large protein complexes; NPs will display size heterogeneity or polydispersity

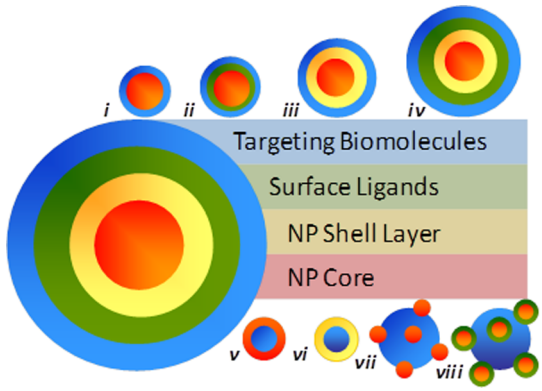

Figure 1. Schematic of some potential NP–bioconjugate components and multilayer configurations (note, not to scale): (i) biomolecule interacting with NP core; (ii) biomolecule interacting with a NP core via intermediate ligands; (iii) biomolecule interacting with NP shell layer that surrounds the NP core; (iv) biomolecule interacting with NP shell layer via intermediate ligands; (v) porous NP core containing entrapped biomolecules; (vi) porous or hollow NP core containing entrapped biomolecules surrounded by a NP shell layer; (vii) NP core (or NP core/NP shell structures) particles smaller in size than the much larger biomolecule; (viii) NP core (or NP core/NP shell structures) particles smaller in size than the much larger biomolecule attached via intermediate ligands. Reprinted from ref 3. Copyright 2011 American Chemical Society.

across a given population; NPs can have a wide range of surface areas, volumes, and corresponding S/V ratios (e.g., spherical NPs with 1–100 nm diameters have volumes of 0.5 yL to 0.5 aL, surface areas of 3 nm^2 to 0.03 μm^2, and S/V ratios ranging between 6 and 0.06 nm^{-1}); NPs do not undergo dissolution but are small enough to be functionalized for colloidal solubility and are still able to diffuse; NP surfaces may not be uniform, or fully available for chemical modification.[14] Additionally, most conjugation reactions involving NPs are not strictly homogeneous reactions. Even with homogeneous dispersion of nonaggregated NPs, the reaction is interfacial in its nature and such interfaces are inherently polydisperse across a population of NPs. Further, with the notable exceptions of silica and silicon NPs, the surface of inorganic NPs does not generally support the formation of new covalent bonds and thus limits the choice of available conjugate chemistries to those that build from coordinated ligands. Effects from nanoscale curvature (e.g., changes in ligand pK_a or differences in reactivity between carbon nanotube tips and sidewalls) further complicate conjugation. Lastly, molecular crowding and other nearest-neighbor interactions, charge density, electrostatic repulsion, and loss of colloidal stability during reactions may result in low yields of conjugates, and conjugates with poorly defined properties or low stability. NPs can thus be described as having a character intermediate between that of bulk interfaces and molecules.[14,16,73–76]

In many cases, the effects of scaling materials into NPs cannot be fully predicted *a priori*. For example, in the development of NP-based MRI contrast agents, a variety of different effects have been observed, including variations in magnetic properties based on size, shape, and volume, along with increased free radical scavenging abilities. Extensive examination has revealed that magnetic properties are extremely sensitive to both variations in physical properties and the character of the NP surface ligands.[77–79] Size and volume have been shown to affect the transition temperature from ferrimagnetism to superparamagnetism in iron oxide (IO) and Co NPs. Magnetic

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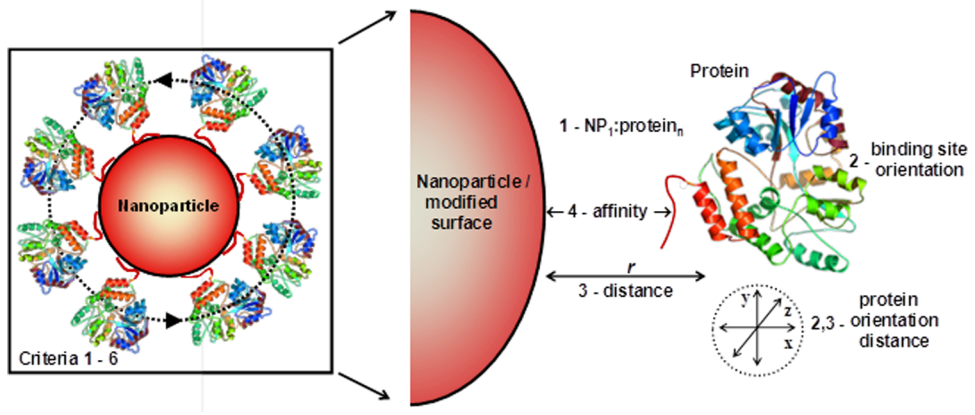

Figure 2. Schematic representation of the six ideal or principle criteria for a universal "toolset" that would allow controlled attachment of any protein or other biomolecule to any NP or surface. These criteria include (1) control over the valence or ratio of biomolecule per NP; (2) control over orientation on the NP; (3) control over relative separation distance from the NP; (4) control over attachment affinity; (5) maintenance of the optimal function or activity of both participants; and (6) ability to be replicated in a facile manner with a variety of biologicals. In this example, the proteins would cover the NP surface in three dimensions and could still have some rotational freedom around the axis connecting them to the NP while still fulfilling the criteria. Reprinted from ref 82. Copyright 2006 Macmillan Publishers Ltd.

coercivity also increases with NP size: nearly 5-fold between 8 and 4 nm Co NPs. Saturation magnetization has also been shown to be directly proportional to the size of magnetism-engineered IO (MEIO) NPs, making them ideal as tunable contrast agents with high magnetic moments and relaxivity.[77]

Perhaps the closest analog to a NP in terms of size and bioconjugation chemistry is a protein. Many proteins are far larger than other biomolecules yet still retain solubility. Within their three-dimensional folded structure, the solvent-exposed residues determine solubility and provide many of the potential sites useful for further labeling. Because protein labeling is well-developed, the techniques from this field were among the first applied to preparing NP bioconjugates and, as will be shown, are still heavily relied upon. Nonetheless, there are conceptual similarities and important differences between protein labeling and NP bioconjugation. Fluorescent labeling of proteins generally utilizes an excess of reactive dye and is effective due to monoreactivity of the dye coupled with the multiple but distributed reaction sites of the protein. Since the size of the dye label is comparable to or slightly larger than that of amino acids, protein perturbations are usually minimal. The degree of labeling can be measured experimentally, and the reactions can be optimized empirically.[80] The same approach is generally not directly applicable to biofunctionalizing NPs, due to their nontrivial size and the display of multiple closely packed target groups on their surface. Further, the attachment of multiple (identical) molecules at adjacent sites on the surface of a NP has a profound impact on the colloidal stability and physicochemical properties of the resulting conjugate.

Large excesses of a limited protein reagent relative to NP, or *vice versa*, can be hard to achieve. Given that the reactive groups on the NP surface may also mediate NP dispersion, overactivation of the surface groups can cause loss of colloidal stability and aggregation. Polydispersity in NP size and surface area also results in variability in the number of sites available for conjugation. Because conjugate reactions may not be well controlled, reaction stoichiometry usually does not translate directly into NP bioconjugate valence.[3,14,72] Efficiently purifying NP bioconjugates from excess biomolecules, especially large protein complexes, without altering the intended biological function can be complicated.[3] Further still, depending upon the chemistry being utilized, protein−protein cross-linking can compete with NP−protein coupling and also mediate NP−NP cross-linking. Proteins often have many residues displaying functional groups that can participate in coupling (e.g., carboxyls, amines), resulting in a distribution of NP attachment points. This, in turn, results in a distribution of protein orientations within the NP−protein conjugates and potential heterogeneity in protein activity, that is, mixed avidity. In contrast, synthetic oligonucleotides and peptides may be monoreactive; however, flexibility in their structure and linkages may allow them to adopt conformations relative to the NP that are unfavorable to the intended application.[81] The interactions between a NP and a biomolecule within a conjugate will be a combined function of the NP, its interfacial chemistry, and the properties of the biomolecule[14,16,73−76] Efforts to control biomolecular display on NPs must consider these factors in addition to that of the conjugation chemistry itself.[14]

2.2. Ideal Criteria and Generalized Considerations

The "ideal" characteristics desired from almost all NP bioconjugation chemistry can be described by six generalized criteria, see Figure 2.[82] In considering a generic, nondescript NP target for bioconjugation, one would like to attach the biomolecules to the NPs with control over the following properties: (1) Control over the valence or ratio of biomolecule per NP. Different applications almost always require different valency; monovalency can be used to identify and correlate single binding events, while higher valency can improve binding interactions and avidity. Conversely, overconjugation can potentially impair binding interactions similar to DNA arrays that are assembled too densely, although NP surface curvature may help mitigate this somewhat.[16] Valency should be easily controlled with minimal variation across the bioconjugate. Being able to accurately predict the number of available conjugation sites on a given NP is an important corollary of this. (2) Control over orientation on the NP. Protein, enzyme, and antibody activities are dependent upon their binding sites having access to the environment. Nonspecific chemistry or electrostatic interactions can result in heterogeneous attachment and impair activity in the final conjugate.[16] (3) Control over relative separation distance from the NP. Some applications necessitate that the NP−biomolecule separation meet rigid distance requirements; for example, sensing schemes utilizing Förster resonance energy

transfer (FRET) or electron transfer, both of which are highly distance dependent.[19,83] (4) Control over attachment affinity. Within some conjugates, the linkage should be permanent, while in others a labile linkage is more desired, such as in the case of NP-mediated drug delivery.[11,84,85] (5) Maintain the optimal function and activity of both participants. In all cases, the chemistry should not alter the properties of each participant, such as causing loss of protein structure or activity or loss of NP stability and optoelectronic properties. (6) Can be replicated in a facile manner with a variety of biologicals. Maximum utility will arise from being able to reproducibly translate the previous five criteria between experiments, between different types or batches of NP, and with different biologicals. These criteria are somewhat self-evident; however, the difficulty in achieving some, let alone all, of these goals quickly arises when trying to implement them. We also note that these considerations apply in almost the same manner to functionalizing metallic and other planar surfaces with biologicals.

2.3. The Nano–Bio Interface

Within the field of NM bioconjugation, the term "nano–bio interface" and related synonyms such as "nanoscale interfaces to biology" and "NP corona" have begun to take on a specific meaning of their own.[16,74–76,86] When introduced to biological environments such as blood, for example, NPs and especially NP–biological materials participate in a complex biophysicochemical interplay with the native molecules, and this is considered the least understood determinant of NP activity in biological environments.[74,87] These interactions and the subsequent effects they have on the NP bioconjugate and its intended application have led to the establishment of this area as an important research field under the loosely defined titles above. Adsorption of biologicals to NPs will be governed by the interplay of hydrogen bonding, London dispersion, acidity/basicity, Coulombic forces, and lone-pair electrons.[88] The complexity of these interactions are highlighted in Table 2, which lists just some of the more dominant influences on the interface between NMs and biologicals.[75] This particular topic has even more implications when developing NP-mediated drug delivery. For example, Dawson has argued, somewhat presciently, that it is not the NPs themselves that drive biological response, but rather the amount and presentation of biologicals (mostly proteins) in an outer layer termed a *corona*, since it is this outer layer that will be presented to a cell or interact with the biological environment.[76] Indeed, recent data from the Chan Lab examining the effects of NP size and surface chemistry on serum protein adsorption and subsequent uptake in macrophages provides evidence to support this concept.[89] This issue is further compounded by the fact that each NP and NP bioconjugate can be expected to undergo very different interactions based upon their own unique physicochemical properties. Of equal concern is that the biomolecule(s) initially attached to the NPs may lose their activity due to sequestration, unfolding, denaturation, or blocking of an active site.[16,74–76]

Depending upon the NP material being utilized, proteins or peptides and the like can undergo both specific and nonspecific interactions with the NP bioconjugates, and these may also be of a permanent or transient nature. For example, serum albumins and fibrinogen may initially cover a NP present in blood, and these may be slowly displaced by lower-abundance proteins with much higher affinities.[75,90] Intracellularly, glutathione (GA) may datively coordinate to noble metal NPs, where its millimolar cytoplasmic concentration may result in dense coverage despite that fact that monothiols can have a dynamic on–off rate with NPs in other circumstances. Alternatively, peptidases or nucleases can damage protein or nucleic acid cargos attached to NPs. The outlook is not completely dire, however, because a full understanding of this complex area may allow development of predictive relationships that can aid in engineering new NP bioconjugates, and indeed concerted efforts in this area appear promising.[88,91] Additionally, these interactions may actually be helpful in developing vaccines or, alternatively, reducing immune activation events along with inducing opsonization; that is, promoting receptor-mediated phagocytosis.[92] In the current context, it is important to consider that well-characterized designer NP bioconjugates may undergo a number of further (bio)interactions and unintended (bio)modifications in biological environments, which may adversely impact their final utility. The interested reader is referred to the work of Dawson, Nel, Hamad-Schifferli, and others for in-depth treatment of the many aspects surrounding this important subject including analytical characterization.[3,16,74–76,86,88,91,93,94]

Table 2. Principle Bio/Physicochemical Influences on the Interface between Nanomaterials and Biological Systems[a]

Nanoparticle
size, shape, and surface area
surface charge, energy, roughness, and porosity
valence and conductance states
functional groups
ligands
crystallinity and defects
hydrophobicity and hydrophilicity
Suspending media
water molecules
acids and bases
salts and multivalent ions
natural organic matter (humics, proteins, lipids)
surfactants
polymers
polyelectrolytes
Solid–liquid interface
surface hydration and dehydration
surface reconstruction and release of free surface energy
ion adsorption and charge neutralization
electrical double-layer formation, ζ potential, and isoelectric point
sorption of steric molecules and toxins
electrostatic, steric, and electrosteric interactions
aggregation, dispersion, and dissolution
hydrophilic and hydrophobic interactions
Nano–bio interface
membrane interactions: specific and nonspecific forces
receptor–ligand binding interactions
membrane wrapping: resistive and promotive forces
biomolecule interactions (lipids, proteins, DNA) leading to structural and functional effects
free energy transfer to biomolecules
conformational change in biomolecules
oxidant injury to biomolecules
mitochondrial and lysosomal damage, decrease in adenosine triphosphate

[a]Reprinted from ref 75. Copyright 2009 Macmillan Publishers Ltd.

2.4. Overview of Conjugation Strategies

Choice of conjugation strategy is initially dictated by a combination of factors including NP size, shape, surface chemistry, and structure, the intrinsic NP material itself, the nature of the NP surface ligands and their available functional groups, the type of biological molecule, its size, its chemical composition,

and the utility desired in the final application. An informative discussion of how NP size, shape, curvature, and structure may influence bioconjugation chemistry and subsequent conjugate activity can be found in ref 16. Additionally, interesting reviews on the functionalization of select inorganic NPs for bioapplications can be found in recent publications from the Ying and Parak groups.[15,95] Most simply, conjugation itself will either be covalent (including covalent coupling to the NP surface or surface ligand) or noncovalent in nature, with the latter encompassing electrostatic attachment, other forms of adsorption, and encapsulation, see Figure 3.

2.4.1. Covalent and Dative Chemistry. Depending upon the particular combination of NP and biological, either the NP surface itself or a surface-bound stabilizing ligand can be directly linked to the biological molecule. Direct conjugation of a biomolecule to a NP surface is usually driven by dative bonds, while attachment to ligands is accomplished with covalent bonds. Dative or coordinate covalent bonds result when two electrons in the bond originate from a single atom and are characterized by their longer bond lengths, lower energies, and greater polarity than covalent bonds. Well-known examples of dative bonding include the chelation of metal ions (discussed below) and Au–thiol chemisorption. In the latter, the sulfur atom of a thiol contributes a lone pair of electrons to the empty orbitals of gold atoms at an interface, and this has formed the predominant basis of AuNP bioconjugation with thiolated proteins, peptides, and DNA.[20,80,96] Dative bonds are not as strong as true covalent linkages and can be compromised by changes in pH, oxidation, and displacement by other similar molecules.[20,80] However, these types of bonds can be strengthened by increasing the number of interactions (i.e., multivalencey). For example, use of multidentate thiols, as opposed to monothiols, is well-known to increase the overall attachment strength of a ligand to both Au and semiconductor NPs or surfaces.[80,97−100] To this end, Stewart demonstrated PEGylated tetradentate thiol ligands that conferred colloidal stability to QDs under extreme conditions, such as near pH 1 or 14.[101] In some cases, biologicals can attach to the NP surface while simultaneously functioning as a ligand that directly mediates NP solubility. For example, Pinaud et al. used phytochelatin-derived polycysteine peptides directly conjugated to ZnS capped semiconductor QDs to promote solubility.[102] Although not strictly covalent, certain electron-donating amino acids, particularly histidine, can form stable coordinate complexes with transition metals such as Ni(II), Cu(II), Zn(II), and Co(II) (see section 3.2.2.1). The search for peptidyl sequences that can specifically bind different NP materials with high affinity through phage display and other selection techniques continues to be a very active area of research (*vide infra*).[103−105] Covalent chemistries also encompass the use of cross-linkers that function as a molecular bridge between the NP and the biological (see section 2.6).

2.4.2. Noncovalent Attachment. In most cases, noncovalent attachment tends to be exemplified by comparatively weak coordination bonding, and thus stability is dictated by equilibrium dissociation constants. Such self-assembled NP bioconjugates can therefore be quite sensitive to the NP and biomolecule concentrations during preparation and in subsequent application. Again, by incorporating or exploiting multiple points of contact, such as through multidentate interactions, the stability of the NP bioconjugates can be improved and facilitate use at lower concentrations that are more typical of biological applications and environments ($<10^{-6}$ M).[14] Since these chemistries often require only stoichiometric mixing of the two components, they are typically referred to as *self-assembly*. These approaches can be advantageous in that they generally offer rapid and facile bioconjugation without the need for additional reagents and can often provide better control as a result.[14]

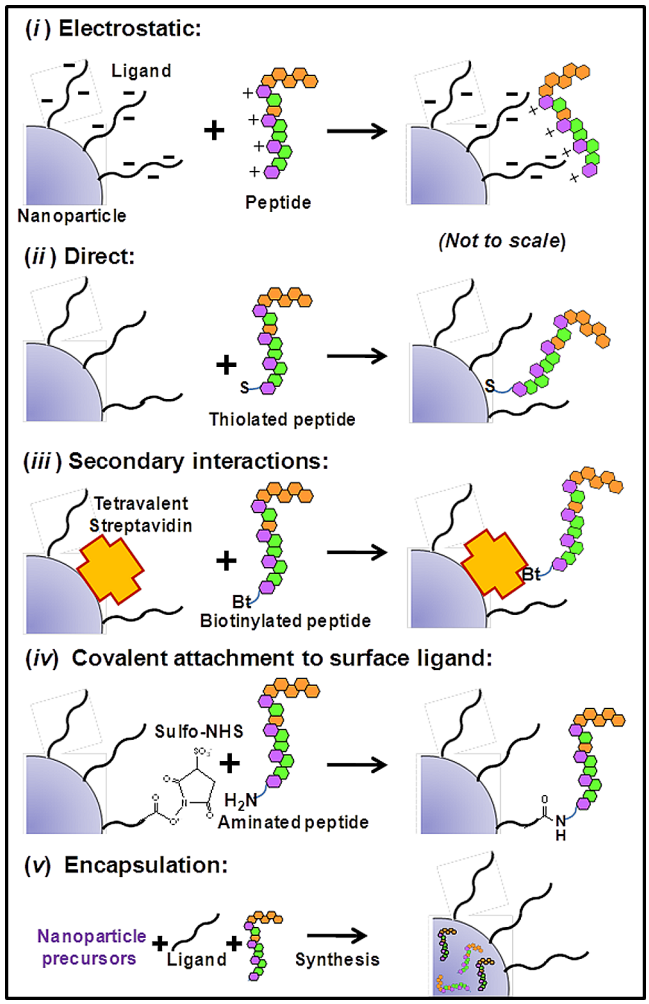

Figure 3. Five general schemes routinely used for bioconjugation to NPs as highlighted by the use of a peptide: (i) electrostatic interaction, opposite charges on the surface of the NP and the peptide are used to mediate charge−charge-based NP-peptide assembly; (ii) direct interaction, certain peptide motifs can bind to or coordinate with the NP surface with high affinity, for example, the interaction of free thiols with the surface of AuNPs and the high-affinity coordination of polyhistidine tracts with NPs (e.g., QDs) displaying Zn^{2+}-bearing surfaces; (iii) secondary interactions, this scheme utilizes specific ligand−receptor interactions as exemplified by biotin−streptavidin (SA) interactions, for example, incorporation of the biotin moiety at the peptide's terminus during synthesis can mediate direct binding of the peptide to the NP−SA conjugate; (iv) covalent chemical attachment, these utilize classical bioconjugation chemistry such as EDC-based coupling of amines to carboxyls or NHS- and maleimide-mediated conjugation to amines and thiols; (v) encapsulation places the peptide inside the NP material, which may happen during synthesis or after the NP material is formed. Concept partially adapted from ref 16. Copyright 2008 IOP Publishing Ltd.

The simplest and most widely used NP bioconjugation approach is electrostatic attachment because it requires no chemical reactions *per se*. The underlying principle behind the electrostatic immobilization of small molecules or biomolecules is the attraction between oppositely charged species: a charged NP will electrostatically attract an oppositely charged species

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and *vice versa*. While this concept is rather intuitive, its implementation presents several complications at the nanoscale since electrostatic effects are amplified, and factors such as ionic strength, the concentration of reagents, and the type and magnitude of charge play an important role in obtaining the desired conjugates.[106−109] The strong negative charge associated with the phosphate backbone of most nucleic acids facilitates conjugation with positively charged NP materials and has formed the basis for many such conjugates; however, pH and ionic strength conditions need to be carefully optimized. In certain cases, NP or protein engineering can even allow regiospecific attachment with this approach. Bayraktar et al. found that AuNPs functionalized with carboxylates bind to cytochrome *c* over a large surface while, in contrast, NPs displaying the amino acid phenylalanine bind a much smaller surface spanning from Tyr67 to Phe82 of the protein sequence.[110] This shows that engineering the surface to be more hydrophobic can increase the selectivity of protein orientation on the NP, provided enough structural information is known about both participants. In another example, a maltose binding protein (MBP) dimer was engineered to display a distal, positively charged leucine zipper region to allow self-assembly to QDs coated with negatively charged ligands. This strategy oriented the MBP binding pocket away from the NP surface.[100]

Other noncovalent chemistries can exploit hydrophobic−hydrophilic or host−guest interactions.[111,112] Secondary interactions between functional groups covalently attached to the NP surface and other moieties are another class of noncovalent interaction. These are typified by biotin−avidin interactions where the NP and biomolecular partner are functionalized to display one of the cognate pair (see below). Alternate approaches can utilize antibody affinity for a ligand attached to the NP (e.g., digoxigenin) or *vice versa*, along with other enzyme−substrate or receptor−ligand interactions. Specific examples of all these are provided in subsections below.

2.4.2.1. Layer-by-Layer Electrostatic Conjugation. Layer-by-layer (LbL) electrostatic complexation is a simple yet powerful emerging methodology for obtaining noncovalent NP bioconjugates.[86,113,114] This technique evolved from the LbL functionalization of surfaces,[115−117] where alternating layers of cationic or anionic polyelectrolytes or both are sequentially deposited on a flat substrate by repeated dip−wash cycles, resulting in multilayer structures. The commonly utilized polymeric polyelectrolytes can, however, be substituted with charged biomolecules, such as oligonucleotides[118,119] and proteins,[120,121] permitting their incorporation within a multilayer structure. More recently, the LbL approach has been adapted to the functionalization of NPs for therapeutic purposes.[113,114,122] A strong rationale for adopting this preparation technique is its potential for higher loading levels since the functionalization of the NP surface is not limited to a molecular monolayer,[123] and multiple layers of biomolecules can be sequentially deposited. In addition, the ratio of biomolecules per NP can, to some extent, be optimized to yield complexes with a desired surface charge.[86,124,125] This is especially helpful when developing nonviral DNA or short interfering RNA (siRNA) transfection agents,[126−129] where a net positive charge, combined with high-loading levels, is essential for cell internalization and intracellular release. In representative examples, poly(ethyleneimine) (PEI) and poly(lysine) have been grafted to Au,[128−133] IO,[126,134] silica,[135−138] and carbon/nanodiamond NPs[139,140] to provide a positively charged substrate that was subsequently loaded with oligonucleotides using the LbL approach. The resulting positively charged complexes were employed as vectors for gene silencing, as illustrated in the above cited references. Proteins have also been conjugated with NPs using this approach.[86] Bovine serum albumin (BSA), for example, has been electrostatically immobilized on native[141] or silanol-derivatized iron oxide NPs (IONPs),[142] without observing protein denaturation, which certainly bodes well for expanding this approach. In a slightly modified approach, IONPs were coated with a positively charged chitosan (CHI) derivative and then used to immobilize BSA.[143] Cytochrome *c* has also been electrostatically immobilized on AuNPs previously functionalized with mercaptopropionic acid in an attempt to develop a bioelectronic sensor.[144] Analogously, heme proteins have been immobilized on PEI-coated silica NPs.[145,146] Polyelectrolyte LbL chemistry has also been used to surface functionalize and render QDs soluble for labeling applications.[147]

Critical to the success of the LbL procedure is the choice of the initial polyelectrolyte layer. For the formation of positively charged surfaces, PEI,[124,126,129] poly(lysine),[148−150] poly(amidoamine) (PAMAM) dendrimers,[151] poly(allylamine),[106,149,152] and CHI[153] are among the most commonly employed polyelectrolytes. For the formation of negatively charged surfaces, hyaluronic acid (HAA),[149,154] poly(lactic acid),[155] poly(vinylsulfonic acid),[151] poly(acrylic acid) (PAA),[156] and other poly sulfonates[106,149,152] are all popular, as reviewed in refs 113 and 122. One issue always to be considered, especially for drug delivery or theranostic applications, is that some of the polyelectrolytes themselves can be quite toxic to cells and other biological systems.[157]

2.4.3. Encapsulation. Sequestering drugs or other biological molecules in a NP matrix is one of the most common chemistries currently being developed for NP-mediated drug delivery.[158] The most obvious benefits of this loading approach include targeted and localized delivery of a high concentration of insoluble materials *in vivo*.[11,159−162] Pertinent NP materials can include metals, oxides, dendrimers, liposomes, micelles, and those derived from both natural or artificial polymeric materials. Formulating these NP constructs to have appropriate size, chemical functionalities, drug loading, biocompatibility, and, most importantly, efficient drug release properties are an incredibly complex undertaking. For details, the interested reader is referred to more focused discussions of these issues.[56−58,84,158,163−165]

2.5. Commonly Utilized Chemistries

2.5.1. Functional Groups and Conjugation Reactions. The functional groups useful for conjugation reactions, or for these purposes *chemical handles*, found on most naturally occurring biomolecules (proteins, DNA, carbohydrates, lipids, etc.) and most currently available NPs are rather limited in terms of their diversity, see schematic in Figure 4. With proteins and peptides, amino acid residues tend to be targeted for conjugation: the ε-amino group found on lysine side chains; the N-terminal primary amine; the less utilized guanidinium group on arginine side chains; the cysteine thiol; the carboxyl groups on aspartic acid, glutamic acid, or the C-terminus; and in some cases, the phenol on tyrosine.[80] Carbohydrates and glycosylated proteins do offer hydroxyl groups or aldehydes as alternative reaction sites. Nucleic acids typically provide ribose sugars, phosphates, and some availability of the bases themselves for modification, although the latter is not often pursued because this can be quite disruptive to native structure and function.[80] This paucity of functionality limits the number and type of reactions that can be used to form a linkage with a target biomolecule. The most common chemistries targeting these groups are illustrated in Table 3, including *N*-hydroxysuccinimidyl (NHS)

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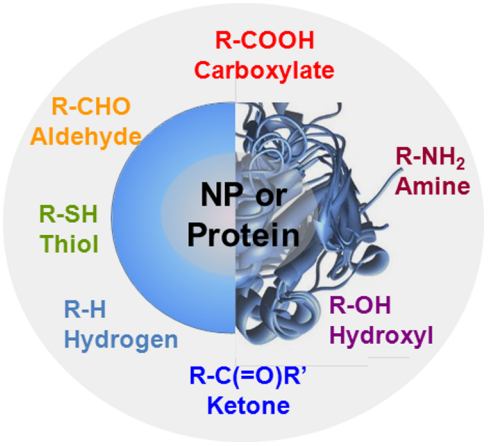


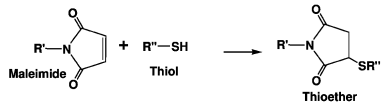
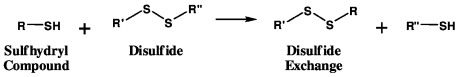
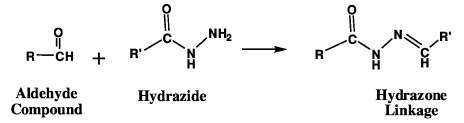
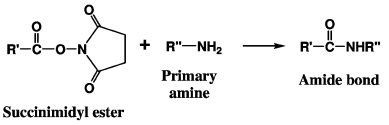
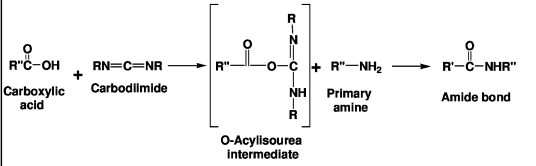
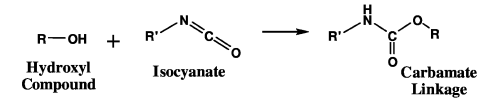
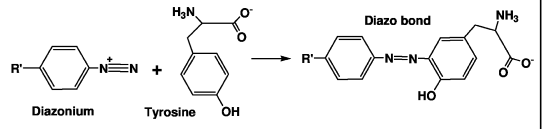
Figure 4. The more common potential functional groups on either a NP or protein structure that can be utilized for subsequent bioconjugation.

ester modification of amines along with carbodiimide-mediated (EDC) condensation of carboxyls with amines, maleimide conjugation to thiols, and diazonium modification of the phenolic side chain on tyrosine. Figure 5 presents a more detailed schematic of many of the chemistries that have already been applied to NPs for functionalization, although not all have been utilized with biologicals.[15] This figure also highlights how many of the chemistries can be applied with reactive groups on either the NP or the biological of interest or both.

It is now very common to recombinantly modify proteins to display unique cysteine residues (i.e., thiol handles) for site-specific labeling and conjugation.[166,167] Protein N-termini can also be specifically modified using multistep N-terminal transamination chemistries, while polyhistidine (His_n) motifs inserted almost anywhere in a protein can act as a site for region-specific interactions with nitrilotriacetic acid (NTA)-modified substrates.[168,169] Clearly, far more chemistries are applicable than presented here, and the interested reader is referred to Hermanson's *Bioconjugate Techniques*,[80] which is perhaps the most comprehensive guide to this subject, along with Haugland's *The Handbook—A Guide to Fluorescent Probes and Labeling Technologies*.[170]

Biomolecules of synthetic origin, particularly peptides and nucleic acids, are far more versatile because virtually any functional group or chemical handle can be site-specifically introduced as needed during initial synthesis or by subsequent modification. The genomic revolution led to the development of a library of chemistries for synthesizing and modifying nucleic acids, and this now allows them to be obtained with a variety of functional groups site-specifically inserted into their structures including amines, thiols, carboxyls, biotin, azides, alkynes,

Table 3. Selected Biological Functional Groups and Representative Reaction Mechanisms[a]

Target	Reactive Group	Product	Example Mechanism[4]
Free Thiol	Maleimide[1]	Thioether	
	Haloacetyl/Alkyl Halide	Thioether	
	Arylating agents	Thioether	
	Aziridine	Thioether	
	Acryloyl derivatives	Thioether	
	Disulfide Exchange[1] – pyridyl disulfides, 5-thio-2-nitrobenzoic acid (TNB)	Mixed disulfides	
Aldehyde/ Ketone	Hydrazine[1]	Hydrazone	
	Amines	Schiff's base (imine)[2]	
Free Amine	*N*-hydroxysuccinimide ester (NHS)[4]	Amide	
	Acyl azides	Amide	
	Isocyanates, Isothiocyanates	Urea, thiourea	
	Sulfonyl chlorides	Sulfonamide	
	Aldehydes, Glioxals	Imine, secondary amine[3]	
	Epoxides, Oxiranes	Secondary amine	
	Carbonates	Carbamate	
	Arylating agents	Arylamine	
	Imidoesters	Amidine	
	Carbodiimides, anhydrides	Amine[4]	
Carboxylate	Carbodiimides[1], Carbonyldiimidazole	Amides[4]	
	Diazoalkanes, Diazoacetyl	Ester	
Hydroxyl	Epoxides, Alkyl halogens	Ether	
	Periodate	Aldehyde	
	Isocyanates[1], Carbonyldiimidazole, *N,N'*-disuccinimidyl carbonate, N-hydroxysuccinimidyl chloroformate	Carbamate or urethane[4]	
Reactive Carbon (On a phenol *e.g.* tyrosine)	Diazonium[1]	Diazo bond	

[a]Adapted from refs 80 and 171. [1]Indicates the example mechanism highlighted. [2]Might be followed by reductive amination to form a stable product. [3]After reduction. [4]Via reactive intermediate.

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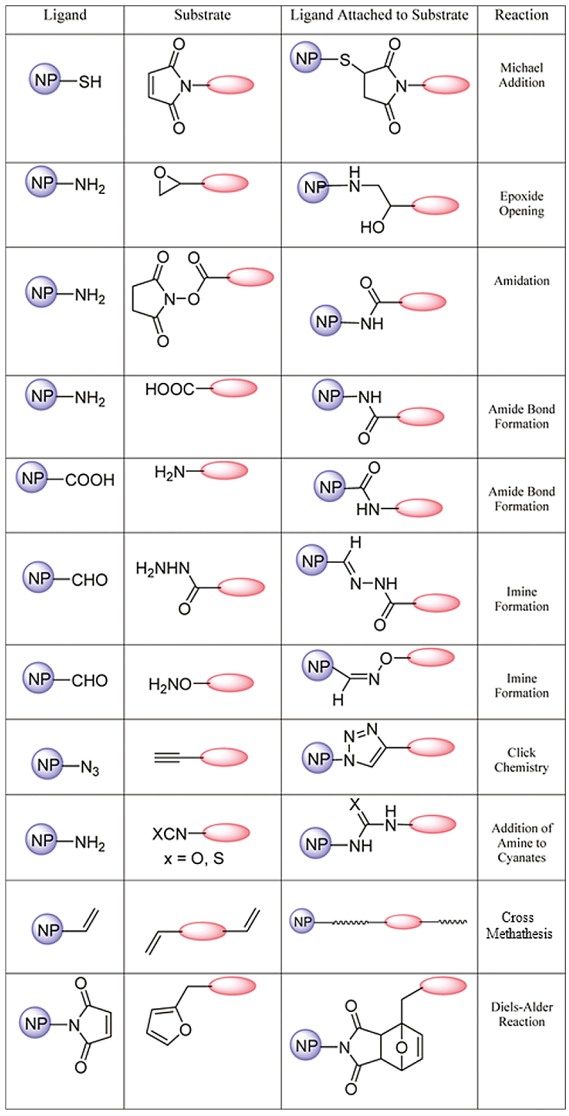

Figure 5. Selected NP functionalization chemistries. Reprinted from ref 15. Copyright 2011 American Chemical Society.

digoxigenin, and a plethora of organic fluorescent dyes.[172−174] Further, the insertion of starter groups such as amines, thiols, or carboxyl groups can allow virtually any other functionality to be achieved using custom or multistep modifications. Synthetic peptides provide even more versatility for site-specific incorporation of functional groups due to the wide availability of literally thousands of modified chemical precursors.[175−181] Again, insertion of unique starter groups such as azides or alkynes can allow incorporation of other functionalities in a bioorthogonal manner (see below), that is, without chemically altering the rest of the peptide.

2.5.2. Biotin−Avidin Chemistry. The number of publications incorporating biotin−avidin chemistry for bioconjugation of diverse NP materials is beyond tracking, see Figure 6 for a schematic overview of the more common application to NPs. Indeed, this chemistry seems to be the first choice for prototyping bioconjugation after synthesizing a new type of NP. The almost overwhelming presence of this bioconjugation strategy within the NP−bioapplication field warrants some discussion so as to understand the underlying motivation. E. E. Snell's 1940s discovery of the protein avidin combined with the structural determination of isolated biotin by du Vigneaud and co-workers

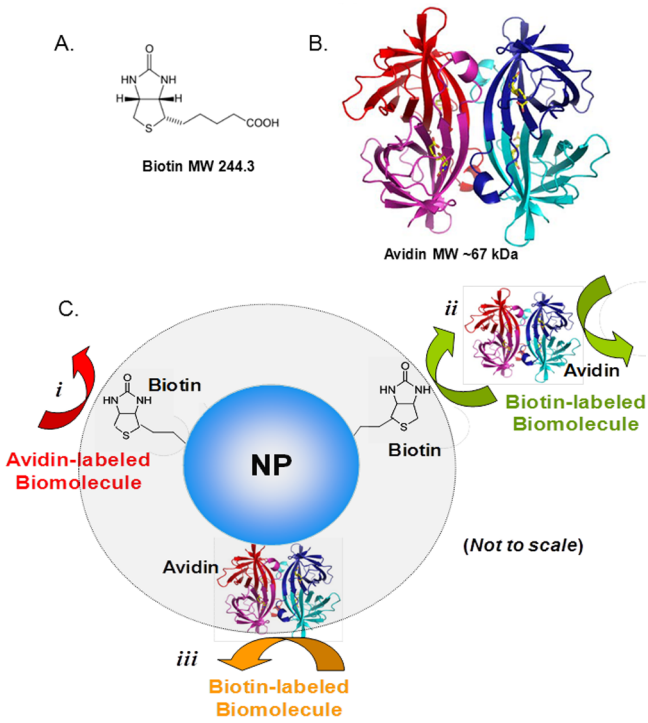

Figure 6. Biotin−avidin chemistry. (A) Chemical structure of biotin along with (B) a ribbon model of tetrameric avidin, showing the monomers in magenta, blue, cyan, and red and the four biotin molecules in yellow. Reprinted with permission from ref 184 with permission from Elsevier Science. (C) Schematic highlighting the three commonly used biotin−avidin strategies for attaching biomolecules to NPs. (i) Biotin-labeled NP coupled to avidin-labeled biomolecule. (ii) Biotin-labeled NP coupled to biotin-labeled biomolecule by an intermediary avidin linker. (iii) Avidin-labeled NP coupled to biotin-labeled biomolecule.

laid the groundwork that established the avidin−biotin system as a powerful tool used in numerous biological and biotechnological applications.[80,182,183] The avidin−biotin complex represents one of the strongest known noncovalent interactions with an association constant (K_a) of 10^{15} M^{-1}, and this, combined with its well-understood interactions and an incredibly wide range of commercially available reagents (see Table 4), has led to its routine application in NM research.

Avidin, originally discovered in egg whites but now found in a number of tissues, is a glycoprotein with a molecular weight of ~67 kDa and comprises a homotetrameric subunit structure, with each subunit capable of binding to one biotin (Figure 6). Structurally the protein is stable to a number of extreme conditions (temperature, denaturants, and pH) and is found to have a high carbohydrate content with a basic isoelectric point (pI) of ~10.[80,184] The groups of Bayer and Kulomaa were instrumental in studying the essential components of the avidin−biotin complex, leading to a better understanding of the overall system and the factors that influenced the interaction.[184,185] The combined basic pI and carbohydrate content, unfortunately, results in the tendency of avidin to display nonspecific binding in a number of applications. Researchers have overcome this issue by recombinantly or chemically deglycosylating avidin without affecting biotin affinity. A number of companies now offer deglycosylated avidin under various product names including NeutrAvidin (Thermo Scientific Pierce), NeutraLite (Belovo), and ExtrAvidin (Sigma-Aldrich). Removal of the carbohydrate functionality along with a reduction in the pI (to ~6.3) has

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Table 4. Selected Biotin and Avidin Materials[a]

category	example materials	comments
Biotin		
functionalized/reactive	functionalized with aliphatic amine, amine, alkyne, azide, carboxylic acid, hydrazide, hydroxylamine, iodoacetamide, maleimide, succinimidyl ester, thiosulfate, iodoacetyl, pyridyl disulfide, or nitrilotriacetic acid (NTA)	range of spacer lengths available including PEG
fluorescently labeled	including fluorescein, AlexaFluor (488, 546, 594), lucifer yellow, Oregon green, tetramethylrhodamine, or rhodamine dyes	range of spacer lengths, custom dye labeling available
bioconjugates	enzymes (phosphatase, peroxidase, galactosidase, lysozyme); antibodies (primary and secondary); rabbit IgG; protein A, G, or L; proteins (BSA, transferrin, annexin V, cholera toxin, EGF, bungarotoxin); other labeled site selective-probes (lipids, nucleotides-dUTP, dextran)	various spacer lengths available
Avidin		
derivatives and homologues	streptavidin, NeutrAvidin, avidin, CaptAvidin, ExtrAvidin, NeutraLite Avidin	
functionalized/reactive	hydrazide and maleimide activated steptavidin and NeutrAvidin	
fluorescently labeled	labeled with numerous Alexa Fluor dyes, fluorescein, Oregon green, phycoerythrin, rhodamine dyes, Texas red, DyLight dyes, Cy dyes, CF dyes, CryptoFluor, Chromeon, atto dyes (425−680 nm)	majority streptavidin-based; some dual dye combinations available
bioconjugates	enzymes (phosphatase, peroxidase, galactosidase); proteins (IgG); other probes (streptavidin−isoluminol, ferritin)	

[a]Sources include Life Technologies, Thermo Scientific, Sigma-Aldrich, Biotium, SouthernBiotech.

largely ameliorated nonspecific binding issues. As an alternative, the analogous protein streptavidin (SA), isolated from the bacteria *Streptomyces avidinii*, is a homologous tetrametric biotin binding protein displaying similar affinity to avidin.[186] SA, like avidin, is extremely stable; however, it is not a glycoprotein and the lack of high carbohydrate content, combined with a much lower pI of 5−6, also results in a lower nonspecific binding. There are now a number of genetically engineered avidin and SA derivatives, which were produced not only to understand the nature of their interaction with biotin but also to improve their properties for different applications.[185,187] For example, monomeric SA has been synthesized, and while it has a much lower affinity for biotin ($K_a \approx 10^8$ M^{-1}), it has lower cross-linking issues than its tetrameric counterpart and has a number of applications where exchange or reversible binding is desirable, such as in purification chromatography. Alternatively, CaptAvidin (Life Technologies) has a $K_a \approx 10^9$ M^{-1} and a nitrated tyrosine present in the biotin-binding site that permits dissociation of the complex at pHs greater than 10.

Biotin is a relatively simple water-soluble organic compound, also known as vitamin B_7 or vitamin H, and functions as an essential coenzyme for several carboxylase enzymes in many different species, including humans. These enzymes control metabolism of fatty acids, amino acids, and glucose and are also thought to be involved in transcriptional gene expression and stability.[188,189] There are a variety of biotin derivatives that make biotinylation of NPs and biological molecules rather straightforward. These are synthesized to display distal amine, thiol, carboxyl, azide, and other functional groups that facilitate conjugation. An extensive range of very effective reactive biotin reagents are available such as maleimide, NHS ester, or other adducts (Table 4), which are intended to "biotinylate" their target cognate group on biologicals, particularly proteins and antibodies, along with NPs.[80] An important issue to consider when conjugating biotin to groups such as the amines distributed around a protein, is that control over the labeling site and ratio is hard to exert given the abundance of targets. Nevertheless, trial and error can usually provide biotin-functionalized proteins and especially antibodies, although some heterogeneity in both labeling and binding affinity should be assumed. It is also quite common to obtain DNA or peptides with biotin groups site-specifically introduced during synthesis. Both of these chemical biotinylation strategies also offer the option of having a PEG, or other extended linker, inserted adjacent to the biotin group to facilitate binding within avidin's relatively deep binding pocket. Alternatively, genetic engineering approaches, where specific biotin labeling sites are recombinantly introduced into fusion proteins, are growing in popularity.[190−192] Inspired by native biotinylation processes, unique peptide sequences, ranging in length from 13 to 75 amino acids, are recombinantly fused to the protein of interest and subsequently biotinylated upon exposure to biotin in the presence of the requisite biotin ligase enzymes (BirA).[190−193] The trademark AviTag technology is offered commercially in a wide range of plasmid expression vectors by Avidity (Aurora, CO) and GeneCopoeia (Rockville, MD). The benefit of this approach is the ability to site-specifically biotinylate a target protein, as required, during (*in vivo*) or after expression (*in vitro*). In addition, many companies now offer biomolecules, such as enzymes and antibodies, along with NPs prelabeled with biotin or avidin species, see Table 4 and Supporting Table 1, Supporting Information.

The high-affinity nature of the avidin−biotin interaction has made it a popular model system for biorecognition applications and many NP researchers developing biosensing platforms or new signal-transduction modalities now routinely use it at the initial developmental stage to demonstrate proof-of-concept.[184,194−197] For example, Roll and co-workers demonstrated an aggregation assay format using AuNPs modified with BSA−biotin by monitoring the UV−visible absorbance spectral shift of the biotin-labeled gold colloids upon addition of avidin.[194] Oh et al. used SA-labeled QDs and biotin-labeled AuNPs to measure avidin using an inhibition assay based on an energy transfer quenching signal transduction.[195] AuNPs labeled with a biotin-functionalized diblock copolymer, in combination with IO magnetic particles, have also been used to extract and concentrate SA spiked into human plasma (as a model biomarker) prior to analysis using lateral flow immunochromatography.[196] For prototyping or troubleshooting the technical aspects of a NP-biosensing platform, the avidin−biotin system is almost an ideal model; however, it also represents a best case scenario because most other biorecognition interactions have much weaker association constants and so will not perform as effectively.

The same affinity also makes the avidin−biotin complex a very popular method of attaching biomolecules, such as antibodies or DNA, onto the surface of a NP. In this case, the interaction is, in essence, functioning as an intermediate linking technique, and, in addition to those examples highlighted throughout this review, some representative examples of this

NP-linking strategy are listed in Table 5. As is made apparent in this table and Figure 6, there are two principle methods of bioconjugation using biotin−avidin chemistry. The first, and probably most common, approach involves an avidin-functionalized NP being mixed with a biotinylated biomolecule. It is important to consider that the attachment of avidin to the NP during initial modification will probably obscure one or more available biotin binding sites and, in conjunction with overall heterogeneity arising from this chemistry, the final avidin-functionalized NPs will probably display many different orientations and an average range of available binding sites. Indeed, this very issue has been noted to be responsible for the heterogeneous orientation of biotinylated DNAs assembled to SA-functionalized QDs.[81] The second approach is often used in conjunction with polymer-based NPs, where researchers take advantage of common organic synthetic procedures to biotinylate the "monomer" unit prior to its incorporation into the polymeric NP. In this case, biotin is preferred over avidin labeling because its small size does not interfere with the self-assembly process. Typically the biomolecule is also biotinylated, and the two are cross-linked via an avidin intermediary. A prominent example of this type of architecture is the attachment of the 240 kDa β-phycoerythrin light-harvesting complex to biotinylated QDs by using SA adducts.[99,198,199] In either case, since each avidin protein can and will potentially bind up to four biotin moieties, care must be taken not to over biotinylate the biomolecule of interest, since this can induce cross-linking of the NPs and subsequent precipitation. Overall, the detailed fundamental understanding of the avidin−biotin interaction, its wide utility, and the extensive library and ongoing evolution of reagents are securing the continued use of avidin−biotin chemistry for NP bioconjugation in the foreseeable future.

2.5.3. Polyhistidine−Nitrilotriacetic Acid Chelation. Polyhistidine or His_n sequences are commonly engineered into proteins as an affinity tag for their subsequent purification using immobilized metal ion affinity chromatography. His_n binds to Co(II), Cr(II), Cu(II), Ni(II), Zn(II), and other divalent metal cations chelated by NTA or functionally analogous structural groups at four of six available sites around the ion, see Figure 7. NTA and other chelation agents are usually attached to some form of solid support or media to facilitate protein isolation and purification. This chemistry was pioneered by Hochuli et al. and the almost universal use of hexahistidine sequences (His_6) on expressed proteins for purification can be traced to his work, although, interestingly, smaller consecutive His_4 sequences may work equally efficiently.[205−207] Subsequent work has optimized the structures of the metal cation-coordinating groups and, although many variants are available, Ni(II)−NTA still remains the most ubiquitous working combination.[206] The His_n motif is exceedingly rare in native proteins and is most often introduced at a protein's terminus. It is usually not detrimental to the expressed protein's activity because its small size helps to minimize disruption of the native protein structure.

The interest in utilizing His_n tags and NTA for NP−biomolecular display borrows directly from immobilized metal-affinity chromatography and surface modification chemistry[208] and is driven by a combination of several inter-related factors including the following: (1) The potency of His_n−NTA cooperative interactions is characterized by dissociation constants on the order of $K_d = 10^{-13}$ M.[205,206] (2) His_n-motifs can be easily synthesized into nascent peptides or genetically engineered into expressed proteins; indeed, a variety of commercial kits are now available and in common use for the latter purposes.[209] (3) Several NTA precursors and analogs are available, some of which

Table 5. Representative Biotin−Avidin Nanoparticle Bioconjugates[a]

nanoparticle	biomolecule	biotin	avidin	utility	ref
gold nanoshell: Si-coated, containing Fe_2O_3 NPs and indocyanine green dye	human epidermal growth factor receptor 2 (HER2) α-body	NHS ester attachment to antibody primary-amines	Si-coating modified with thiol exposed to streptavidin−maleimide	multimodal therapeutic nanocomplex targeting breast cancer	200
adenovirus: genetically fused to biotin acceptor peptide	antibodies and oligonucleotides; other ligands	adenovirus metabolically biotinylated; ligands also biotinylated	avidin, NeutrAvidin used to bridge biotin-labeled ligand and NP	potential gene therapy applications	193
quantum dots	ricin, shiga toxin and transferrin	prelabeled or biotinylated in-house via primary amines	commercial streptavidin QDs	investigate intracellular trafficking of ligands	201
gelatin nanoparticles: cross-linked with glutaraldehyde; surface amines thiolated with 2-iminothiolane	anti-CD3 antibodies	biotin-labeled anti-CD3 antibody	NeutrAvidin coupled to thiols on NP surface via MBS bifunctional cross-linker	investigating receptor mediated cellular uptake in lymphocytic cells	202
polymer nanoparticles: PLGA−PEG	tetanus toxin C fragment (TTC)	biotin-labeled PLGA−PEG polymer; TTC biotinylated using an NHS−PEG_4−biotin	avidin, streptavidin, or NeutrAvidin used to bridge biotin-labeled ligand to NP	targeted drug delivery to neurons	203
polymer nanoparticles: PLA−PEG paclitaxel loaded nanoparticles	transferrin	biotin-labeled PLA−PEG polymer; transferrin obtained biotinylated	NeutrAvidin used to bridge the biotin-labeled ligand and NP	targeted drug delivery to brain tumor cells	204

[a]Abbreviations: PLA = poly(lactic acid), PEG = poly(ethylene glycol), PLGA = poly(lactic-*co*-glycolic acid), MBS = *m*-maleinimidobenzoyl-*N*-hydroxysulfosuccinimide, NHS = *N*-hydroxysuccinimide.

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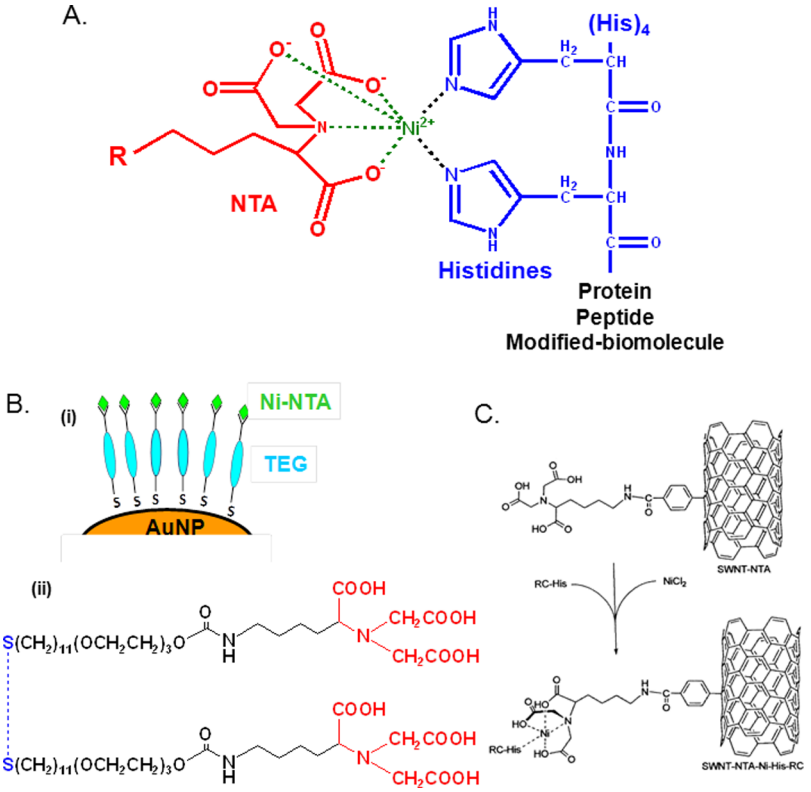

Figure 7. Metal-affinity coordination. (A) Coordination of polyhistidine residues to Ni(II)-chelated nitrilotriacetic acid (NTA). The polyhistidines usually consist of a hexahistidine motif (His_6), and this can be attached to protein, peptide, or other biomolecule such as DNA. (B) Schematic (i) of a AuNP surface displaying NTA disulfide, as exposed to the NPs, whose chemical structure (ii) is shown as the disulfide. Reprinted from ref 213 with permission. Copyright 2006, Institute of Electrical and Electronics Engineers, New York City, NY, USA. (C) Reaction scheme for the nickel functionalization of SWCNT following reaction with 4-carboxybenzene diazonium salt and N_{α},N_{α}-bis(carboxymethyl)-L-lysine hydrate for assembly with polyhistidine-tagged reaction center (RC) protein. Reprinted from ref 215 Copyright 2008 American Chemical Society.

are in a reactive form, to allow direct coupling to other common chemical handles. (4) The His_n tags allow access to what can be essentially considered a single point of attachment. Most often, two-adjacent histidine residues coordinate with each individual metal–NTA complex. The His_n sequence, however, does not span a long distance. This helps eliminate undesirable cross-linking and even potentiates some control over biomolecular orientation.[14] (5) The system has applicability beyond proteins and peptides. A variety of biomolecules have been chemically modified to display a His_n tag for both purification and immobilization purposes. These include nucleic acids and other materials such as PEG.[210−212]

The strategy most commonly utilized to facilitate His_n-mediated biomolecular assembly to NPs is the use of Ni(II)–NTA modified coatings as highlighted in Figure 7.[14,213−215] This is usually accomplished by chemically reacting the NP coatings toward a nucleophilic derivative of NTA. Representative examples include AuNPs, magnetic NPs, and semiconductor QDs displaying surface carboxyl groups that are activated with EDC/NHS toward N_{α},N_{α}-bis(carboxymethyl)-L-lysine. The latter is an amine-terminated lysine derivative of NTA most often used for this type of modification.[216,217] Precomplexing the NTA moiety with Ni(II) prior to the covalent modification step may help mitigate undesired cross-linking by blocking many of the pendant carboxyls from participating in the EDC reaction. Alternative approaches have used succinic anhydride or glutaraldehyde to activate polyglycerol-coated magnetic NPs and amine-coated silica NPs, respectively, toward the same lysine–NTA derivative.[218,219] Silane derivatives of NTA have also been directly reacted to silica NPs.[220] Thiolated-NTA derivatives (Figure 7B) have been used to chemisorb directly to the surface of AuNPs[221] and ZnS overcoated QDs[222] or, alternatively, reacted with aminated polymer-functionalized QDs premodified with a reactive maleimide.[223] NTA-coated single wall carbon nanotubes (SWCNT) (Figure 7C) have also been generated for controlled protein conjugation based on prior observations that proteins denatured when attached directly to the hydrophobic surface of SWCNTs; this was significantly minimized by using NTA for conjugation.[215]

NTA and other structurally analogous chelating agents function by relying on the ability of multiple closely displayed carboxyl groups to simultaneously coordinate the metal ion. It appears that the density of displayed carboxyl groups associated with some types of NP coatings may be sufficient to coordinate divalent metals in a similar manner.[224,225] This may essentially negate the need for an NTA group if the NP displays a functionally equivalent configuration at its periphery. Because many of the divalent metal cations coordinated by NTA are intrinsic components of some NP materials, it has recently been demonstrated that His_n can effectively coordinate directly to these inorganic surfaces as well, again bypassing the need for an NTA group (discussed below in section 3.2.2.1). Utilizing His_n–Ni(II)–NTA interactions for NP biomolecular display is rapidly growing in popularity. Excess NTA is easier to separate from NPs, less prone to undesired cross-linking, and more amenable to controlling biomolecular orientation than the more common biotin–avidin approach.

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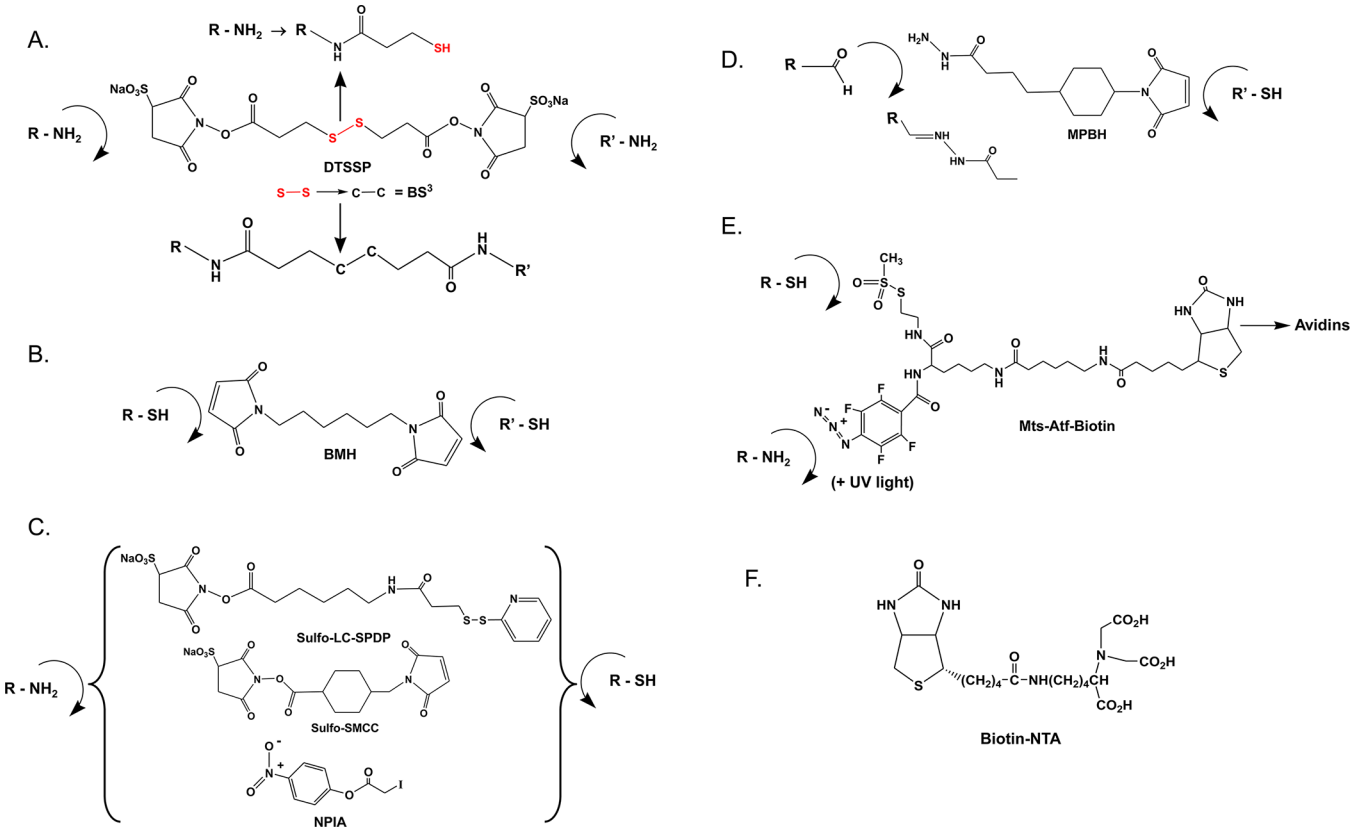

Figure 8. Representative homo-, hetero-, and trifunctional chemical cross-linkers. (A) Structure of amino-reactive DTSSP and BS^3 highlighting the central dithiol in the former. Replacing the dithiol with an alkane bond yields the BS^3 homobifunctional linker. (B) Structure of thiol-reactive homobifunctional bis-maleimidohexane (BMH) linker, which displays two maleimide groups. (C) Structure of three heterobifunctional linkers that join amines and thiols. (D) MPBH structure, which targets a thiolated group with a maleimide while also displaying a carbonyl-reactive hydrazide group. (E) Heterotrifunctional Mts−Atf−biotin linker, which can target thiols with a reactive methanethiolsulfonate and amines or other functional groups with a photoreactive perfluorinated phenyl azide group. The internal MTS dithiol can be reduced to allow later release of that particular group, while the biotin facilitates capture by avidin. (E) Structure of biotin−NTA; note that this molecule is available with extended linkers separating the two functional ends.

2.6. Functional Cross-linkers

The paucity of diverse chemical functional groups available on native proteins led to the development of functional cross-linkers, see Figure 8. These are most easily defined as a bifunctional reactive molecule meant to join two (bio)macromolecular entities together; however, they can also serve to introduce a new functional group, for example, converting an aminated-NP to also display free thiols.[80] Because cross-linkers form a large part of the NP bioconjugation chemistry repertoire highlighted below, some discussion of their capabilities is warranted. The simplest form is a homobifunctional cross-linker, which consists of the same reactive entity displayed on opposite ends of an alkyl spacer. Bis(sulfosuccinimidyl) suberate (BS^3) and its cleavable functional analog 3,3′-dithiobis(sulfosuccinimidylpropionate) (DTSSP), shown in Figure 8A, illustrate the functional properties available to these first-generation linkers. BS^3 displays amine-reactive sulfo-NHS esters at each end of an octyl spacer arm, allowing it to covalently couple between two amines ideally associated with two separate entities, for example, proteins. DTSSP is almost identical to BS^3 except for the replacement of the two central carbons with a disulfide, which provides a bond that is labile in the presence of suitable reducing agents. This modification can allow, for example, interacting proteins that are cross-linked *in situ* to be later separated under mild reducing conditions.[80] In the current context, these molecules most often allow amine-functionalized NPs and proteins to be conjugated,[165] and strategies where BS^3-linked antibodies have been incorporated into PLGA NPs for cancer targeting have also been demonstrated.[226] Figure 8B shows bis-maleimidohexane, which is a similar bireactive cross-linker that targets thiols. Many other homobifunctional cross-linkers are commercially available and principally target the amine and thiol groups. Some homobifunctional epoxide and hydrazide species are also available, extending the reactive species to hydroxyl and aldehyde groups, respectively.[80]

Heterobifunctional cross-linkers contain two *different* reactive groups, allowing coupling of two *different* functional targets on proteins, other macromolecules, and NPs. Again, most versions target the amine and thiol group combination. Figure 8C presents the structure of three of the most commonly utilized molecules. *p*-Nitrophenyliodoacetate (NPIA) is perhaps the smallest heterobifunctional cross-linker available and results in only an acetyl bridge joining the conjugated molecules. It consists of an iodoacetyl group that has been activated with a *p*-nitrophenyl ester at its carboxyl end. The ester is strongly reactive toward amines in slightly basic buffers while the iodoacetate reacts with thiol groups. Its principle drawback is its insolubility in water, which necessitates predissolving in organic solvent.[80] Sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) is one of the more popular heterobifunctional cross-linkers in current use and consists of a maleimide attached to a sulfo-NHS by an intervening cyclohexane ring. Because the

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sulfo-moiety provides for water solubility at relatively high concentrations, this linker can be added directly to reaction mixtures in aqueous environments. In an illustrative example, Lockney et al. recently used sulfo-SMCC to attach the unique terminal cysteine thiols on cell targeting peptides to label red clover necrotic mosaic virus for testing as a potential cellular cargo delivery platform.[227] Sulfosuccinimidyl 6-[3′-(2-pyridyldithio)-propionamido]hexanoate (sulfo-LC-SPDP) is a water-soluble cross-linker displaying sulfo-NHS and thiol-reactive 2-pyridyldithiol groups separated by a longer chain length linker. Reaction with thiol displaying molecules via thiol exchange results in the release of a detectable byproduct that can be monitored by its absorption band at 343 nm, allowing for evaluation of labeling efficiency and reaction kinetics. Alternatively, sulfo-LC-SPDP can be used to introduce a thiol to an aminated-NP or protein. For this purpose, treatment with reducing agents such as dithiothreitol or β-mercaptoethanol after the first amine-reactive step can release the pyridine-thione leaving group and generate an available thiol. In a strategy similar to that demonstrated with the virus above, Naczynski et al. used sulfo-LC-SPDP to couple thiolated cyclic Arg-Gly-Asp (RGD) cell membrane binding peptides to the amines on rare earth doped $NaYF_4$ NPs encapsulated in human serum albumin nanoshells; these NPs were subsequently used to label the membrane integrins on cancerous cell lines.[228] It is important to note that since amines and thiols are both good nucleophiles, amine−thiol cross-linking is usually done in two steps, starting with conjugation to the amine. 4-(4-*N*-Maleimidophenyl)-butyric acid hydrazide (MPBH), shown in Figure 8D, represents a modification on the more common amine−thiol cross-linking motif by displaying a carbonyl-reactive hydrazide group along with the maleimide. Shi et al. recently used MPBH to functionalize antibody-displaying polymeric NPs with the chemotherapeutic doxorubicin (DOX) for testing of intracellular delivery.[229] The hydrazide specifically allowed covalent coupling to DOX's ketone group.

Because they possess *three different* reactive groups, trifunctional cross-linkers are far more complicated to design and synthesize and thus represent a much smaller group of available heterolinking reagents.[80] Nevertheless, given the long-term needs of NP bioconjugation chemistry, they may also have the most to offer for assembling far more complex multifunctional biomolecular structures. Methanethiosulfonate−azidotetrafluoro−biotin (Mts−Atf−biotin), shown in Figure 8E, highlights the potential of these reagents. It consists of a biotin on an extended linker attached to a thiol-reactive methanethiolsulfonate along with a UV-light/photoreactive, perfluorinated phenyl azide group that can be covalently linked to many different target groups including amines, sulfides, sulfoxides, alkenes, aromatic rings, and in some cases, unactivated CH bonds.[230] This broad reactivity means the latter can be potentially used to capture primarily proteins or other biomolecular targets of interest that are interacting with a NP assembly *in situ* in an externally controlled manner. The biotin handle allows the complex to be captured and recovered, while the methanethiolsulfonate thiol can be further reduced to release one of the original captured moieties or provide a free thiol group. Such a label-transfer approach may enable the application of *in vitro* selection principles to NP bioconjugation or, alternatively, the use of NPs as scaffolds and labels for pull-down assays in protein discovery. Another heterobifunctional cross-linker that is growing in popularity is biotin−NTA, see Figure 8F, which can allow any His_n-displaying protein to display biotin in a noncovalent manner. Alternatively, this same cross-linker can allow any of the avidins, either in solution, on surfaces, or as displayed on many NPs, to complex His_n-proteins and display them.[231] Several versions of this linker are commercially available and these usually vary by the length of the intervening linker placed between the terminal biotin and NTA moieties.

Another relevant consideration is that, in many cases, analogues of selected homo-, hetero-, and trifunctional cross-linkers are available where the differences consist of just variations in linker length. Linkers consist primarily of either an alkyl or a PEG chain; the latter in many cases helps provide solubility. Different linker lengths can allow for some level of control over separation distances and orientation between cross-linked entities, which may help optimize subsequent binding interactions. Again, Hermanson is an excellent resource and source of advice on important considerations for using cross-linker chemistry.[80]

2.7. Bioorthogonality

An important and growing concept in bioconjugation chemistry is that of bioorthogonality. The definition of *bioorthogonal* is a subclass of "click" chemistries that, besides meeting all of the requisite "click" reaction parameters (see section 2.8), can be applied in a biological environment (where diverse biomolecular functional groups are present) without cross-reactivity. Reactions are considered bioorthogonal when the two reactive functional groups are highly chemoselective and unreactive to the other functionalities present in biological systems and when the reaction proceeds in water at or near neutral pH, between 25 and 37 °C, and do not involve cytotoxic reagents or byproducts.[232−235] Endogenous functional groups typically present in biological environments include amine, carboxyl, hydroxyl, and thiol groups along with alkenes, amides, disulfides, esters, phosphodiesters, and a host of other less common functional groups. The development of bioorthogonal chemistry has been driven by the desire to selectively label or conjugate to target molecules, such as proteins, within cells or tissues or even model organisms. Because the bioorthogonal functional groups providing the high reaction specificity are not naturally occurring, application of such chemistry typically requires introduction of the functional groups to biomolecular targets, sometimes *in vivo*, using reagents and methods that do no significant damage to biological molecules and systems. A common aspect found in many of the examples described below is that the NPs and biomolecules are either synthesized (e.g., peptides, oligonucleotides) or expressed (e.g., proteins) displaying one of the requisite bioorthogonal functional groups needed. Alternatively, these modifications are introduced chemically, often using heterobifunctional cross-linkers (e.g., NHS ester−azide) or other conjugate reactions at amine, carboxyl, and thiol groups. Additionally, bioorthogonal chemistries must be aqueous, resist nucleophilic attack, reducing environments, and enzymatic degradation, and not require extensive heating (usually ≤37 °C) or a high molar excess of a reagent, while still having relatively short reaction times. Although these combined criteria appear quite daunting, several click (and enzymatic ligation) chemistries have been developed that satisfy these requirements. More importantly, many have already been demonstrated for NP bioconjugation.

2.8. Click Chemistry

The concept of "click" chemistry, as outlined by Kolb, Finn, and Sharpless in 2001,[236] is generally applied only when a reaction meets several defining criteria: (1) modular, (2) wide in scope, (3) high yield, and (4) producing nontoxic or inoffensive byproducts. Additionally, the chemistry itself should utilize (1) simple reaction conditions attainable in any

chemistry laboratory, (2) readily available starting materials or reagents, and (3) no solvent or a benign solvent and (4) should allow simple product isolation. The reactions themselves should all have an intrinsically high thermodynamic driving force (>20 kcal mol^{-1}) to favor product formation under appropriate conditions.[236−241] The term *click chemistry* is correctly used to describe a class of reactions that meet the above criteria and currently includes certain cycloadditions, nucleophilic substitutions, nonaldol carbonyl formation, and additions to carbon−carbon bonds such as epoxidation. Click reactions provide a highly desirable quality in their *orthogonal reactivity*, which refers to the mutually exclusive coupling of species bearing specifically paired functional groups. Simply stated, orthogonal reactivity is the controlled chemical coupling between two targeted functional groups regardless of the presence of other functional groups.

Orthogonal reactivity and concomitant application of click reactions have become critically important to a variety of fields, and click chemistries have already been demonstrated for functionalizing various biologicals, polymeric materials, surfaces, and inorganic NMs.[240,242−251] In labeling or modifying biologicals, exploiting click reactions to achieve selective or site-specific conjugation is always the desired goal. In most cases, site-specificity allows intrinsic bioactivity to remain relatively unperturbed, minimizes side reactions and nonspecific labeling, and provides the researcher with *a priori* knowledge of the chemistry sites.[80,242,252] Click reactions have shown great potential for rapidly and specifically modifying targeted biological species for subsequent *in vivo* tracking under physiological conditions in their native environment where many other functionalities are ubiquitous.[235] Click chemistry also has growing utility in labeling and modifying myriad biomolecules including proteins, peptides, and nucleic acids, as recently reviewed.[232,235,242] For these same reasons, the application of click chemistry to biofunctionalizing NPs is clearly desirable and rapidly moving beyond proof-of-concept. An overview of representative click chemistries with direct applicability to NP bioconjugation is briefly provided below.

2.8.1. 1,3-Dipolar Cycloaddition. The Huisgen 1,3-dipolar cycloaddition of azides and alkynes to form 1,2,3-triazoles has become, perhaps, the most popular and frequently employed click reaction. This also means that this particular cycloaddition reaction is sometimes inadvertently referred to solely as the "click" reaction. The popularity of this reaction can be directly ascribed to the discovery of Cu(I) as an efficient, relatively benign catalyst that accelerates the reaction ~10^8 fold and regioselectively forms the 1,4-disubstituted 1,2,3-triazole, see Figure 9.[253−255] This chemistry is now commonly referred to as Cu(I)-mediated [3 + 2] azide−alkyne cycloaddition (CuAAC). Several methods for generating the active Cu(I) species *in situ* during the reaction have been investigated, including the direct addition of Cu(I) salts (such as CuI). This often requires the use of organic solvents, addition of base, and O_2-free, anaerobic reaction conditions.[254] To avoid working under such extreme conditions to maintain the Cu(I) valence, reactions can be performed under aerobic conditions that typically utilize ascorbic acid to reduce Cu(II) species such as $CuSO_4$ to Cu^I.[253] Triazole- and benzimidazole-based ligands have also been utilized to stabilize Cu(I) species and enhance the reaction kinetics.[256,257] CuAAC is highly specific (no byproducts), chemically orthogonal, and compatible with aqueous media and biological components and utilizes mild reaction conditions. Although some concerns have been raised about *in vivo* toxicity

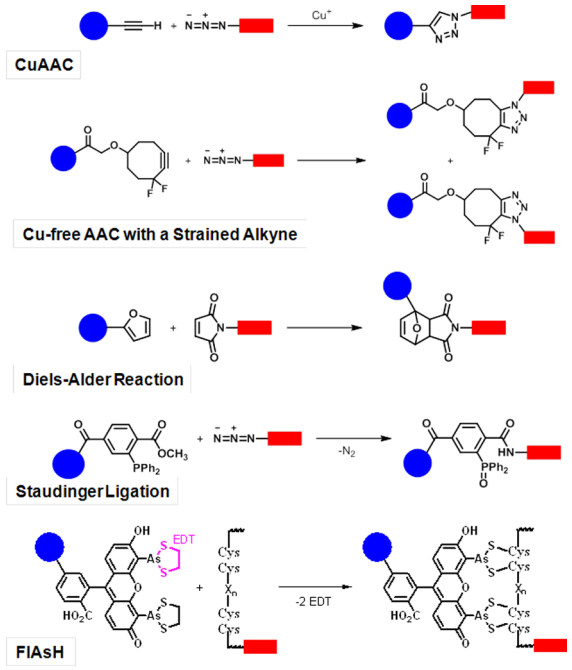

Figure 9. Schematic highlighting CuAAC, Cu-free AAC, the Diels−Alder reaction, Staudinger ligation, and FlAsH chemistries. The blue circle and red square represent the different molecules to which these functionalities would be attached.

from the Cu(I/II) species,[258] the CuAAC has become the most widely investigated click reaction for assembling NP bioconjugates.

2.8.2. Diels−Alder Reactions. The Diels−Alder reactions are typified by a [4 + 2]-cycloaddition between a diene and a dienophile, see Figure 9.[259] These reactions meet the key click chemistry criteria because they can be utilized for bioconjugation in aqueous buffers, at or near room temp, and in a highly selective, efficient manner that produces no byproducts. Despite the reputation of CuAAC as the premier click reaction, Diels−Alder chemistry is increasingly finding application in biofunctionalizing NP materials.[229,260]

2.8.3. Staudinger Ligation. The classic Staudinger reaction involves the nearly quantitative combination of an azide with a triaryl phosphine to form an iminophosphorane (aza-ylide),[261] an intermediate with diverse utility in synthetic chemistry.[262,263] The Staudinger ligation is an adaptation of the classic Staudinger reduction that utilizes a strategically placed ester on one of the phosphine aryl groups to induce the iminophosphorane to undergo spontaneous intramolecular cyclization to form an amide bond.[264,265] Saxon and Bertozzi demonstrated the "traceless" Staudinger ligation in 2000, where the aza-ylide is trapped by an adjacent electrophilic carbonyl group to yield an amide bond after hydrolysis.[265] The Staudinger ligation achieves chemoselectivity in a biological environment by utilizing the orthogonal reactivity of azide groups with phosphines, see Figure 9. This has made the Staudinger ligation a powerful covalent bond-forming reaction in aqueous media with demonstrated biological applications, including fluorescent labeling of proteins and DNA, protein immobilization, and targeted labeling of non-natural glycoproteins on cell surfaces.[266] The potential shortcoming of this chemistry is the oxidation of phosphines under ambient conditions or by metabolic enzymes, both of which can be overcome through the use of excess reagents. This strategy has already been used in some preliminary applications with NPs.[267,268]

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2.9. FlAsH/CrAsH and Other Chemistries

The desire to provide a fluorescent label that could selectively label a protein *in vivo* and allow its subsequent monitoring in a native cellular environment led the Tsien Laboratory to develop the fluorescein arsenical helix binder (FlAsH) fluorescent tag in 1998.[269] The first iteration was a fluorescein derivative with As(III) substituents on the 4′- and 5′-positions. The two As moieties of FlAsH selectively interact with proteins encoded to express the complementary tetracysteine tag. Each As atom of FlAsH binds a pair of vicinal thiols from two cysteine residues. FlAsH labels are synthesized, isolated, and employed with 1,2-ethanedithiol coordinated to the As atoms (FlAsH−EDT_2). FlAsH−EDT_2 is membrane-permeable and nonfluorescent until it binds with high affinity and specificity to the tetracysteine domain *in vivo*, see Figure 9. Although mainly applied for intracellular labeling, the latter properties allow it to be considered as a functional click-like or bioorthogonal chemistry in many respects. FlAsH−EDT_2 offers several advantages over other fluorescent labeling methodologies (e.g., fluorescent protein fusions): (1) the use of a small, synthetic organic dye; (2) the specificity of the dye to tetracysteine motifs (cf. NHS ester and isothiocyanate activated dyes); (3) the ease of genetically encoding small tetracysteine tags into protein sequences; (4) the turn-on fluorescence from FlAsH after coordination to the tetracysteine tags (50 000-fold increase); and (5) retention of fluorescence even after the protein is denatured.[270] A modified version of the FlAsH probe containing a second carboxy group (CrAsH) has also been prepared, which is more hydrophilic than FlAsH.[270,271] Compared with FlAsH, CrAsH also has stronger fluorescence upon binding to the tetracysteine motif at physiological conditions (pH 7.1−7.4) and reduced nonspecific fluorescent interaction with serum albumin, providing enhanced signal-to-noise ratios when used *in vivo*.[271]

Beyond click chemistry, there are other diverse chemistries with similar properties, including several chemoselective ligations, and these are discussed below. In cumulatively examining the various click chemistries highlighted here, along with several others in development, the enormous potential for bioorthogonal NP modification is clearly evident. The primary liability for almost all these chemistries, however, remains the incorporation of the requisite bioorthogonal functional groups onto either the NP or a biological substrate at site-specific positions. This sometimes requires a multistep process incorporating several different bioconjugation chemistries to achieve the desired placement of one of the reactive groups, as exemplified by the Francis group when applying the hetero-Diels−Alder reaction to viral capsids.[260] Although technically biocompatible, some of the conditions or catalysts required to achieve these linkages have been shown to be detrimental for either NP function or further *in vivo* applications. As most commonly applied, the CuAAC reaction has the additional requirement of removing the excess Cu catalyst and the ascorbic acid if utilized. Cu and other metal ions are known to quench the luminescence of semiconductor QDs, which would be problematic for CuAAC bioconjugation to QDs.[272−275] The recent development of Cu-free azide−alkyne cycloaddition chemistry, which exploits strained cycloalkynes to drive the reaction (see Figure 9), suggests that these issues can be circumvented, although cycloalkynes require intensive, multistep syntheses that are quite challenging.[232,276,277] However, this development does highlight how continuing research can overcome potential barriers to implementing these chemistries, and we can expect similar improvements to other click chemistries. Indeed, Life Technologies now provides a Click-iT product line that includes azide- and alkyne-modified fluorophores for CuAAC-based labeling applications, including flow cytometry, high-throughput screening, and cellular or whole animal studies. The application of click reactions to interfacing NPs with biologicals will continue to expand as more "clickable" heterobifunctional cross-linkers and biomacromolecule precursors (e.g., non-natural amino acids and nucleotides) become available and as novel reactions are developed and optimized for mild aqueous conditions, allowing them to function as bioorthogonal conjugate chemistries.

2.10. Native and Chemoselective Protein/Peptide Ligation Chemistry

A strict definition of chemoselective ligation would be "targeted-covalent coupling of mutually and uniquely reactive functional groups under mild, aqueous conditions."[278] Although such chemistries have long been established in organic chemistry, as the names imply, some select thioester and imine chemistries originating primarily from Dawson's work are described here. These have been recently applied to joining peptides together to chemically synthesize proteins *in vitro*, for peptide modifications in general, and, more recently, to modifying a complex assortment of biomacromolecules including nucleic acids, proteins and carbohydrates.[279−281] These chemistries are characterized by a host of properties that make them of direct interest in NP bioconjugation: (1) the reactions occur at or near physiological pH, in dilute aqueous solution, (2) they can be applied to many other biologicals beyond proteins and peptides, and (3) the underlying chemistry displays many chemoselective and bioorthogonal properties similar to "click" chemistries.

2.10.1. Native Chemical Ligation. The reaction between an N-terminal cysteine on one peptide and a C-terminal thioester on a second peptide to yield an amide bond, as illustrated in Figure 10A, is referred to as native chemical ligation (NCL).[280,282] The reaction proceeds via a transthioesterification intermediary step, which links the peptides through a thioester that, in turn, undergoes a highly favored, spontaneous intramolecular S → N acyl shift rearrangement to form an amide peptidyl bond while regenerating the cysteine side-chain thiol. Of particular relevance is that the reaction is highly chemoselective, it does not require protection of any other amino acid side chains including nonterminal cysteine residues because they do not form stable amides, and the C-terminal thioester may be associated with any amino acid including non-natural and highly modified residues. NCL can be considered a synthetic adaptation of the naturally occurring intein-mediated protein ligation (see section 5.2.1.2). Because both reactions proceed through a similar thioester intermediate, reagents activated for intein ligation can be directed through NCL as well. As will be highlighted below, NCL in combination with intein chemistry is beginning to see preliminary use in NP bioconjugation.

2.10.2. Hydrazone and Oxime Ligation Chemistry. Reaction between hydrazino/hydrazido groups and select carbonyls (e.g., aldehydes or ketones) will yield hydrazone bonds as illustrated in Figure 10B. The reaction can be reversible; however, the equilibrium favors the hydrazone under aqueous conditions (K_{eq} = 10^4−10^6 M^{-1}).[283] The forward and reverse reaction rates are typically slow at neutral pH but are significantly accelerated at acidic pH with the use of aniline as a nucleophilic catalyst, providing an efficient ligation that proceeds in minutes to hours.[284] Aniline reacts to form an imine group that is a key intermediary in the catalysis of this reaction.

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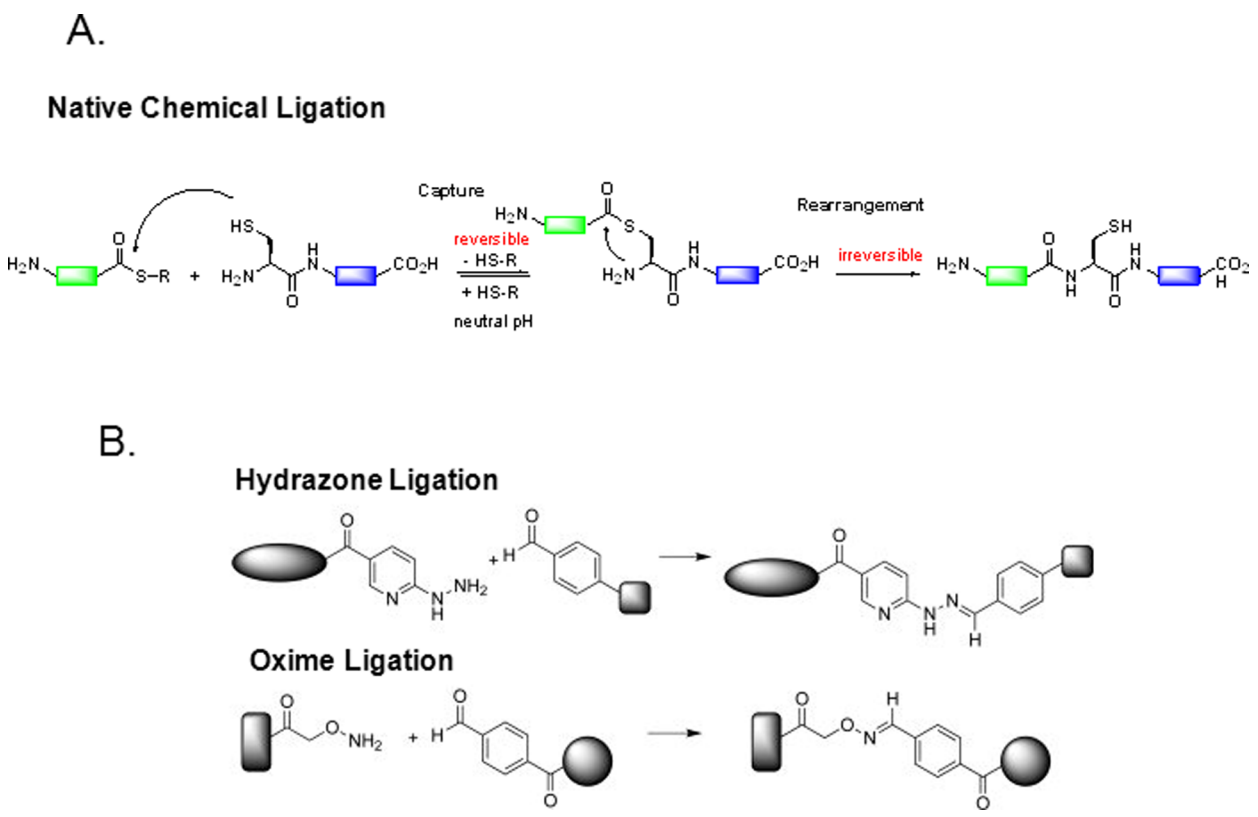

Figure 10. Chemical ligation. (A) Mechanistic pathway of native chemical ligation. Unprotected peptide segments are reacted by means of reversible thiol/thioester exchange to give thioester-linked initial reaction products. The N-terminal cysteine thioester-linked intermediate undergoes nucleophilic rearrangement by an irreversible, highly favored, intramolecular mechanism; this step yields a polypeptide product that is linked by a native amide (i.e., peptide) bond. Only a single reaction product is obtained, even in the presence of additional cysteine residues in either segment. Reprinted from ref 281. Copyright 2008 Wiley VCH. (B) Hydrazone ligation between a 6-hydrazinonicotinic acid (HYNIC) and a benzaldehyde. Oxime ligation between an aminooxy group and benzaldehyde to generate a stable oxime linkage. Reprinted from ref 279. Copyright 2010 Wiley Periodicals Inc.

Moreover, the aniline can be easily removed postreaction from the bioconjugates by dialysis or chromatography. Reacting aromatic aldehydes with 6-hydrazinonicotinic acid (HYNIC), in particular, can allow direct monitoring of the reaction progress through formation of a UV absorbing hydrazone product. The slow uncatalyzed rate of back hydrolysis at neutral pH ($\sim 10^{-6}$ s^{-1}) allows for relatively stable linkages over the time course of most experiments.[283] Hydrazone ligation can be considered bioorthogonal to most of the functional groups in proteins, oligonucleotides, and carbohydrates, with the only possible exception being the reducing ends of sugars in the latter. Hydrolysis of hydrazone bonds becomes appreciable at a slightly acidic pH range (pH 5–6); however, this pH sensitivity makes it potentially attractive for use in controlled intracellular drug delivery. This slightly acidic pH range is normally associated with endosomes and lysosomes, and thus NP bioconjugates prepared through hydrazone bonds and sequestered in endosomes following cellular delivery can potentially be triggered to release their biological cargo as the environment becomes more acidic during the change from early to late endosomes.

The ligation reaction between an oxyamine and an aldehyde or a ketone produces an oxime, see Figure 10B. Oximes are more stable toward hydrolysis than hydrazones ($K_{eq} \geq 10^8$ M^{-1}); however, they are similarly characterized by slow reaction rates at neutral pH and the requirement for aniline catalysis to achieve efficient ligation rates.[283,285] Both of these chemistries are starting to see significant application in NP bioconjugation as described below.

2.11. Non-natural Amino Acids

The desire to overcome the lack of bioorthogonal functional groups in peptides and proteins has led to the development of methods for site-specifically inserting non-natural amino acids into their sequences. The added presence of these new reactive groups creates many new possibilities for NP biomodification.[286] The versatility of synthetic chemistry along with myriad commercial precursors also allows such groups to be site-specifically incorporated into nascent peptide strands during synthesis.[179,180] Site-specific insertion of non-natural amino acids into proteins is far more challenging and is accomplished by either semisynthetic or entirely recombinant methods. Semisynthetic methods rely primarily on chemoselectively modifying proteins and other peptides to accomplish the insertion.[286] In contrast, recombinant methods usually require more complex strategies for modifying codons along with tRNAs and some of the requisite expression enzymes in auxotrophic *Escherichia coli* strains.[286−289] Codons for phenylalanine, leucine, isoleucine, proline, tryptophan, and methionine have all been "reassigned" to allow non-natural amino acid incorporation. Methionine, in particular, is the most frequently targeted because it is among the rarest of amino acids found in proteins. Incorporation is further facilitated by overexpression of the appropriate tRNA transferase in conjunction with simultaneous relaxation of its proofreading and substrate specificity.[286−289] Figure 11 highlights the structure of several such amino acids along with the potentially reactive or functional groups that they introduce into proteins. As a

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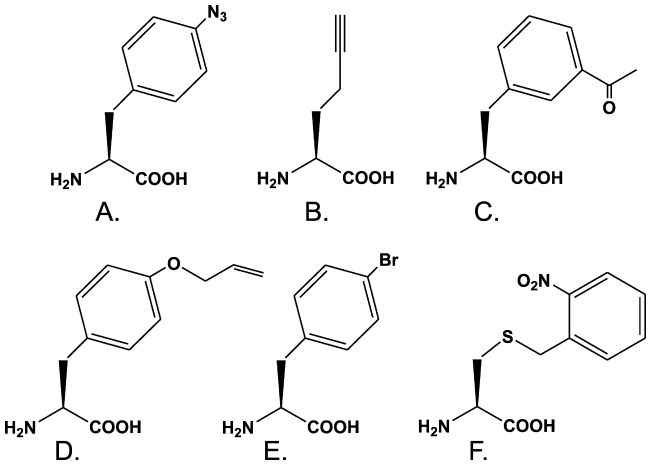

Figure 11. Structures of several non-natural amino acids that have been site-specifically introduced into proteins: (A) *p*-azidophenylalanine; (B) homopropargylglycine; (C) *m*-acetylphenylalanine; (D) *O*-allyltyrosine; (E) *p*-bromophenylalanine; (F) photocaged cysteine analogue blocked with a substituted nitrobenzyl. The protective group can be cleaved by 365 nm irradiation, allowing the protein to be "switched on" with light.[286]

generalized technique, non-natural amino acid substitution into proteins is still in its infancy, and many issues need to be addressed, including efficiency of incorporation, overall yield, and toxicity. However, the expected growth and optimization of such techniques in the near term will perhaps be the strongest facilitator of bioorthogonal chemistry being applied to more generalized *in vivo* protein-labeling along with NP modification. Relevant examples where such residues have been incorporated into proteins along with their far more common use in synthetic peptides for NP modification are described where applicable.

2.12. Peptide-Driven Recognition

Conjugation methods based on the formation of covalent bonds between NPs and organic or bio-organic moieties are often the method of choice to access nanobioconjugates.[19] However, the formation of bonds between an inorganic substrate and an organic or bio-organic molecule is often impaired by physical or chemical incompatibility between the two reactive partners. Recently, an alternative approach based on the formation of noncovalent bonds has emerged, relying on specific recognition and binding to NMs using peptide sequences identified through combinatorial methods.[103,290−292] This approach is an extension of the naturally occurring biomineralization process, where polypeptides mediate the synthesis of nanostructures with well-defined morphology.[293] For instance, in nature, the formation of hybrid organic−inorganic nanostructures is driven by specific interactions between specialized polypeptides and inorganic precursors. This process is characterized by an exquisite level of control over the synthesis of the biogenic inorganic NMs[294,295] and their binding to bio-organic components.[104] Although the interactions driving this recognition process are mostly noncovalent, they can nonetheless be very strong and quite specific. Classic examples of this level of control are the silaffin-mediated formation of nanostructured silica in diatoms[296] or the synthesis of magnetite NPs in magnetotactic bacteria.[297] This remarkable interplay between biomolecules and inorganic structures is the result of selection processes occurring on an evolutionary time scale.[298] In order to identify peptides that specifically bind a material of choice, combinatorial methods have been adapted to permit similar selection processes on a laboratory time scale.[103,291,299]

A simple example of an inorganic substrate-binding peptide identified in the laboratory is the previously mentioned His_6-motif (see section 2.5.3), albeit a nonspecific peptide sequence known to bind to a variety of metal NMs including Ni[300] and AuNPs[301,302], as well as semiconductor QDs, see section 3.2.2.1.[303] Although unable to discriminate between different materials, His_6 has been successfully utilized for the conjugation of QDs to nucleic acids[212,303] and proteins[304] and represents a robust linker that also permits a certain degree of control over the stoichiometry of the resulting nanobioconjugates. A multitude of more specific peptide sequences selected for their binding affinity toward various materials have been identified using phage display and cell surface display combinatorial methods. A technical description of these evolutionary techniques is beyond the scope of the present review, and the reader is referred to some excellent discussions on the subject.[103,292,305] Surprisingly, while many peptide sequences have been evolved to recognize macroscopic inorganic surfaces, only a few sequences have been evolved specifically for binding NMs. Table 6 highlights some representative peptide sequences selected for their biomineralization abilities. It is worth pointing out that peptide sequences that bind to the surface of a macroscopic material might not necessarily show high affinity for the same material in NP form. This is because certain conformational restrictions and surface effects are amplified at the nanoscale, and these special requirements should be included as part of the screening assay.

Although fundamental for the surface interactions themselves, very little experimental effort has been put toward understanding the molecular scale recognition and binding processes, which appear to be dictated by the interactions of specific residues located at key sites within the peptide sequence.[105,343−348] This is somewhat reflected by the diversity of sequences showing affinity toward some of the same materials listed in Table 6. Computational approaches have been explored to increase the understanding of peptide−material interactions, but they only provide limited information, because they cannot model all the interactions between a polypeptide and an inorganic surface. For this reason, most theoretical models are built on simple peptides interacting with well-characterized inorganic surfaces.[349−352] Although, many of the sequences reported in Table 6 are biomineralizing peptides, it is important to point out that, at least in principle, sequences that promote the synthesis of a given NM might also show significant binding affinity for the NM itself. In some instances, this concept has been validated. For instance, one of the earliest reports of a peptide sequence used to bind a NM is the patterning of AuNPs on a Au(111) substrate mediated by the 42-mer gold binding peptide sequence $[MHGKTQATSGTIQS]_3$.[353] After covering the Au(111) surface with a reactive self-assembled monolayer, the Au-binding peptide was patterned by microcontact printing and exposed to AuNPs, resulting in the formation of ordered arrays of AuNPs. Although the Au-binding sequence was originally envisioned to bind to macroscopic Au(111) surfaces,[307] the binding was apparently retained also for AuNPs, indicating that it is perhaps not specific for that surface. There was, however, no indication of the relative binding affinity of the peptide sequence for the two different forms of Au, and the experimental setup did not entirely rule out the possibility of nonspecific binding.

Dual-binding peptides are probably the most interesting application of NM−peptide binders because they could permit the facile conjugation of a macroscopic surface with specific NMs. For instance, peptide sequences binding to silica substrates (HPPMNASHPHMH) were tailed with sequences that

Table 6. Representative Peptide Sequences Selected for Binding to or Biomineralization of Nanoparticles

material	sequence
Au	MHGKTQATSGTIQS, SKTSLGCQKPLYMGREMRML, QATSEKLVRGMEGASLHPAKT;[306−308] VSGSSPDS, LKAHLPPSRLPS, TGTSVLIATPYV[309]
Ag	AYSSGAPPMPPF, NPSSLFRYLPSD, SLATQPPRTPPV;[310] IRPAIHIIPISH, WSWRSPTPHVVT[311]
Pt	DRTSTWR, QSVTSTK, SSSHLNK;[103,292] SLATQPPRTPPV, CPTSTGQAC, CTLHVSSYC[312]
Pd	QQSWPIS;[313] NFMSLPRLGHMH;[314] SVTQNKY; SPHPGPY; HAPTPML[103,292]
SiO_2	MSPHPHPRHHHT, RGRRRRLSCRLL, YSDQPTQSSQRP, TYHSSQLQRPPL, SPLSIAASSPWP;[296,315−317] RGRRRRLSCRLL;[315] RLNPPSQMDPPF, QTWPPPLWFSTS;[318] HPPMNASHPHMH, HTKHSHTSPPPL;[319] CHKKPSKSC[320]
ZnO	YDSRSMRPH, NTRMTARQHRSANHKSTQRA;[321] RIGHGRQIRKPL;[322,323] PASRVEKNGVRR, EAHVMHKVAPRP;[324] VRTRDDARTHRK;[325] GLHVMHLVAPPR[326]
Cu_2O	RHTDGLRRIAAR, RTRRQGGDVSRD, RPRRSAARGSEG[322]
$CaCO_3$	HTQNMRMYEPWFG, DVFSSFNLKHMRG
Cr_2O_3	VVRPKAATN, RIRHRLVGQ[327]
GaAs	AQNPSDNNTHT, RLELAIPLQGSG, TPPRPIQYNHTS[328]
InP	SVSVGMKPSPRP[329]
Fe_2O_3	RRTVKHHVN;[330] LSTVQTISPSNH[331]
ZnS, PbS, CdS	NNPMHQN;[332] QNPIHTH, CTYSRLHLC[333]
Al	VPSSGPQDTRTT, YSPDPRPWSSRY[334]
Ti/TiO_2	RKLPDAPGMHTW;[335,336] YPSAPPQWLTNT, STPLVTGTNNLM, QSGSHVTGDLRL, ATTLHPPRTSLP;[337] SCSDCLKSVDFIPSSLASS;[338] LNAAVPFTMAGS;[325] ATWVSPY;[339] RKKRTKNPTHKLGGGW, KSLSRHDHIHHHGGGW, TQHLSHPRYATKGGGW[340]
IrO_2	AGETQQAM[341]
GeO_2	TGHQSPGAYAAH, SLKMPHWPHLLP[340]
stainless steel	MTWDPSLASPRS;[325] ATIHDAFYSAPE, NLNPNTASAMHV[334]
FePt alloy	HNKHLPSTQPLA, SVSVGMKPSPRP,VISNHRESSRPL[342]

bound to AuNPs (MHGKTQATSGTIQS) or carbon nanotubes (HSSYWYAFNNKT), permitting some control over the patterning of these nanocomponents.[354] In a notable example, Kuang et al. have demonstrated the use of fusogenic peptides consisting of a SWCNT binding domain linked to a trinitrotoluene (TNT) binding domain. These sequences could be immobilized on a SWCNT and retained their affinity for TNT, as demonstrated by assembly of a field effect transistor sensor for TNT.[355] Cui et al. have also reported on the selection of peptides that bind to nanometer-sized strips of graphene and used a similar fusogenic peptide approach to create a TNT sensor.[356] The fact that peptides could be derived and used against such hydrophobic carbon allotrope materials suggests that similar approaches may indeed work for many other NMs. In the subsequent discussion, further examples are highlighted where peptide-driven recognition of NPs is used as a means of bioconjugation. Clearly, the strong interest in NP bioconjugation coupled to ongoing work in this area will help drive expanded use of this approach.

2.13. Oligonucleotide-Mediated Recognition and Nanoparticle Synthesis

Oligonucleotides have demonstrated potential for both recognizing and binding to inorganic NMs and functioning as mediators for the synthesis of NPs.[357−359] Similar to the polypeptide-mediated recognition discussed above, the presence of moieties with electron-donating properties in the oligonucleotide backbone and on the nucleobases allows for coordination of empty orbitals on the NM surface, resulting in strong bonding interactions in some cases.[360−363] The chemical diversity displayed in oligonucleotides is not as varied as that available to polypeptides; however, the ease, speed, and low cost of synthesis, the possibility of introducing modified nucleobases and other reactive appendages, and its self-recognition properties still make oligonucleotides attractive candidates for use in biomolecule-mediated recognition of inorganic NMs.[364] Although there are some reports on the exploration of oligonucleotide binding to NMs,[357,360,365,366] this field is still mostly focused on assisted synthesis of NMs, which is usually achieved by controlled precipitation of inorganic salts in the presence of the oligonucleotides.[367−369] Evidence indicates that both oligonucleotide sequence[359,370−374] and length[359,372] can influence the physicochemical properties of the resulting NPs; however, a definitive relationship linking the oligo sequence with the ensuing NM properties still has not been established. Thus, speculation on the role different nucleic acid moieties contribute to the synthetic outcome continues.[361,372,375]

Nanocrystals derived from Ag,[371,372,375−377] Cu,[378] Au,[367] CdS,[361,362,368,373,379,380] CuS,[369] PbS,[379] and Fe_2O_3[381] have all been assembled via oligonucleotide-mediated synthesis. More notable are the reported RNA-mediated synthesis of Pd[382−385] and IO nanocrystals,[386] where specific RNA sequences were suggested to facilitate the formation of crystals with specific shapes and morphologies. These findings were, however, considered somewhat controversial and are still being evaluated.[387−389]

Introduction of a phosphorothioate modification on the oligo backbone has been reported to facilitate the synthesis of semiconductor nanocrystals compared to a comparable sequence with a standard phosphodiester backbone.[374,390] This differential reactivity has been recently exploited to synthesize semiconductor nanocrystals passivated with oligonucleotides that still retained recognition properties and could be arranged in organized superstructures (*vide infra*).[370] This example also reflects one of the more desirable benefits of this approach: namely, synthesizing a defined NP that simultaneously displays DNA directly available for further utility. While oligonucleotide-mediated synthesis and recognition of an inorganic NM is an intriguing and potentially useful concept, this field is still in its infancy. The focus remains on synthesis of NMs where desirable physicochemical properties are tuned by the selection of sequence, and success in this endeavor will be clearly predicated on a full understanding of the link between specific sequence properties and structural outcomes.

2.14. Scope of the Text

The majority of the text will focus on reviewing available bioconjugation chemistries by NP type or specific NM. A brief summary of each NP, NM, or nanostructure's unique nanoscale, photophysical, chemical, or quantum confined properties of interest will be presented so as to highlight the motivation behind developing bioconjugates with that particular material. Some information is also provided on synthesis, where applicable, because this, in many cases, is an important factor in the subsequent bioconjugation process. NP material groupings do not strictly follow the periodic table or the chemistry of their nanoparticulate form but are rather based more on a theme of common functionality. For example, if an element is most commonly used in semiconductor QDs such as Te, then that is where it will be covered rather than in its more rigorous classification of metalloid or metal oxide. There are also cases of overlap between materials in different sections. Some metals are covered in the oxide section while others are found with the transition or rare earth metals, although for biological applications, both are usually utilized as oxides. Our intention is to keep elements used within a similar biological role or context together. Again using the example of semiconductor QDs, rather than doing a strict parsing based on semiconductor classifications such as III–V, II–VI, or group IV, we describe most semiconductor QDs (III–V, II–VI) together while providing separate sections for silicon and germanium(IV) and silicon dioxide NP materials.

For each type of NP or NM, we use representative examples from the literature to review the conjugation chemistries available to that material, the types of biological molecules that have been conjugated, any necessary modifications of those biomolecules, and any important characteristics of the final conjugates. The benefits and liabilities of each approach will be discussed, including the role of the bioconjugation chemistry in determining the effectiveness of the conjugates in their intended application. The reader will also be referred to any pertinent reviews on specific types of chemistry or bioapplications of a particular NP material, if available. For materials where functionalization is mostly accomplished by direct surface interactions, we focus predominantly on this aspect. Where multiple routes have been taken, we extend our discussion to different classes of biologicals (peptides, DNA, etc.) within each route as applicable. For well-established and well-characterized materials (e.g., dendrimers or lipid NPs in drug delivery), we must necessarily limit ourselves to a representative overview. We also focus predominantly on NP bioconjugation chemistry instead of biotemplated growth of NMs (i.e., biomineralization), although there are many similar interfacial interactions in common. The exception is where biotemplated growth is incorporated directly as part of the subsequent bioconjugation strategy.[104,105,359,391,392] Overall, this review aims to imbue the reader with knowledge of the types of bioconjugation chemistries that can be used to biofunctionalize a particular NP or NM and the important considerations for each combination of NP or NM and bioconjugation chemistry and also provide an overview of what has been accomplished in this field as a whole to this point, along with insight into new and developing methods. The use of chemistries originally developed for labeling proteins (i.e., EDC or reactive NHS esters and maleimides) are sometimes referred to as *classical* or *standard* bioconjugation chemistries for the purpose of differentiation from recent developments. Because we know of no other predecessor reviews, the publications covered herein extend back over several years as warranted. Given the vast breadth and complexity of this field, we are unable to cover all materials and chemistries or mention all pertinent examples to date; our apologies are extended for any and all omissions.

3. NANOPARTICLE BIOCONJUGATION CHEMISTRIES

3.1. Noble Metal Nanoparticles

3.1.1. Gold and Silver Nanoparticles. The noble metals are elements characterized by an electron configuration that imparts a certain chemical inertia and resistance to corrosion and oxidation. Elements considered to belong to the noble metal family include rhenium, ruthenium, rhodium, palladium, silver, osmium, iridium, platinum, and gold. However, in this section we restrict ourselves to palladium (Pd), platinum (Pt), silver (Ag) and, most prominently, gold (Au). Colloidal AuNPs, perhaps the first man-made NM, are also one of the most thoroughly studied and applied. The use of colloidal gold as a colorant dates back to the 4th or 5th century B.C. and continues even today. The earliest scientific accounts of colloidal AuNPs were written in the 1600s, and highlighted preparation and medicinal uses through the Middle Ages, including a test for syphilis (see ref 20 for an interesting historical account). The paradigm shift to the more contemporary view of colloidal AuNPs is often attributed to Faraday in 1857,[393] and the development of Mie theory in 1908.[394] In modern medicine, treatment of rheumatoid arthritis with colloidal Au dates back to the 1920s.[73,395] The 1970s also saw colloidal gold emerge as an immunostaining and contrast agent for electron microscopy.[396,397] It was, however, advances in synthetic methods and characterization tools in the 1990s that laid the foundation for 21st century materials and applications that now extend far beyond AuNPs. It is an interesting parallel with the Middle Ages that our current, and infinitely greater, understanding of the properties of AuNPs continues to lead to new diagnostic and therapeutic applications. Many of these applications are predicated on the ability to reliably bioconjugate AuNPs.

3.1.1.1. Properties and Applications of Gold and Silver Nanoparticles. AuNPs exhibit a strong localized surface plasmon resonance (LSPR) that results from the collective coherent oscillation of conduction band electrons across the NP upon interaction with light at a specific resonant wavelength. As shown in Figure 12, the frequency (or wavelength) of the LSPR depends on the size and shape of the particle, as well as the dielectric properties of the medium surrounding the NP. Spherical AuNPs are often characterized by a bright red or purple color (see Figure 12A) and an absorption maximum between 517 and 575 nm for particle diameters between ~9 and 99 nm (see Figure 12B).[20] Particle sizes <2 nm do not exhibit an LSPR band (*vide infra*). While the LSPR in spherical AuNPs has only a weak size dependence, the LSPR is very sensitive to NP anisotropy. For example, rod-shaped AuNPs, or "nanorods", are characterized by two LSPR bands: a transverse oscillation with a visible resonance that corresponds to that of a sphere with analogous dimensionality and a longitudinal oscillation, which exhibits a NIR resonance and shifts to longer wavelengths and stronger intensities as the aspect ratio increases (Figure 12C).[398] More exotic shapes, such as various polyhedra, plates, and hollow "nanoshell" structures, can also be prepared and demonstrate interesting optical properties.[399] The latter, for example, exhibit a strong LSPR that depends on shell thickness. Useful catalytic effects have also been demonstrated with AuNPs.[55,400]

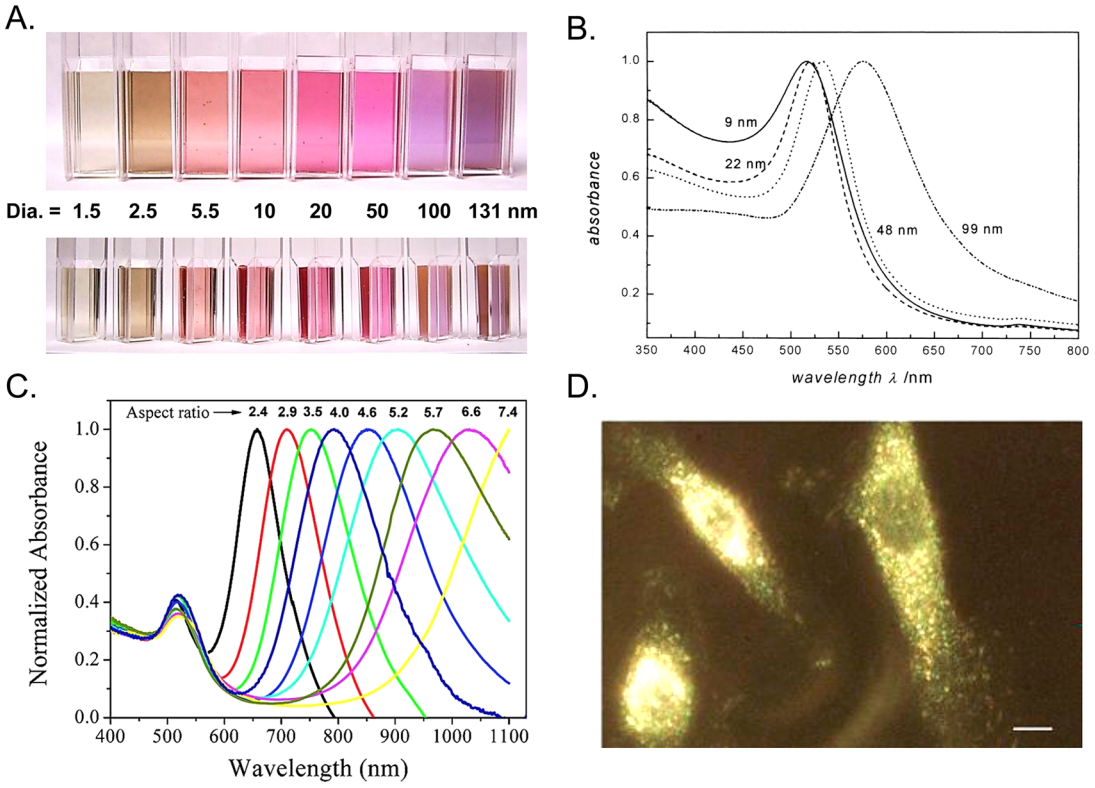

Figure 12. Optical characteristics of AuNPs. (A) Photograph of different sizes of PEG-stabilized AuNPs. Image provided by E. Oh (NRL). (B) LSPR frequency of 9, 22, 48, and 99 nm AuNPs. Figure reproduced from ref 2079. Copyright 1999 American Chemical Society. (C) The longitudinal mode LSPR frequency of Au nanorods with different aspect ratios. Figure reproduced from ref 2080. Copyright 2006 American Chemical Society. (D) Dark-field microscopy/light scattering images of human stem cell cancerous cells stained with antibody-conjugated AuNPs. The scale bar is 10 um. Figure reproduced from ref 2081. Copyright 2005 American Chemical Society.

The optical properties of AuNPs are useful in many bioapplications. For instance, the sensitivity of the LSPR to the surrounding dielectric medium has been exploited by using shifts in the LSPR band or the corresponding color change as an analytical signal. This utility is epitomized by the seminal work of Elghanian et al. on the use of AuNPs for ultrasensitive DNA detection through hybridization-driven aggregation,[401] although this general format has been adapted to the colorimetric detection of several other bioanalytes.[402] Alternatively, AuNPs also exhibit efficient, proximity-dependent fluorescence quenching, and this has been widely used to develop assays and probes that generate "on/off" signaling.[195,403−408] Due to the local electric field enhancement from the LSPR, AuNPs are also potentially useful substrates in surface-enhanced Raman spectroscopy (SERS)-based assays,[398] although AgNPs are often preferred. In imaging applications, the strong light scattering by AuNPs approaching 100 nm in size can provide contrast in dark-field microscopy that is comparable to or even better than fluorescence microscopy, see Figure 12D.[409,410] AuNPs in the 10−50 nm size range are more strongly absorbing than most molecular dyes and have negligible quantum yields. The fast thermalization of absorbed light energy, combined with the NIR resonance of Au nanorods and nanoshells, is ideal for photothermal therapy.[409]

Analogous to their Au counterparts, AgNPs exhibit a strong LSPR; however, the resonance is stronger, narrower, and shifted to shorter wavelengths than that of AuNPs (ca. 400−525 nm for dimensions ca. 10−100 nm). In addition, AgNPs are generally considered to be superior substrates for SERS, providing larger enhancements when compared with Au, Cu, and other metals.[411,412] AgNPs also have interesting and potent antimicrobial properties.[413] Aside from this latter property, the utility of AgNPs in biological applications largely mirrors that of AuNPs. Their application, however, has been limited by more challenging synthesis and susceptibility to oxidation[414] (Au does not form surface oxides[96]), which can hinder access to the potentially superior optical properties of AgNPs.

3.1.1.2. Synthesis. AuNPs are typically synthesized by the reduction of Au(III) precursors in the presence of a stabilizing ligand. The most popular methods are based on those of Turkevitch and Frens, wherein citrate is used as both a reductant for $HAuCl_4$ and a stabilizer for NPs in aqueous solution.[415−417] NPs with sizes between ca. 10 and 100 nm can be prepared using this general approach. A recent variation using a hydroquinone as a selective reductant for the further growth of citrate-stabilized AuNP seeds produced larger NPs (50−200 nm) with better monodispersity compared with citrate reduction alone.[418] Another popular method is the Brust−Schiffrin two-phase approach,[419] and variations thereof.[420] In this approach, AuNPs ca. 1−6 nm in diameter can be synthesized using an aqueous reductant (e.g., $NaBH_4$), phase transfer reagents such as tetralkylammonium salts (e.g., $[CH_3(CH_2)_7]_4NBr$), and a stabilizing thiolate ligand. Brust et al. also devised a similar one-phase approach in methanol using a thiolate stabilizer.[421] Both of these general strategies are still widely used, and new mechanistic insights into the underlying chemistry are even being gleaned today.[422] For example, in a recent variation of the one-phase method, a PEGylated disulfide stabilizing ligand was shown to yield a much broader size range of AuNPs (1.5−18 nm).[423] A large number of other bottom-up, wet chemical synthetic methods have also been developed, and primarily vary

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in the choice of reductant or stabilizing ligands. In addition to thiols, other common ligands used in variations of the Brust method include amines, carboxylates, phosphines, and phosphine oxides.[20] Further, different polymers[424] such as PEI[130] and polyvinylpyrrolidone (PVP),[425] dendrimers such as PAMAM,[426] surfactants (e.g., cetyltrimethylammonium bromide, CTAB[427]), ionic liquids (e.g., imidazolium chloride derivative[428]), and even biomolecules (e.g., BSA[429]) have been used to stabilize AuNPs during synthesis. Methods for shape control are reviewed elsewhere and rely on manipulation of the growth conditions, the use of seed particles, or the selection of surfactants or stabilizing ligands.[399,430] The biosynthesis of AuNPs has also been recently reviewed.[431]

AgNPs are typically synthesized by reduction of silver salts (e.g., $AgNO_3$). One common strategy is the use of the Lee–Meisel citrate reduction[432] (an analog of the Turkevitch AuNP method) or variations thereof. Another popular strategy is the polyol thermal synthesis that relies on a polymeric stabilizer (e.g., PVP) and ethylene glycol as both solvent and source of reductant.[425] Good control over both size and shape is possible. The polyol methodology can be used to synthesize other noble metal NP materials (e.g., Au, Pd, Pt, FePt) as well. Monodisperse AgNPs of reproducible size and shape with good optical properties have generally been more difficult to synthesize than AuNPs,[433] but high-quality AgNPs are gradually becoming more facile to synthesize.

3.1.1.3. Bioconjugation Strategies. Although AuNPs are perhaps the most studied, best characterized, and most widely applied NP material to date, the bioconjugation chemistries are actually quite limited and are primarily driven by the NP surface itself, the stabilizing ligand, or some functional intermediary. Given this, we focus in this section more on the bioconjugation chemistry rather than the type of bioconjugate. Analogous to virtually all NP materials, one strategy for the bioconjugation of Au and AgNPs is to utilize specific chemical handles introduced by the coating of stabilizing ligands or polymer. This chemistry is almost always enabled by the formation of an intermediate monolayer coating of bifunctional thiol ligands on the surface of the Au or AgNP. A second strategy is to bind thiol-terminated biomolecules directly to the AuNPs. A third and common strategy, the adsorption of biomolecules on hydrophilic NPs, is also discussed. We first focus on the Au–thiol and Ag–thiol interactions and address these three general bioconjugate strategies thereafter.

3.1.1.3.1. Thiolate Chemisorption on Gold (and Other Noble Metals). Because this interaction forms the first and most critical step in bioconjugation of this class of NP materials, the underlying chemistry is briefly discussed. A variety of organosulfur compounds spontaneously form monolayers on noble metal substrates. These include but are not necessarily limited to alkyl thiols, dialkyl disulfides, dialkyl sulfides, alkylxanthates, and dialkylthiocarbamates.[434] In NM-related applications, the use of alkyl thiols and dialkyl disulfides predominates. Both of these two organosulfur compounds chemisorb to Au as gold(I)-thiolates.[96,434] In the case of thiols, chemisorption likely occurs with reductive elimination of the thiol hydrogen as either H_2 or, with subsequent oxidation, as H_2O. In the case of disulfides, chemisorption is thought to occur with S–S bond cleavage. The Au–S homolytic bond strength is estimated at ca. 40–50 kcal/mol.[96,434] Monolayers of alkyl thiols deposited on clean, planar Au substrates have been extensively characterized, and readers are referred to two excellent reviews.[96,434] Figure 13 provides a descriptive schematic of a self-assembled monolayer (SAM) of thiolates on a Au surface. Thiols will also chemisorb on Ag surfaces as a thiolate.

In the context of Ag and AuNPs, thiols are usually only used as a stabilizing ligand in the synthesis of small sized NPs (<5 nm) since their strong affinity prevents growth to larger particle sizes. The use of bifunctional ligands (e.g., thioalkyl acids or thioalkyl amines) can enable further modification chemistry through the distal functionality. Larger particles synthesized with, for example, alkyl phosphines or surfactants such as CTAB do not afford this capability, see Table 7. Citrate ligands, although suitable for bioconjugation, are also less than ideal due to their weak binding to the NM surface. Fortunately, these synthetic ligands can be displaced by thiol ligands without disrupting NP structure or function.[96,435] The distal functional group of the thiol or disulfide derivative can be specifically chosen to impart aqueous solubility or a chemical handle for bioconjugation, and multiple functionalities can be introduced using mixed compositions of ligands. While the process of forming thiol monolayer protected AuNPs is, to a first approximation, analogous to that on planar Au substrates, the curvature of the NP structure does create important differences.[96] The outermost part of the thiolate ligand layer is more loosely packed since the distal portion of the ligand structure cannot occupy the volume of a cone defined by its point of attachment to the surface of the NP. As the NP size increases, the properties of the thiolate ligand layer approach those of a planar SAM. For a AuNP grown as a truncated octahedron beyond a diameter of ca. 4.4 nm, most of the NP surface atoms comprise flat terraces, rather than edges and vertices.[96] Here, the thiolate ligands are well packed, akin to a planar SAM, albeit with disorder remaining at edges and vertices between facets.[96]

Undoubtedly, thiolate chemisorption on Au, whether at a bulk or NP substrate, is the most studied and thoroughly characterized thiolate interaction among noble metals. However, significant study has also been done on thiolate chemisorption on planar substrates of Ag, Pd, and Pt.[96,434] These studies, and parallels drawn with thiolate–AuNP systems, offer a useful framework for the preparation and application of thiolate-coated Ag-, Pt-, and PdNPs.

3.1.1.3.2. Covalent Bioconjugation to Surface Coatings. We first consider bioconjugation to the ligand or polymeric coatings of Au and AgNPs. Here, the noble metal core of the NP is a spectator in bioconjugation; its role is to support the organic coating. With very few exceptions, heterobifunctional thiol ligands are chemisorbed to the Au- and AgNPs exclusively via the thiol group. Polymer coatings, which are less common, can associate with the NP through a variety of functional groups and coordination mechanisms. Although there is a trend toward the use of a larger variety of chemoselective and bioorthogonal conjugate chemistries,[14] many methods tend to directly or indirectly utilize more standard bioconjugation protocols (e.g., carbodiimide- or maleimide-mediated coupling) that rely on the most common NP coatings that display available amine or carboxyl groups. However, as many of the following examples will illustrate, it is often preferable to decouple NP solubility and reactivity. Thioalkyl acids or thioalkyl amines have frequently given way to the use of amino-PEG or carboxy-PEG ligands or mixed coatings that also incorporate an unreactive PEG ligand. In either case, the PEG mediates the solubility and dispersion of the NP.

Carbodiimide coupling is perhaps the easiest and most common approach to preparing Au- and AgNP bioconjugates. One possible strategy starts by coating the AuNPs with thioalkyl amines. For example, Tsai et al. prepared AuNPs

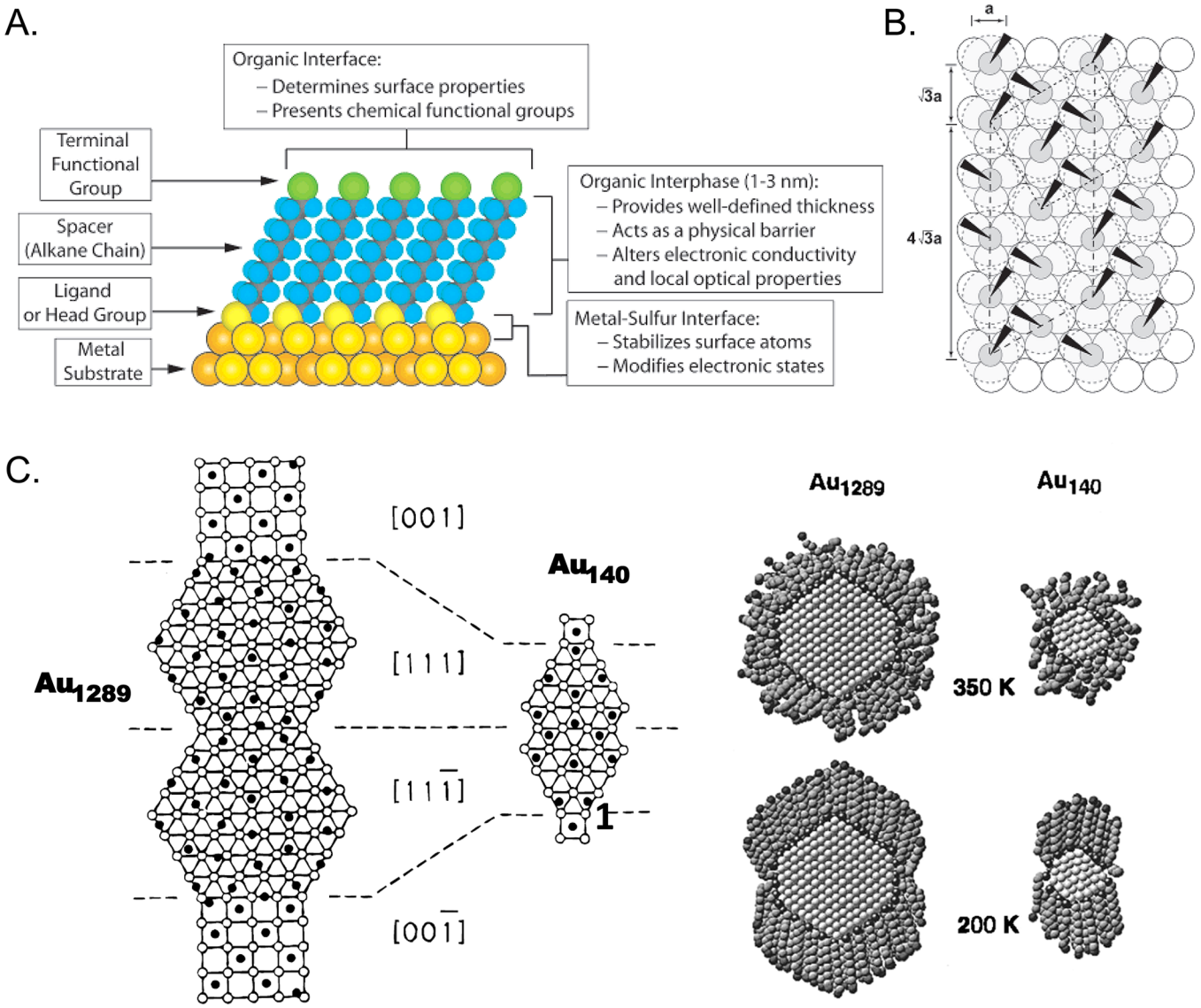

Figure 13. Thiolate self-assembled monolayers (SAMs) on Au. (A) Schematic illustration of a bifunctional alkylthiolate SAM on a planar Au (or other noble metal) surface. (B) Geometric configuration of alkylthiolate ligands on Au(111). (C) Arrangement of thiolate ligands on Au_{1289} and Au_{140} NPs. Cross sections are shown on the right, and "unfolded" NPs are shown on the left (the open circles represent Au atoms, and the closed circles represent sulfur atoms). Figure adapted from ref 96. Copyright 2005 American Chemical Society.

stabilized with citrate and CTAB, exchanged these ligands with 2-mercaptoethylamine, and coupled folic acid (FA, vitamin B9) to the distal amine via EDC activation of the former.[436] In another example, AuNPs were modified with PAMAM dendrimer and conjugated with NHS-activated biotin.[195] However, a more common strategy is to prepare AuNPs with thioalkyl acid ligands. Recent examples include tiopronin-coated AuNPs that were conjugated to DOX using EDC activation[437] and 4-mercaptobenzoic acid-coated AuNPs that were conjugated with the Fe-binding transporter transferrin (Tf) using EDC/NHS activation.[438] Because this protein's normal function is to transport Fe intracellularly, it is often attached to different types of NPs to facilitate intracellular uptake. Cell-penetrating TAT peptides have been coupled to tiopronin-coated AuNPs analogously.[439] However, given the potential liabilities of EDC activation,[14] as well as the limited colloidal stability of thioalkyl acid coated NPs in acidic, high salt, or complex media, several groups have adopted alternative approaches. As mentioned, one common approach to improve the colloidal stability of the conjugate is the use of a mixed monolayer. For example, Zheng et al. prepared AuNPs with a mixed monolayer of PEG–thiolate and tiopronin; the latter was then EDC-activated and coupled with commercially available biotin–PEG–amine.[440] As a further alternative, several groups have used bifunctional carboxy-PEG–alkylthiolate ligands and EDC/NHS activation to conjugate, for example, immunoglobulin G (IgG),[441] single-chain variable fragments (scFv),[442] and peptides (see Figure 14).[443,444] Skewis et al. adopted a particularly unique approach to conjugating AgNPs with antibodies, completing all manipulations within a 1% agarose matrix, therefore bypassing potential aggregation issues that arise during modification.[445] For this, commercial AgNPs were modified with carboxy-PEG–alkylthiolate but could not be reliably purified by centrifugation. Instead, the NPs were then EDC/NHS-activated and conjugated to antibodies within the gel matrix. The conjugates were then recovered by electroelution and used to label A431 cell membranes for dark field microscopy.[445]

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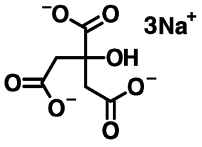
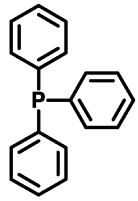
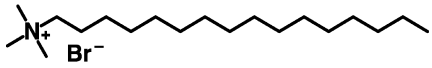
Table 7. Selected Examples of AuNP Stabilizing and Capping Agents

Non-thiol stabilizing ligands

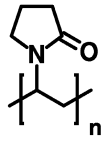
Sodium citrate 

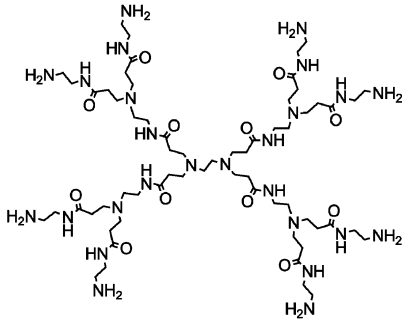
Phosphines, PR_3 (*e.g.* R = Ph) 

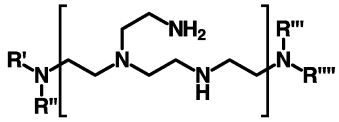
Tetraalkylammonium bromides, $Br^- R_3N^+(CH)_nCH_3$ (*e.g.* R= CH_3, n = 15; CTAB)



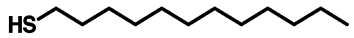
Polymer stabilizers

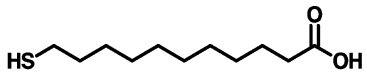
Poly(vinyl pyrrolidone) 

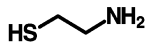
Poly(amido amine) (G2) [far right] 

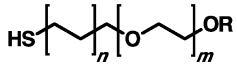
Polyethyleneimine 

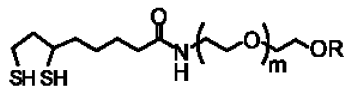
Thiol ligands

Alkylthiols, $HS(CH_2)_nCH_3$ (*e.g.* n = 11) 

Thioalkyl acids, $HS(CH_2)_nCOOH$ (*e.g.* n = 10) 

Thioalkyl amines, $HS(CH_2)_nNH_2$ (*e.g.* n = 2) 

Thioalkyl-PEG-R (R = H, CH_3, CH_2COOH, NH_2) 



In addition to EDC, other common cross-linkers can be used to bioconjugate AuNPs with multifunctional ligands. As illustrated in Figure 15, Kim et al. used SMCC to couple cRDGyC (c = cyclic) peptides to AuNPs coated with an amino-PEG−thiolate ligand.[446] The AuNP was also directly labeled with chemisorbed ^{125}I. The AuNP−cRDGyC conjugates, which were stable from pH 2 to 8 and at salt concentrations ≤1 M, selectively targeted and were taken up by tumor cells through integrin $\alpha_v\beta_3$ receptor-mediated endocytosis without appreciable cytotoxicity. The multivalent display of the cRDGyC peptides on the AuNP resulted in a 150-fold greater avidity for $\alpha_v\beta_3$ integrin compared with cRDGyC peptide alone. The conjugates were used as a tumor contrast agent in single-photon emission computed tomography (SPECT) and computed tomography (CT) imaging imaging of a mouse model.[446] AuNPs have been similarly conjugated with conantokin-G peptide to target *N*-methyl-D-aspartate receptors; here, a PEGylated SMCC ester analog was used with a mixed film of carboxy-PEG−thiolate and amino-PEG−thiolate.[447] Rather than adopting a cross-linker or displacing the surface stabilizing ligand, Oh et al. synthesized a maleimido-PEG derivative of thioctic acid that could be incorporated into a mixed surface with an unreactive PEG derivative.[448] The resulting AuNPs were colloidally stable and could be coupled with different numbers of cysteine-terminated peptides (see Figure 15B) by controlling the mole fraction of maleimido-PEG in the mixed coating. This strategy also suggests itself for coupling to bulky thiolated proteins, for example, where the PEG ligands would remain on the NP surface providing colloidal stability. Additionally, attaching thiolated DNA to the AuNP in this manner obviates the need for a long linker on the DNA to extend it out from the PEG layer and make it fully available for subsequent function.

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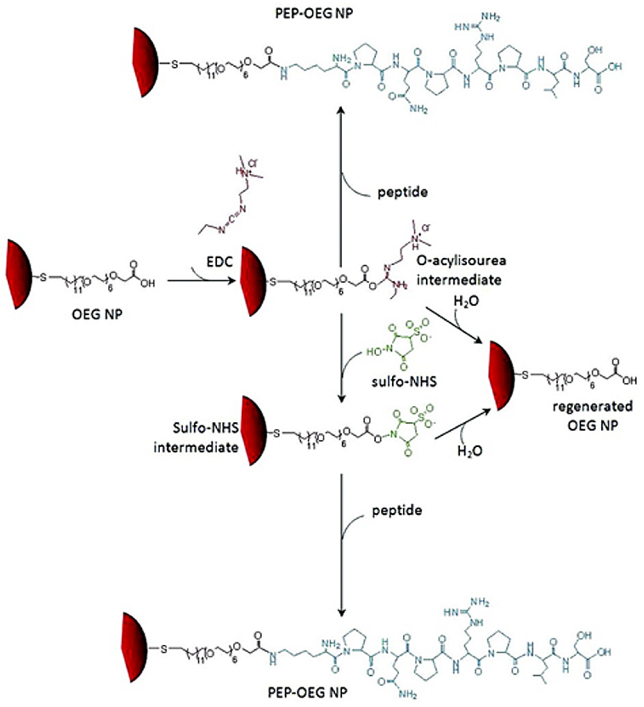

Figure 14. EDC/NHS coupling of peptides (PEP, shown in blue) to AuNPs coated with carboxy-PEG−thiolate ligands, illustrating reactive intermediates and competing hydrolysis pathways (OEG = oligo(ethylene glycol)). Figure reproduced from ref 443. Copyright 2011 American Chemical Society.

Chemoselective ligation chemistries have also been adopted with AuNPs. For example, hydrazone ligation is often used to prepare acid-labile DOX conjugates with a variety of NP materials, and AuNPs are no exception. Aryal et al. prepared 23% w/w DOX-modified AuNPs using a mixed coating of PEG−thiolate and methyl thioglycolate.[449] The methyl ester was reacted with hydrazine to give the corresponding hydrazide, which was coupled to the DOX keto group using a hydrazone linkage. DOX that was intracellularly released by these NPs accumulated in the perinuclear/nuclear region, analogous to unconjugated DOX.[449] Multifunctional AuNPs have also been prepared starting from an aminoethane thiolate coating using the multistep conjugation strategy illustrated in Figure 16.[450] The distal amine of the ligand was reacted with an *N*-carboxyanhydride derivative of *β*-benzyl-L-aspartate to yield a protected polyaspartate sequence, the N-terminus of which was coupled to carboxy-PEG using DCC (dicyclohexylcarbodiimide)/NHS activation. The distal hydroxy terminus of the PEG was further modified with FA using a Steglich esterification. DOX was then conjugated via a hydrazone bond after reaction of the benzyl ester-protected polyaspartate side chains with hydrazine. When applied to a mouse mammary carcinoma cell line, the conjugated FA was able to induce higher NP uptake, resulting in greater cytotoxicity by the DOX.[450]

Given the growing interest in the control offered by bioorthogonal conjugation chemistries,[14] it is not surprising that the CuAAC reaction has been extensively applied to AuNPs with azido ligands. For example, AuNPs were modified with a bis-azide derivative of GA, which was then clicked with DNA incorporating alkynyl nucleotide analogs.[451] Brennan et al. clicked AuNPs coated with azido-PEG−thiolate to a recombinant lipase protein (see Figure 17), which was modified with 4-pentynoic acid (via EDC) at its only solvent-accessible lysine residue.[452] Similarly, Gole and Murphy used CuAAC

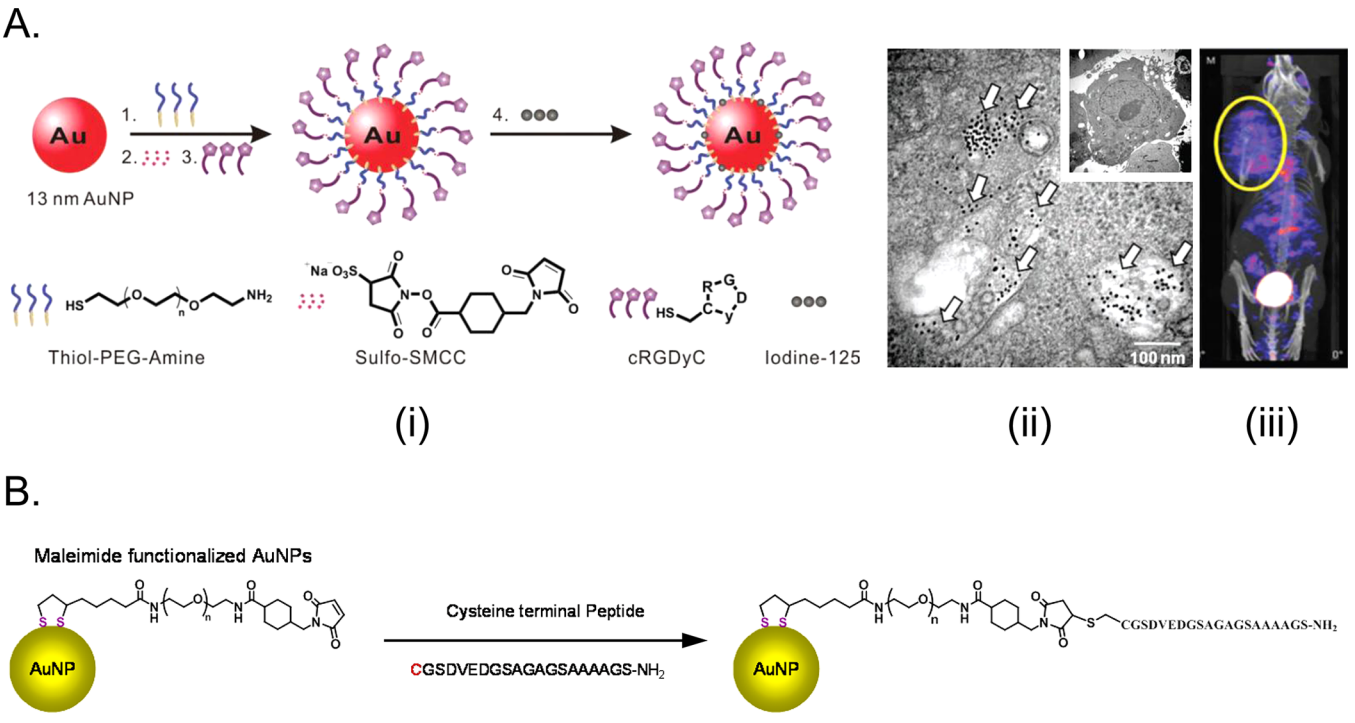

Figure 15. Bioconjugation of AuNPs using maleimide activation. (A) (i) cRDGyC conjugation using sulfo-SMCC as a heterobifunctional cross-linker between the cysteine (C) residue and amino-PEG−thiolate ligands. (ii) TEM image of AuNP uptake in U87MG cells. The inset shows the whole cell. (iii) Small-animal SPECT/CT of ^{125}I−cRGDyC−AuNPs 5 h postinjection, showing tumor contrast. Figure adapted from ref 446 with permission. Copyright 2011 WILEY-VCH Verlag GmbH & Co. (B) Peptide conjugation using maleimide functionalized AuNPs (cofunctionalization with an unreactive PEG−thioctic acid derivative is omitted for clarity).

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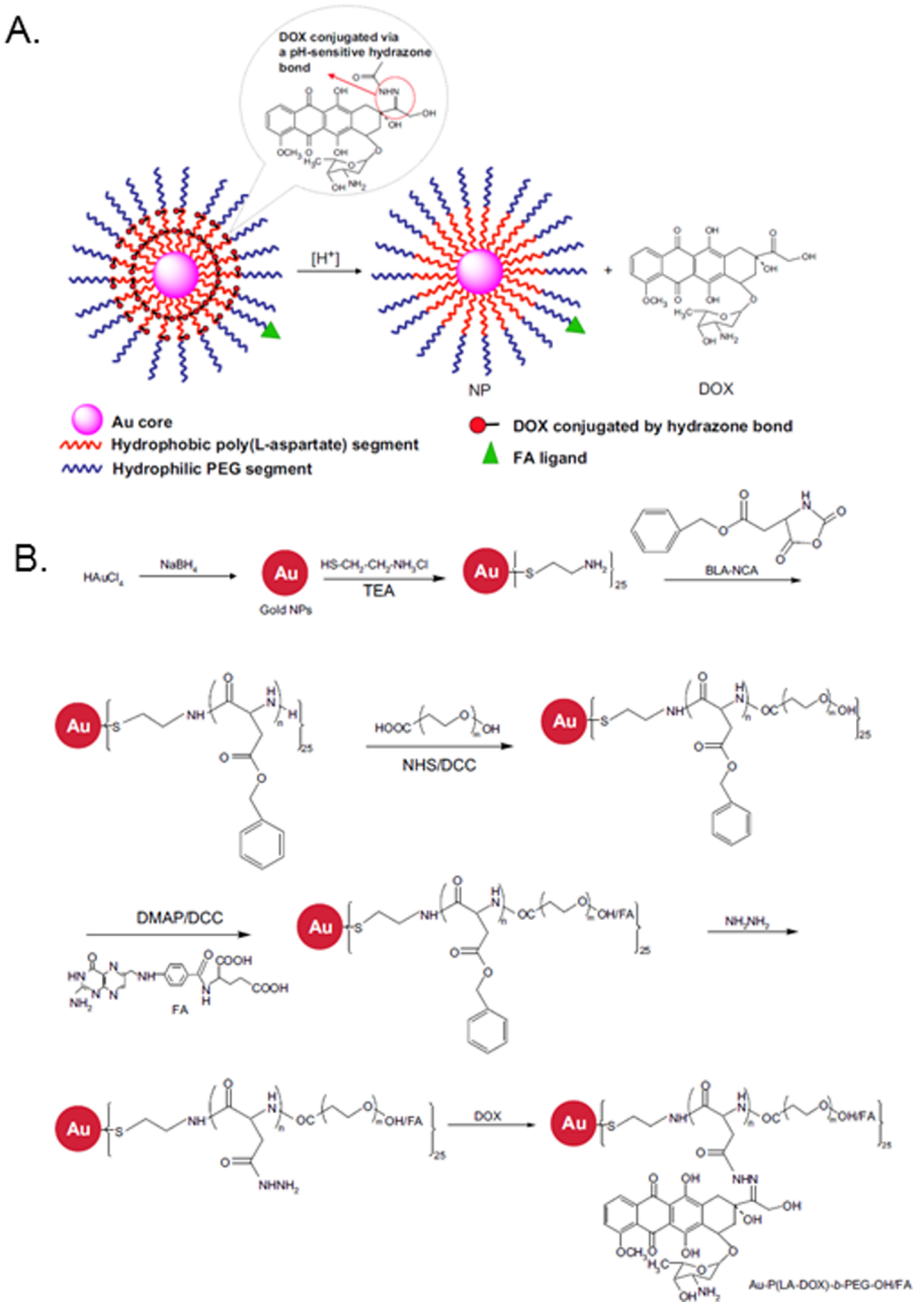

Figure 16. DOX conjugates for intracellular drug delivery using a pH-sensitive hydrazone bond. (A) Schematic representation of the AuNP vector. (B) *In situ* synthesis of an amphiphilic coating on AuNPs with subsequent DOX and folic acid conjugation. Starting from aminoethane thiolate ligands, the AuNPs were modified using *N*-carboxyanhydride ring-opening polymerization, carbodiimide/DCC coupling, Steglich esterification with folic acid, FA (DMAP = dimethylaminopyridine), hydrazinolysis, and chemoselective hydrazone ligation with DOX. Figure reproduced from ref 450, Copyright 2009, with permission from Elsevier.

to conjugate trypsin to Au nanorods coated with poly(4-styrenesulfonic acid-*co*-maleic acid).[453] Through EDC activation, the trypsin was modified with 4-pentynoic acid, and the NP was subsequently modified with amino-PEG−azide, as shown in Figure 17. The clicked trypsin retained 57% of its native activity, which was more than 3-fold better than trypsin conjugated to Au nanorods using EDC coupling or electrostatic adsorption.[453] Beyond ligand coatings, Zhang et al. used the CuAAC to modify AuNPs coated with an amphiphilic polymer.[454] These AuNPs were synthesized with didecyldimethylammonium bromide and dodecylamine as stabilizers, then coated with an amphiphilic polymer comprised of PAA modified with octylamine and 3-azidopropyl-1-amine. EDC activation was similarly used to label horseradish peroxidase (HRP) with 2-propynoic acid, which was then clicked to the AuNP while still retaining catalytic activity.[454]

In an elegant example of utilizing two chemoselective bioconjugate reactions, Kim et al. prepared AuNPs for protease sensing using a combination of intein-mediated ligation and click chemistry.[455] Starting with mixed monolayer PEG−thiolate and a bifunctional carboxy-PEG−thiolate, propargylamine was coupled to the AuNP through EDC activation. Separately, a bioluminescent luciferase protein was expressed with a C-terminal peptide substrate for matrix metalloproteinase-2 (MMP-2) and

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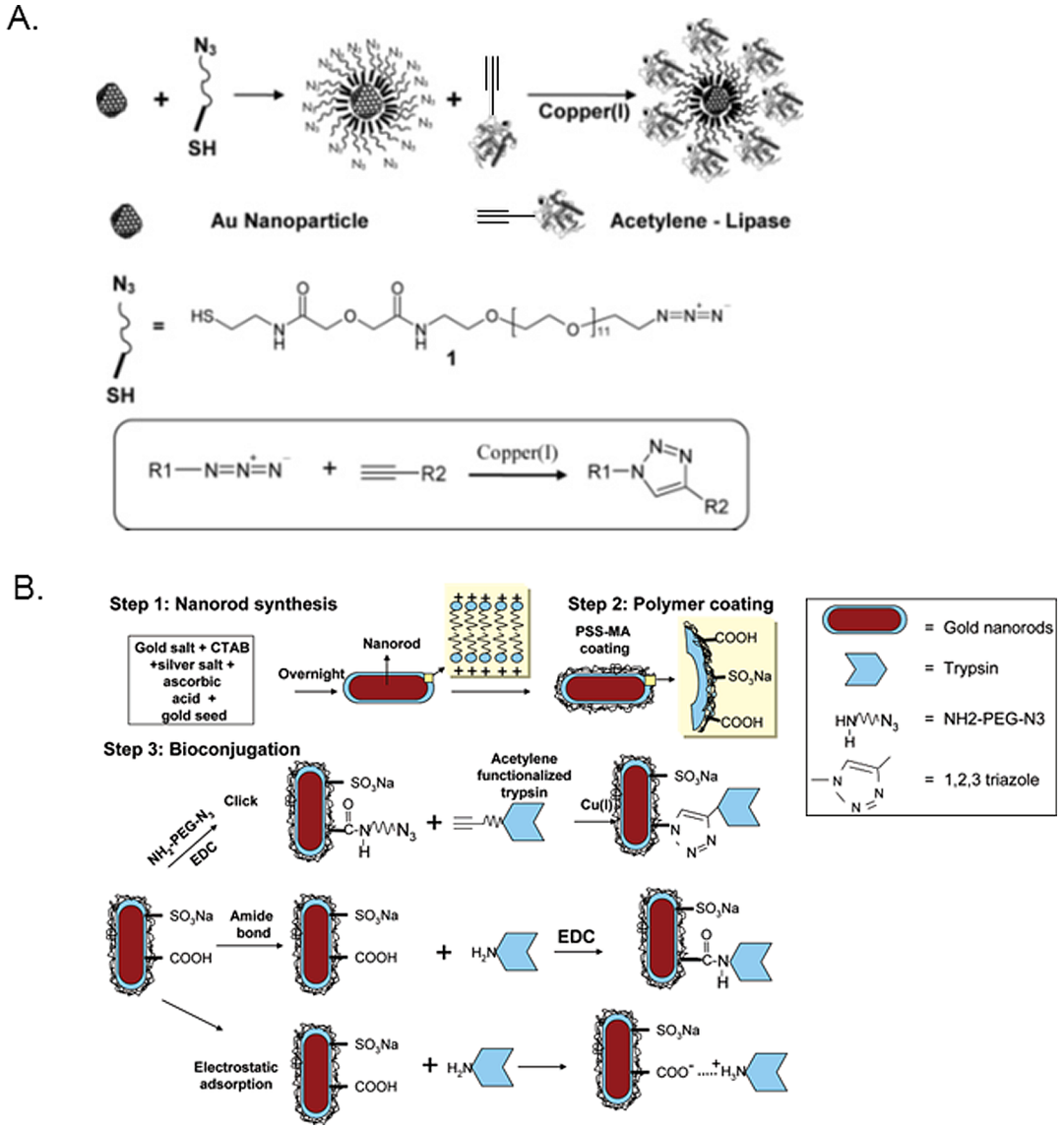

Figure 17. Conjugation of AuNPs with enzymes via CuAAC click chemistry. (A) Modification of AuNPs with azido-PEG−thiolate ligands and CuAAC click conjugation to lipase enzymes. Figure reproduced from ref 452. Copyright 2006 American Chemical Society. (B) Modification of Au nanorods with poly(4-styrenesulfonic acid-*co*-maleic acid) and conjugation with trypsin using three different strategies. The "click" route yielded the highest activity. Figure reproduced from ref 453. Copyright 2008 American Chemical Society.

a *Mex* GyrA intein. Intein-mediated ligation was then used to C-terminally couple an azide-cysteine derivative to the luciferase fusion protein. CuAAC completed the bioconjugation, and the resulting AuNPs were able to detect the hydrolytic activity associated with 50−1000 ng mL^{-1} of MMP-2 using the recovery of bioluminescence that had been quenched by the AuNP in the conjugate, see scheme in Figure 18.[455]

Finally, we note a noncovalent method for conjugating proteins and peptides to a specific organic moiety displayed on Au- and AgNPs: the association of polyhistidine tags with Ni(II)−NTA. Again, this strategy offers selective and oriented immobilization on the NP. In one recent example, citrate and tannic acid ligands from AuNP synthesis were exchanged with a bisNTA−disulfide and coordinated with Ni(II).[456] Human kinesin 1 was then fused with N-terminal polyhistidine and SA-binding peptide motifs; the latter was used for purification, while the former bound to the Ni(II)−NTA−AuNPs.[456] Alternatively, a carboxylate ligand such as thioctic acid can be modified with an amino derivative of NTA using EDC and then used to bind polyhistidine tagged proteins.[457] These two examples are illustrated in Figure 19.

3.1.1.3.3. Self-Assembly to the Inorganic Surface. When Au and AgNPs are coated with weakly bound ligands, it is usually possible to conjugate the NP with thiol-modified biomolecules via direct chemisorption to the inorganic surface. This methodology has the advantage of being spontaneous and occurring without activation or competing hydrolysis reactions and is both stable and broadly applicable: the biomolecule of interest need only be modified with a thiolated linker.

The Mirkin group's development of polyvalent AuNP−oligonucleotide conjugates is the most prominent example of this strategy. Much of this work, which is far too detailed to discuss here, has been recently reviewed.[458] Overall, a wide array of bioanalytical assay and probe technologies have been developed around combining the unique properties of AuNPs and the physicochemical characteristics of DNA. The enabling bioconjugate chemistry is the modification of citrate-stabilized AuNPs (~15 nm) with an excess of alkylthiol-terminated oligonucleotides

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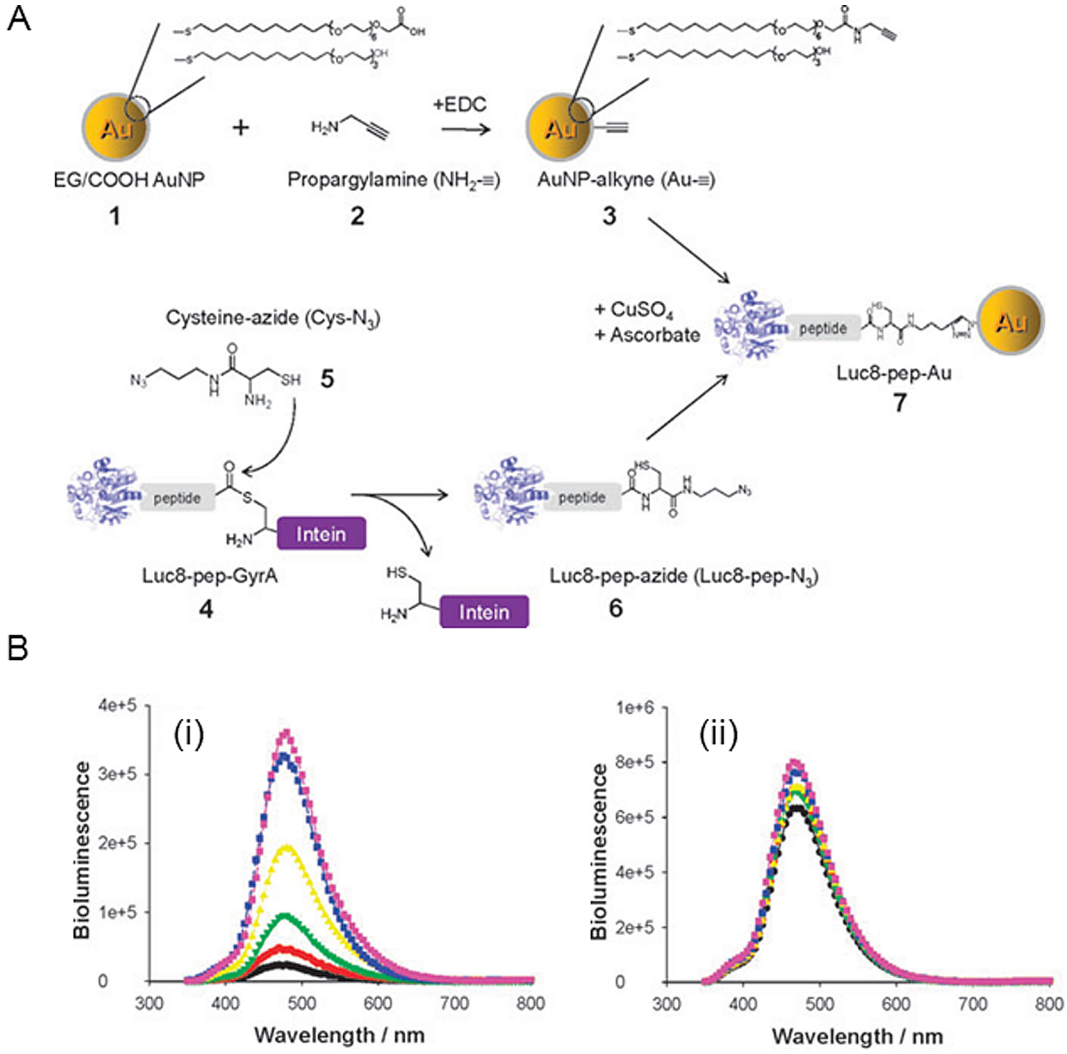

Figure 18. Luciferase–AuNP bioconjugates for protease sensing. (A) Bioconjugation of AuNPs with luciferase using a combination of intein-mediated ligation and CuAAC click chemistry. (B) Comparison of the bioluminescence recovery (from quenching induced by the proximal AuNP) with MMP-2 catalyzed hydrolysis of the peptide using (i) intein ligation and click chemistry and (ii) randomly coupling mediated by EDC. The former provided superior control and a more sensitive response. Figure reproduced with permission from ref 455. Copyright 2010 Royal Society of Chemistry.

at moderate ionic strength (ca. 0.1 M) to yield conjugates with ca. 50–100 oligonucleotides per NP.[459,460] Gradually increasing the ionic strength up to 1.0 M or "salt aging" can yield up to 250 oligonucleotides per NP.[461] Under these conditions, the maximum loading can be predicted based on NP size and a geometric footprint for the oligonucleotide.[460] The process is depicted in Figure 20A. Multivalent AuNP–oligonucleotide conjugates are useful in highly sensitive and selective DNA hybridization assays and as biobarcode assays for other analytes (see, for example, refs401 and 462–464). Indeed, these conjugates are part of a series of FDA-cleared *in vitro* diagnostic tests for warfarin metabolism, respiratory viruses, and some Gram-positive bacteria and are utilized within the Verigene system marketed by Nanosphere Inc. The Mirkin group has also found that multivalent AuNP–oligonucleotide conjugates have strong cellular uptake and resistance to nuclease activity and can be applied to monitoring intracellular gene regulation[465] and detection, see Figure 20B.[466]

Beyond oligonucleotides, thiol chemisorption on AuNPs has been applied to the conjugation of a variety of other biomolecules. For example, Thygesen used an oxime ligation to couple a heterobifunctional aminooxy-PEG linker with a terminal, trityl-protected thiol to the reducing end of glycans (e.g., glucose, maltose).[467] Following thiol deprotection, the thiol linker-modified glycans were chemisorbed to citrate-stabilized AuNPs. Alternatively, Kumar et al. periodate-oxidized the glycan chains of glycosylated antibodies to yield aldehyde groups at the nonbinding antibody F_c region, which were then coupled to a heterobifunctional hydrazido-PEG–dithiolate linker.[468] For applications, AuNPs were comodified with anti-actin and anti-biotin antibodies using this chemistry, coated with PEG–thiolate for added stability, and the anti-biotin antibody bound to biotinylated TAT–HA2 peptide. These conjugates provided four intrinsic functions: targeting (anti-actin), endosomal uptake (cationic TAT peptide), endosomal release (pH-sensitive HA2 peptide), and contrast (AuNP).[469] As shown in Figure 21, Choi et al. used a similar strategy and labeled Tf with an acetyl-protected mercapto-PEG–NHS linker; in the final step, the thiol was deprotected for assembly with AuNPs.[470] In an interesting alternate approach, Xiao et al. modified commercially available sulfo-NHS–AuNPs with N^6-(2-aminoethyl)-flavin adenine dinucleotide, which then bound to apo-glucose oxidase as its cofactor, forming a AuNP–glucose oxidase conjugate.[471]

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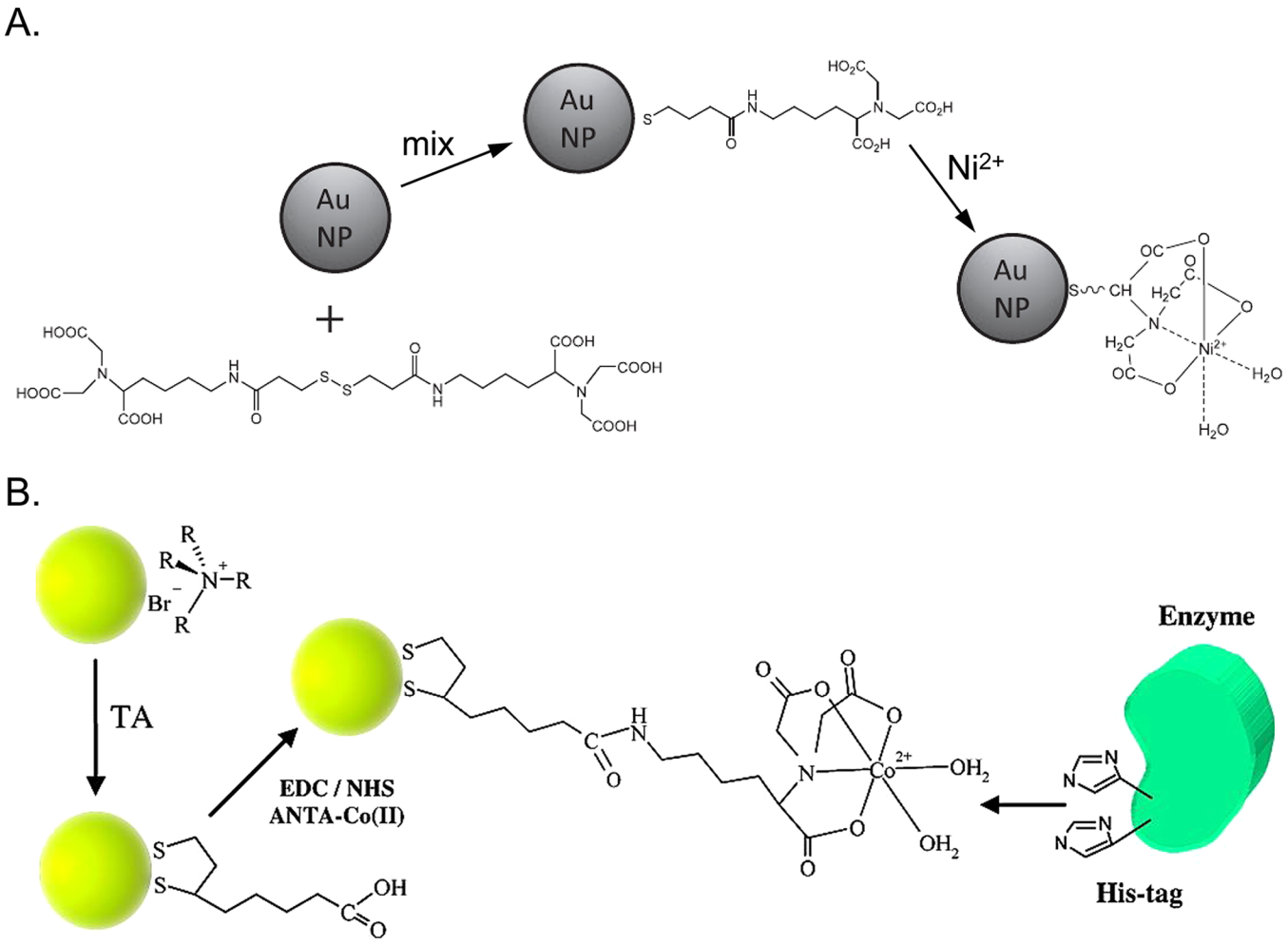

Figure 19. Bioconjugation to AuNPs using the affinity of polyhistidine toward Ni(II)−NTA. (A) Chemisorption of a bis-NTA−disulfide and activation with Ni(II). Figure adapted with permission from ref 456. Copyright 2011 The Japan Society of Applied Physics. (B) Modification of AuNPs with thioctic acid (TA) coupling to N_{α},N_{α}-bis(carboxymethyl)-L-lysine using EDC/NHS activation. Figure reproduced from ref 457. Copyright 2005 American Chemical Society.

Intrinsic or recombinantly engineered thiol groups can also be used to attach peptides and proteins to Au- or AgNPs. Citrate-stabilized AuNPs have been densely coated with CALNN peptides through the thiol side chain of the cysteine residue.[472] Chemisorbed antibody−AuNP conjugates can be prepared by the reduction of disulfide bridges at the hinge region of IgG to yield available thiol groups for assembly.[473] Further, Sandros et al. have assembled cysteine-rich metallothionein fusion proteins to AuNPs.[474] Site-directed cysteine introduction and metallothionein protein fusions are expected to be applicable to a diverse range of proteins for conjugation to Au- or AgNPs.

Interestingly, the Strouse group has found that His_n-tagged peptides and proteins can also self-assemble to 1.5 nm AuNPs stabilized with phosphine ligands or CAAKA peptides.[301,475] In the case of the latter, the ability to displace the gold−thiolate interaction was attributed to a multichelate effect.[475] While 1:1 mixing of a thiol-terminated peptide with AuNPs is expected to yield a distribution of NP valences (i.e., a mixture of zero, one, and two peptides per NP), polyhistidine tags may be suitable for preparing strictly monovalent populations of 1.5 nm AuNPs by using an excess of peptide (and subsequent purification).

In certain special cases, AuNP bioconjugates can be directly prepared during NP synthesis. For example, BSA has been used to stabilize Au- and AgNP growth.[429] Cationic disulfidyl peptides (KKC∼CKK) have also been used to template the growth of AgNPs from the reduction of $AgNO_3$ with ascorbate,[476] and such a method may be extendable to longer cationic peptides with cell-penetrating properties. Kemp et al. synthesized AuNPs stabilized with either hyaluronan or a 2,6-diaminopyridine derivative of heparin, both of which acted as simultaneous reducing agents and stabilizers.[477] The resulting NPs had anticoagulant and anti-inflammatory properties.

3.1.1.3.4. Adsorption. Nonspecific affinity between AuNPs and proteins is well-known.[478] The adsorption of proteins and other biomolecules on Au- and AgNPs can be mechanistically quite complex. Intrinsic amine, carboxyl, hydroxyl, imidazole, phosphate, and thiol groups, etc., all of which have different degrees of binding affinity with inorganic Au and Ag surfaces, may be found in abundance depending on the biomolecule of interest. The organic coating on the NP will determine the relative availability or access to the NP surface, and it will also reciprocate polar functional groups, potentially supporting an array of electrostatic, hydrogen bonding, and van der Waals interactions. The desolvation and association of hydrophobic surfaces with the NP can provide an additional entropic driving force for adsorption. In many cases, the adsorption of native biomolecules on Au- and AgNPs can therefore be strictly

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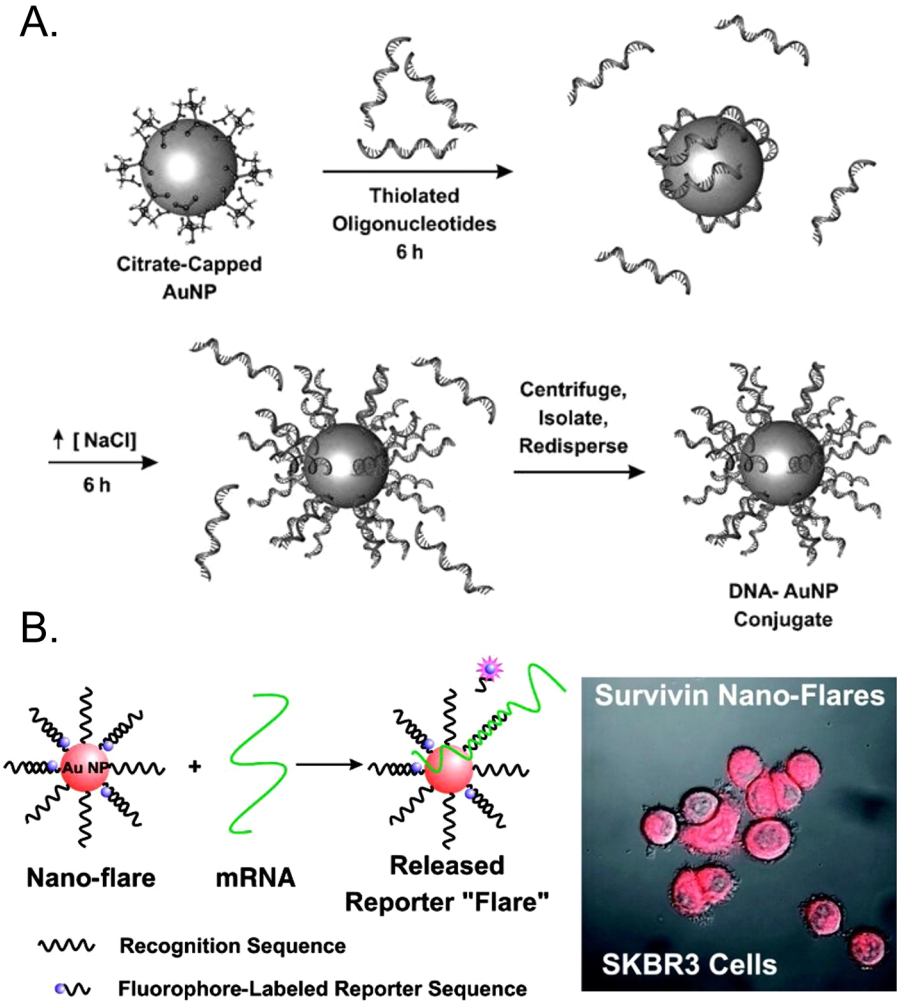

Figure 20. Multivalent AuNP−oligonucleotide conjugates. (A) Preparation via thiolate self-assembly on the AuNP. Figure reproduced from ref 458 with permission. Copyright 2010 WILEY-VCH Verlag GmbH & Co. (B) "Nano-flare" concept applied to intracellular mRNA detection. The nanoflares enter cells, and hybridization with complementary mRNA displaces a partially complementary fluorophore-labeled "flare" oligonucleotide. Release of the "flare" from the AuNP restores its fluorescence, resulting in a light-up signal. Figure reproduced from ref 466. Copyright 2007 American Chemical Society.

defined as neither chemisorption to the inorganic NP surface nor physisorption to the organic coating of the NP (although adsorption to AuNPs with a dense coating of thiolate ligands will often be dominated by the latter). We thus loosely define adsorptive methods as bioconjugation without any special modification of a native biomolecule or the use of cross-linkers during the attachment process.

The Rotello group has extensively studied the adsorption of cytochrome *c* and chymotrypsin (ChT) to AuNPs stabilized with different X−PEG−alkylthiolate ligand (Figure 22), where X is varied between anionic, cationic, neutral, hydrophilic, and hydrophobic.[110,479−483] The X group was tuned by amide coupling L-amino acids via their N-terminus to a parent carboxy-PEG−thiol molecule. ChT was found to adsorb primarily through an electrostatic interaction between the anionic NP and lysine groups around the protein's binding pocket.[480] Several interesting observations were correlated to the selection of the X group and its effect on ChT adsorption: (1) the ChT could be inhibited and denatured upon adsorption to the NP;[480,481] (2) restoration of ChT activity and release from the NP was possible through the addition of cationic surfactants that competed for the NP surface;[481] (3) ChT adsorption could be additionally stabilized through hydrophobic X groups or destabilized through hydrophilic X groups (see Figure 22);[483] and (4) the ChT could selectively turnover cationic and neutral substrates but not anionic substrates.[482] In further studies, it was shown that the NP−anionic, −cationic, and −neutral X groups could selectively bind cytochrome *c*, cytochrome *c* peroxidase, or neither, respectively.[484] Moreover, the orientation of cytochrome *c* binding could be controlled with the "front" face bound to AuNPs modified with hydrophilic, anionic X groups, whereas another protein face bound to AuNPs modified with a hydrophobic, anionic X group.[110] The Rotello group has even developed a protein sensing array based on such tunable adsorption.[479] Six different proteins and a fluorescent polymer competed to adsorb to the surface of AuNPs coated with $R(CH_3)_2N^+$−PEG−alkylthiolate ligands (R = methyl, ethyl, hexyl, cyclohexyl, benzyl, propanoyl) and generated a multivariate optical response that could be correlated to a specific protein by linear discriminant analysis.[479] In a related report, Hamad-Schifferli showed that, in general, glucose oxidase (GOx) activity was negatively impacted by AuNP interactions.[485] It can be seen from these studies that engineered, optimized bioconjugation to AuNPs comprises not only oriented attachment

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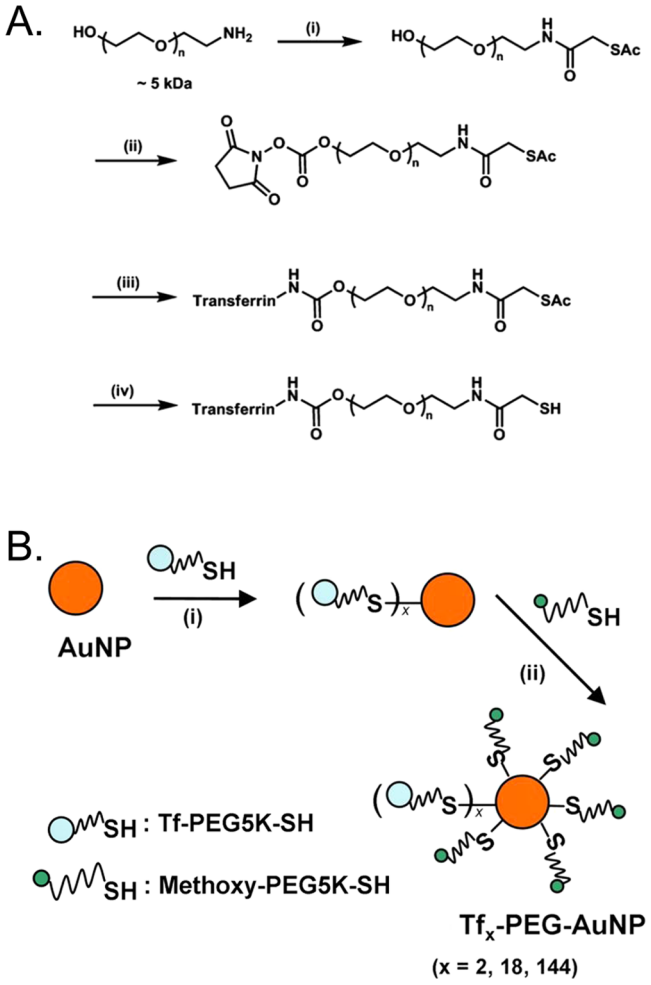

Figure 21. Conjugation of proteins to noble metal NPs using thiol-terminated tethers. (A) Synthesis of a bifunctional NHS−PEG−thiol linker and conjugation to transferrin, followed by deprotection. (B) Assembly to a AuNP and PEGylation of the AuNP−transferrin conjugate (source, ref 470).

at a well-defined site and through a well-defined linkage but also an underlying NP interface that is suitably tuned. More importantly, this has to be carefully evaluated for each protein on a case by case basis.

Several other groups have also used adsorption to bioconjugate Au- and AgNPs. For example, to prepare Au- and AgNP probes for SERS-based assays, Raman active dyes were initially adsorbed to the NPs, which were then modified with thioalkyl acid ligands for EDC coupling to antibodies[442] or assembled with thiol-terminated oligonucleotides.[412] In another study, myoglobin and hemoglobin were adsorbed to citrate-stabilized Au- and AgNPs in a manner that retained both structure and activity.[486] Concanavalin A (ConA) has also been directly adsorbed to citrate-stabilized AuNPs and was able to bind dextran-conjugated QDs.[408] More generally, protein A was adsorbed to commercially available AgNPs, which were further passivated with BSA and conjugated to antibodies via protein A binding.[487] SA was similarly adsorbed to citrate-stabilized AgNPs and used to bind a biotinylated aptamer.[488] The adsorption of protein A and SA on Au- and AgNPs provides a simple route to further conjugation with a large variety of immunoglobulin and biotinylated biomolecule conjugates, respectively. Aside from relying on such linker/intermediary proteins, even generic proteins such as BSA will have an exposed face with potentially diverse chemical functionality (e.g., amines, carboxyls, thiols, phenols) suitable for further modification.

It is important to note that secondary adsorptive interactions are not necessarily trivial even when, for example, Au−thiolate chemisorption or covalent coupling is intentionally used to prepare Au- or AgNP bioconjugates. Although attachment may be better defined with these methods, the biomolecule can still potentially collapse onto the NP surface. For example, the Mirkin group has found that spacer regions between an alkylthiol linker and oligonucleotide recognition sequence affect probe orientation and hybridization efficiency.[461,489] While a poly dT spacer has lower affinity for the AuNP than other homodeoxyribonucleotide sequences and provides greater probe loading on the NPs,[459,489] the use of a PEG linker provides the highest level of probe loading. This example illustrates that adsorption is a potentially important consideration even when not the primary method of bioconjugation. The aforementioned work by the Rotello group would also seem to suggest that when "shotgun" conjugate methods (such as EDC coupling between carboxylated NPs and protein lysine residues) are used, nonspecific interactions between the NP surface and the protein could potentially direct covalent conjugation to a preferred residue(s) based on the surrounding protein topology and physicochemical properties, thus influencing the overall orientation on the NP.

3.1.1.3.5. Commercial Materials. It is important to note that AuNPs are available commercially from a variety of suppliers. This includes generic citrate-, tannate-, PVP-, or CTAB-stabilized AuNPs or nanorods (e.g., Sigma-Aldrich, Ted Pella, Inc., nanoComposix, Inc.). Such materials, which range in size from Au_{11} clusters to 1.4−200 nm AuNPs, are readily modified with custom ligands and other coatings for bioconjugation by the end-user. While these types of products are the most versatile, several different suppliers also market AuNPs premodified with functionalized ligands or polymer coatings. These include amine-, carboxyl-, or hydroxyl-functionalized coatings, as well as PEG, dextran, BSA, silica, and other modifications (e.g., Minerva Biotechnologies, Inc., nanoComposix, Inc., Nanocs, Inc., NanoPartz, Inc., Nanoprobes, Inc., Ocean Nanotech). Further still, numerous bioconjugation-ready products are also sold by these and other suppliers. Maleimido- or NHS-activated AuNPs are convenient for covalent bioconjugation, while Ni(II)−NTA, biotin, NeutrAvidin, SA, and protein A modifications are useful for preparing a diverse range of antibody and other bioconjugates noncovalently, see Supporting Table 1, Supporting Information.

3.1.2. Palladium and Platinum Nanoparticles. *3.1.2.1. Properties and Synthesis.* While the primary interest in Pd- and PtNPs is catalysis,[411] it is important to note that they can also have optical properties of interest,[490] just as Ag and Au can have catalytic properties in addition to their optical properties,[20,491] but that this "secondary" utility is currently much less explored in bionanotechnology. Pt and Pd have similar catalytic activities[492] and are used in several important applications including, but not limited to, hydrogenation and dehydrogenation, petroleum cracking, and catalytic converters. Pt is also important in fuel cells, catalyzing the reduction of oxygen and oxidation of fuel (e.g., hydrogen, methanol, ethanol, formic acid).[493,494] Pd materials are the catalyst of choice in many C−C coupling reactions (e.g., Heck, Negishi, Sonogashira, Stille, and Suzuki coupling).[495] Since both Pt and Pd are precious metals, there is significant interest in increasing their catalytic efficiency so as to obtain greater activities with less

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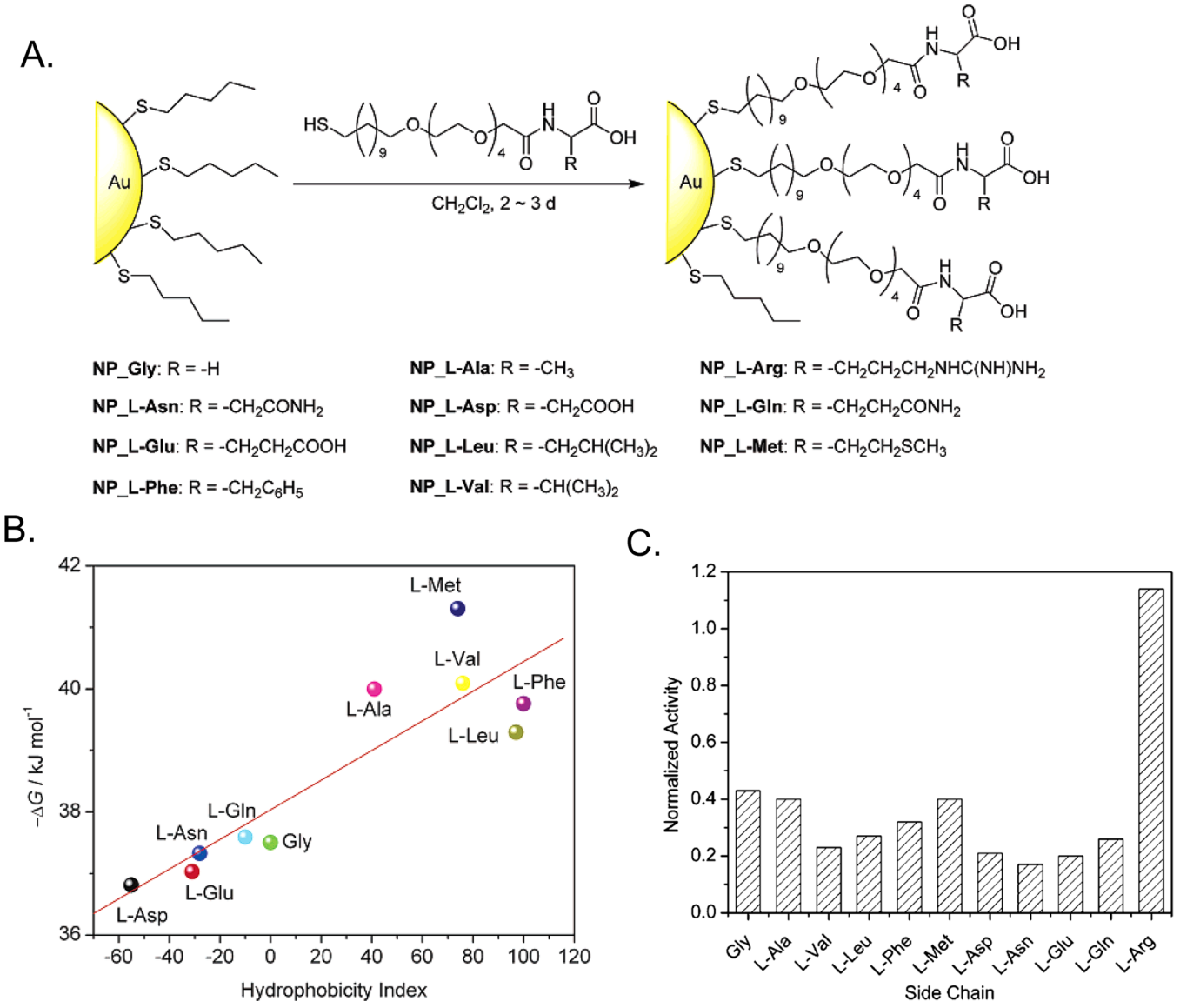

Figure 22. Tuning the adsorption of chymotrypsin on AuNPs using amino acid-modified, carboxy-PEG−alkylthiolate ligands. (A) Ligand exchange to yield the modified NPs. The different amino acid side chains, R, are tabulated below the general schematic. (B) Correlation between chymotrypsin adsorption free energy and R group hydrophobicity. (C) Chymotrypsin activity as a function of R group (side chain). Figure adapted from ref 483. Copyright 2005 American Chemical Society.

material. The use of Pt- and PdNPs is highly advantageous in this respect: NPs offer high S/V ratios, high surface free energies, and catalytic activity that can be tuned by controlling their size or shape (i.e., arrangement of surface atoms) and stabilizing ligands.[494,496,497] Pd- and PtNPs are synthesized similarly to Ag- and AuNPs,[498] and shape control methods have been reviewed extensively.[399,494,499,500] Precursor salts (e.g., $PdCl_4^{2-}$, $PtCl_4^{2-}$) are reduced using common reductants (e.g., $NaBH_4$, glycols) in the presence of suitable stabilizers (e.g., PVP). Analogous to Au and Ag, bioconjugation to Pt- and PdNPs will typically be predicated on a labile stabilizer place-exchanging with a thiol associated with either a linker on a biomolecule or bifunctional ligand suitable for covalent coupling.

Although many potential applications for Pt- and PdNPs are entirely abiotic and bioconjugation is not a consideration, there are some exceptions. In a biological context, Pt has electrocatalytic activity toward nonenzymatic glucose oxidation,[493,501] and PtNPs can efficiently catalyze the chemiluminescent reactions between hydrogen peroxide and luminol or lucigenin.[497,502] Further, PtNPs are potential enzyme mimics by virtue of their catalytic decomposition of peroxide and superoxide.[503,504] PtNPs have also been shown to be potent antioxidants.[505] Although biological application of PdNPs has been quite limited to date, PtNPs may yet prove to be particularly useful as bioconjugates in assay signal amplification mechanisms that rely on chemiluminescent reactions, which are not dissimilar to enzyme-linked immunosorbent assays (ELISAs).

3.1.2.2. Bioconjugation. Overall, the methods for bioconjugation to Pd- and PtNPs are analogous to those used with Ag- and AuNPs, although much less frequently reported in the literature. To date, the predominant methods have been coordination to the inorganic surface of the NP or simple adsorption. Covalent coupling to stabilizing coatings is scarce, although ligand exchange of glycol or alkylamine stabilizers, for example, is certainly possible.[506,507] We do not describe electrode composites with noble metal NPs and biomolecules here.[19,508−510]

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Adsorption has been used to prepare PtNP conjugates for use in immunoassays. Song et al. modified two different antibodies with ferrocene and thionine as redox probes and then sequentially adsorbed the labeled antibodies, HRP, and BSA to hollow PtNPs.[511] The NPs served as a localized scaffold for several HRP molecules and provided a synergistic ability to reduce peroxide, leading to an electrochemical signal amplification. Similarly, He et al. adsorbed antibodies to polystyrene sulfonate-coated Au nanorods that were speckled with Pt nanodots (catalytic activity being a function of the latter); however, in this case the NPs were used to catalyze the oxidation of indicator dyes in a colorimetric sandwich immunoassay.[512]

Biomolecules have also been used to template PtNP synthesis and yield the final NP as a bioconjugate. Examples of templating/stabilizing biomolecules have included GOx,[22] BSA,[429] and peptide sequences that provided PtNPs between 2 and 8 nm with a variety of shapes.[513] Dopamine has been used to synthesize PtNPs in the presence of antibodies, yielding a PtNP−poly(dopamine)−antibody composite.[514] Conjugation of additional antibodies by adsorption or coupling via a glutaraldehyde cross-linker was also explored. The 8 nm spherical apoferritin cage (see section 4.2.4) has also been used to template PtNPs, yielding bioconjugates that exhibited better cellular uptake and biocompatibility than PVP-stabilized PtNPs.[515]

Chemisorption to the inorganic surface of PtNPs has further been used to assemble bioconjugates. Oligonucleotides with a thiol linker were chemisorbed to PtNPs, which were then subsequently modified with HRP and ferrocenemonocarboxylic acid using adsorption and EDC/NHS coupling, respectively.[516] The conjugates were used as a reporter in an electrochemical, rolling-circle-amplification-linked sandwich immunoassay, wherein the Pt provided a catalytic enhancement. In a very elegant study, Grimme et al. chemisorbed a photosystem I (PS-I) complex for use in photochemical hydrogen production, see Figure 23.[517]

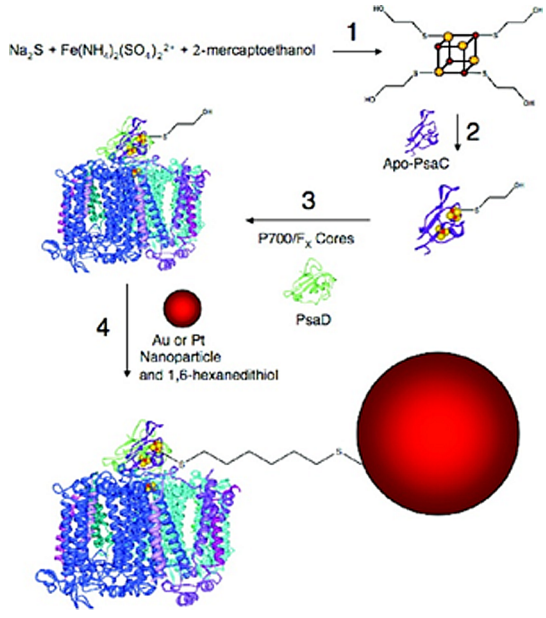

Figure 23. Protein chemisorption to the inorganic surface: (1) synthesis of an Fe−S cluster with mercaptoethanol ligands; (2) association of the cluster with apo-PsaC; (3) assembly of PS-I; (4) exchange of the remaining mercaptoethanol ligands with hexanedithiol and chemisorption of PS-I to PtNPs. Figure reproduced from ref 517. Copyright 2008 American Chemical Society.

F_B is the terminal electron transfer cofactor of PS-I and a cluster of the PsaC subunit, which is a chloroplast-encoded, highly conserved 10 kDa Fe−S binding protein. A variant of PsaC developed by this group[518] could transfer an electron from the F_B cluster to a covalently bound, external acceptor. The PsaC variant lacks a solvent-exposed cysteine residue that would normally bind to the F_B [4Fe−4S] clusters; however, the resulting unoccupied ligand binding site can be chemically rescued by an external thiolate. Grimme synthesized the cluster with mercaptoethanol ligands for reconstitution with the PsaC variant, which was incorporated into a rebuilt PS-I.[517] Finally, the mercaptoethanol ligands on the F_B cluster were exchanged with 1,6-hexanedithiol, resulting in a free thiolate to bind to PtNPs stabilized with mercaptosuccinic acid.[517] The conjugates were able to photocatalyze H_2 production, which was limited by the intrinsic properties of PS-I, rather than electron transfer to the PtNP.

As an alternative to thiol chemisorption, PtNPs have been bioconjugated using a Pt-binding peptide sequence. Kim et al. designed a peptide with a Pt-binding sequence adjacent to an HIV-1 TAT-derived cell-penetrating peptide sequence.[519] The PtNPs, which scavenge superoxide and peroxide as artificial mimics of superoxide dismutase and catalase, were delivered to *C. Elegans* and found to extend its lifespan and minimize oxidative stress at 100-fold lower concentrations than the PtNPs without the TAT peptide.[519] Even with the success of thiol linkers, noble metal-binding peptide sequences can clearly provide a promising and alternative approach to bioconjugation.

3.1.3. Noble Metal Nanoclusters. *3.1.3.1. Properties and Synthesis.* Noble metal nanoclusters, although still particles at the nanometer size scale (<2 nm), are quite different than their NP counterparts. Nanoclusters, which typically consist of ≤100 atoms, have properties that are intermediate to atoms and larger NPs.[520] This arises from dimensionality less than the Fermi length of electrons in the metal, resulting in discrete electronic states rather than a continuum (i.e., quantum confinement). Perhaps the two best structurally understood nanoclusters are $Au_{25}L_{18}$ (where L is a ligand)[521] and Schmid's cluster,[20,522,523] $Au_{55}[P(C_6H_5)_3]_{12}Cl_6$. It has been suggested that the latter may represent the approximate boundary between the regime of discrete energy levels and collective electron oscillations.[524]

The most interesting property of metal nanoclusters, including Ag, Au, and Pt in particular, is their size-dependent luminescence and related properties, such as good photostability, large Stokes shifts, and high emission rates.[411,520] Noble metal nanoclusters exhibit molecular-like absorption and emission bands, with quantum yields typically on the order of 10^{-4}−10^{-1} (albeit continuously improving with synthetic refinements). Another promising feature are the large two-photon absorption cross sections, which are reported to be as large as 2700 GM (GM = Goeppert-Mayer units) at 1290 nm excitation for Au_{25} in hexane or 189 740 GM for Au nanoclusters[525,526] and 34 000 GM at 890 nm excitation to 50 000 GM at 830 nm excitation for Ag nanoclusters with emission at 680 and 700 nm, respectively.[527]

Nanoclusters are often synthesized similarly to their larger NP counterparts; however, more care is required to restrict growth and avoid contamination with NPs. Ligands and stabilizers with low affinity for Ag or Au cannot stop the aggregation and growth of nanoclusters into NPs.[528] Hydroxy-terminated second or fourth generation PAMAM dendrimers, as popularized by the Dickson group,[529−531] are an effective template for nanocluster synthesis. Fluorescent Au_8 nanoclusters can be synthesized in this manner using $HAuCl_4$ with

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1 equiv of $NaBH_4$,[529−531] whereas $AgNO_3$ only forms NPs under these conditions, necessitating UV photoreduction to synthesize Ag_2−Ag_8 nanoclusters.[529] Tanaka et al. similarly demonstrated that Pt_5 nanoclusters could be prepared with these dendrimers via H_2PtCl_6 and $NaBH_4$. Shang et al. have used nondendritic poly(methacrylic acid) (PMAA) to template Ag nanoclusters from the photoreduction of $AgNO_3$.[532]

3.1.3.2. Bioconjugation. As anticipated, dendrimer-templated noble metal nanoclusters can be ligand exchanged with bifunctional thiols for coupling. For example, Tanaka et al. exchanged a PAMAM dendrimer for mercaptoacetic acid, which was then EDC-activated to couple protein A.[533] Similarly, Lin et al. exchanged a PAMAM dendrimer with 11-mecaptoundecanoic acid and coupled it via EDC to a SV40 nuclear localization signal peptide for cell delivery.[534] Au_{23} nanoclusters synthesized with GA ligands have also been EDC coupled to SA, offering a wealth of further bioconjugate possibilities.[535] It follows from these examples that nanocluster bioconjugates can be prepared using bifunctional thiolate ligands and the bioconjugate chemistries previously described for their noble metal NP analogs.

Nanocluster templating by proteins is also common. The reduction of Ag(I) to Ag nanoclusters using BH_4^- has been templated by both ChT[536] and BSA,[537] where the former even retained catalytic activity. Denatured and disulfide-reduced BSA, which displays approximately 35 thiol groups that can bind metals (or potentially serve as further sites for conjugation), has been used to template Ag nanoclusters.[538] BSA has also templated the synthesis of Au nanoclusters via ascorbic acid reduction.[537]

In an elegant study, Sun et al. used the two ferroxidase active sites of apo-ferritin to template the reduction of $HAuCl_4$, resulting in the *in situ* synthesis of paired fluorescent Au nanoclusters.[539] The ferritin−Au nanocluster conjugates were used as an *in vivo* fluorescent probe following ferritin-receptor-mediated cellular uptake. This is not the only work to avoid the use of a chemical reducing agent; biomineralization-like processes utilizing BSA[540] and HRP[541] at strongly basic pH (∼12) have also been described, with the latter retaining catalytic activity. Alternatively, Dickson developed an interesting strategy that avoids the potentially harsh and denaturing effect of $NaBH_4$ and other reducing agents on proteins.[542] Ag(I) complexed with 3-(2-aminoethylamino)propyltrimethoxy silane was reduced with BH_4^- to Ag nanoclusters using PAA as a stabilizer. Separately, antibodies were activated with SMCC then coupled to a $(deoxycytidine)_{12}$ oligonucleotide modified with a thiol linker. The $(deoxycytidine)_{12}$ was able to extract the Ag nanoclusters from the silane/PAA stabilizers, effectively creating an antibody $(deoxycytidine)_{12}$−Ag nanocluster conjugate that was useful in cellular labeling.[542] Beyond the Ag nanocluster "shuttling" described above, the Dickson group has done extensive work developing oligonucleotides as templates for the synthesis of Ag nanoclusters.[377,542−544] The N_3 (nitrogen atom) of dC has a particularly strong interaction with Ag, and different sequences of DNA can template different fluorescent Ag nanoclusters with various excitation and emission characteristics. They also found that a peptide sequence rich in asparagine, aspartic acid, cysteine, lysine, and histidine can template fluorescent Ag_2−Ag_5 nanoclusters bound to single peptides, which were useful for cellular labeling.[545]

Yeh et al. developed a hybridization assay scheme using oligonucleotide-templated Ag nanoclusters.[546] The oligonucleotide comprised both a template sequence and influenza-related probe sequence. As synthesized, the Ag nanoclusters were weakly fluorescent. However, incorporation into a sandwich assay where probe−target hybridization also brought a dG-rich reporter oligonucleotide into close proximity to the Ag nanoclusters resulted in a strong fluorescence enhancement and a "turn-on" signal for detection.[546] Although a promising approach to bioconjugation, it is not yet clear that this strategy will be generally applicable. Even a single base-pair mismatch adjacent to a hairpin structure has been shown to inhibit the templating of fluorescent Ag nanoclusters.[547] Oligonucleotide conformations can also change drastically with small changes in Ag nanocluster size.[548] Thus, other oligonucleotide probe sequences and aptamers appended to templating sequences may inadvertently alter the properties of the as-synthesized Ag nanoclusters.

3.2. Semiconductor Quantum Dots

The terms *luminescent* or *colloidal quantum dots* (QDs) are often used to describe nanocrystalline particles commonly synthesized from binary mixtures of III−V or II−VI semiconductor materials including ZnS, ZnSe, CdS, CdSe, CdTe, InP, and others.[549,550] Their nanoscale size, which is smaller than the Bohr radius associated with the exciton of the bulk constituent materials, gives rise to unique quantum-confined photonic and electronic properties.[551,552] QDs are physically larger than organic dyes and fluorescent proteins and were initially envisioned to only have electronic applicability. However, their cumulative optical properties provided far more capability than previously available fluorophores and suggested the possibility of biological utility.[30,199,551,553−555] Pertinent photophysical properties of relevance to biology include broad absorption profiles that increase nearly continuously toward the UV, narrow, size-dependent, and symmetric PL spectra spanning from the UV to the IR that are a function of the constituent materials, strong resistance to photobleaching and chemical degradation, high quantum yield (QY), remarkable photostability, a large effective Stokes shift, and some of the highest multiphoton action cross sections known.[30,551,553,554,556−558] The seminal 1998 publications from the Nie and Alivisatos groups were the first to demonstrate their potential as biological labels and spurred widespread interest for bioapplications.[559,560] Over the last 12 years, there has been a continuous drive to adapt, improve, and apply the unique properties of QDs for myriad bioapplications, including use as *in vitro* and *in vivo* fluorescent probes, engineered biosensors, photodynamic therapy agents or sensitizers, and theranostic platforms, along with light harvesting and active nanoplatforms. These applications are extensively reviewed in refs 13,24, 551, 553−556, and 561−563 and references therein.

In biological applications, QDs are most commonly utilized either as core-only or as core/shell structures, where the overcoating shell consists of a wider band gap material that serves to protect and passivate the core and prevent leaching, while also increasing the QY.[551,564] Core/shell/shell QDs, core/alloy/shell QDs, and other synthetic permutations have also begun to see biological utility recently.[551] Here, we focus predominantly on type II−VI CdSe and CdTe core or core/shell QDs since these have been most commonly applied in biology. Other materials, such as type III−V InP or InAs/ZnCdS are also mentioned, but not as frequently, because these are still currently undergoing concerted development in pursuit of near-IR emitting materials that do not contain Cd.[565−567] The synthesis of high-quality, monodisperse QDs is consistently

achieved utilizing the pyrolysis of organometallic precursors in the presence of hydrophobic coordinating ligands in organic media.[549,550,564] Hydrophilic QDs have also been directly synthesized in aqueous solutions; however, the quality of these "greener" nanocrystals for bioapplications has not reached the same level as those synthesized in hot organic solvent.[568] While many groups still synthesize their own materials or collaborate with a group that does, QDs are also available commercially (Supporting Table 1, Supporting Information) with surfaces that display amines, carboxyls, biotin, avidin, and other proteins for subsequent chemical modifications or targeted bioconjugation, and this has contributed significantly to their widespread use.

Hydrophobic alkane ligands are typically used to solubilize QD precursor materials as monomers and to stabilize and mediate nanocrystal growth during synthesis. However, the resulting or *native* QDs are insoluble in aqueous media and must undergo further processing to yield hydrophilic QDs. The two most common strategies utilized are (1) wholesale, mass-action driven *cap exchange* (a.k.a. *ligand exchange*) of the native surface with bifunctional ligands that attach to the QDs at one end, typically by thiol interactions, and provide solubility at the other through functional groups such as carboxylates or PEGs and (2) encapsulation of the native surface with, for example, amphiphilic block copolymers that interdigitate the hydrophobic surface and provide a hydrophilic outer layer, see Figure 24, reviewed in refs 551 and 569. These surface preparations directly affect subsequent bioconjugation. For example, cap-exchange approaches can still allow access to the QD surface whereas polymer encapsulation may completely preclude this. Further, the size and hydrodynamic diameter of the final hydrophilic QD is highly dependent on which strategy is used. Cap exchange can provide QDs with a small hydrodynamic diameter but typically lower QY, while encapsulation typically results in larger sizes with higher QYs.[570] It is important to consider these factors when designing bioconjugated QDs because they can impact the intended utility, such as FRET interactions, cellular delivery, and *in vivo* circulation, distribution, and clearance.[571,572]

Although numerous strategies have been developed for bioconjugating QDs, almost all can be subdivided into six functional subclasses based upon the chemistry utilized or the mechanism of interaction with the QD or its surface ligand. These include (1) covalent conjugation between QD ligands and target functional groups on biomolecules, (2) electrostatic interactions between charged QDs and oppositely charged biomolecules, (3) direct dative interactions between the inorganic surface of the QDs and biomolecules containing thiol groups or polyhistidine sequences, (4) high-affinity secondary binding such as that between QDs prefunctionalized with SA and biomolecules labeled with biotin or *vice versa*, (5) enzyme-catalyzed bioconjugation, and (6) biological templating. It is important to note that all these approaches also have inherent benefits and liabilities that should be considered in terms of the final application. For example, reagents for EDC/NHS chemistry are broadly available, and standardized protocols have been developed for application to commercial QDs displaying amines/carboxyls. However, the presence of the same ubiquitous target groups on target proteins such as antibodies means that this approach can often result in uncontrolled heterogeneous orientation and valence on the QD and undesirable cross-linking.[14] Furthermore, QD conjugates formed with this chemistry may require some purification to remove both the vast excess of chemical reactants used and uncoupled proteins. In the subsequent overview, we highlight examples where each approach was utilized to functionalize QDs with major types of biomolecules including proteins, peptides, and DNA along with other relevant targets such as carbohydrates or drugs. Note, enzyme-catalyzed bioconjugation of QDs and other NP materials is discussed in section 5.2.2.

3.2.1. Covalent Chemistries. *3.2.1.1. Proteins.* Given its ubiquitous presence, EDC/NHS chemistry is often used as the only chemistry to attach either proteins or antibodies to QDs, typically by direct amide bond formation between terminal carboxyls on the QD ligands and ubiquitous amines on proteins, although the reverse configuration may work equally well in some cases. This chemistry also often serves as the first in series of multistep modifications that add other functionalities to the QD surface via standard bioconjugation techniques with heterobifunctional linkers, see Figure 8.[80] This was elegantly demonstrated by Wu et al., who utilized CdSe/ZnS QDs made hydrophilic with a carboxylated amphiphilic polymer (40% octylamine-modified PAA) for cellular imaging applications.[573] EDC chemistry mediated the initial attachment of goat anti-mouse IgG to 535 or 630 nm emitting QDs, which were used to image breast cancer SK-BR-3 cells incubated with monoclonal anti-HER2 antibodies. QDs functionalized with SA via EDC were also used to image the same biotinylated marker. Similarly prepared QD−SA and QD−IgG bioconjugates were then used to label microtubules and F-actin, respectively, in the cytoplasm of mouse 3T3 fibroblast cells.[573] This approach was further extended to a two-color format where green QD−IgG and red QD−SA conjugates were used to label the cell surface and nuclear antigens of SK-BR-3 cells. The targeting of multiple different cellular antigens with the same QD conjugates highlighted the versatility of this approach. Importantly, the authors unequivocally demonstrated that QD labels were far brighter and more resistant to photobleaching in comparison to AlexaFluor organic dyes; the latter result greatly stimulated subsequent interest in their use as biological probes.[573]

During fluorescent bioimaging, external illumination often causes strong background autofluorescence and is ineffective for exciting fluorophores at depth within the tissue due to absorption and scattering. Seeking to overcome reliance on external illumination, Rao's group used EDC chemistry to attach a mutationally optimized *Renilla reniformis* luciferase enzyme (Luc8) to QDs. This yielded self-illuminating QD−Luc8 bioconjugates that were driven via chemically induced bioluminescence resonance energy transfer (BRET).[574] A variety of differentially emissive commercial CdSe/ZnS and CdTe/ZnS QDs coated with the same carboxylated amphiphilic polymer as above were coupled to the Luc8 solvent-accessible amino side chain groups. Enzymatic oxidation of added coelenterazine substrate by Luc8 generated an excited state product that sensitized emission from the proximal QD through BRET. The excited state product emits at ca. 480 nm, which offers excellent spectral overlap with QDs. Multicolor BRET emission from QDs extending well into the near-IR was observed from QD−Luc8 bioconjugates tested in mouse blood and whole serum and from subcutaneous and intramuscular injections in nude mice. Further EDC coupling of QD−Luc8 assemblies to a polycationic TAT-based $(Arg)_9$ cell-penetrating peptide also facilitated cellular uptake and BRET-sensitized QD emission in C6 glioma cells. Several other factors contributed to make both this QD bioconjugation approach and its application successful. Internal amine groups that were potentially critical to Luc8 catalysis were not permanently modified during QD attachment by

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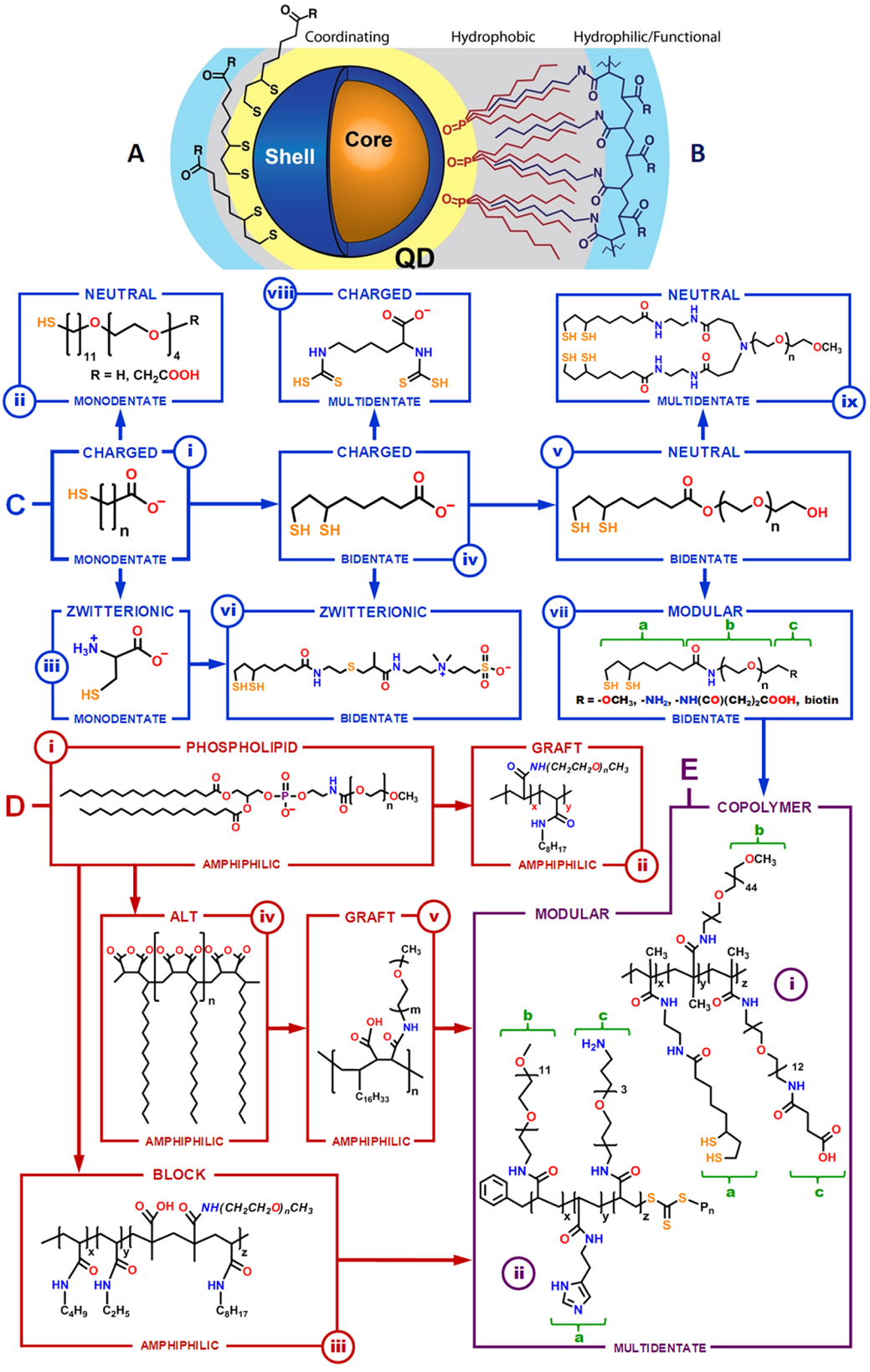

Figure 24. Quantum dot surface chemistries. (A) Ligand binding at a QD surface. (B) Association of an amphipol (blue) with the native QD ligands (red). (C) Ligand chemistries: (i) thioalkyl acids; (ii) PEGylated ligands; (iii) zwitterionic ligands; (iv) dihydrolipoic acid ligands and (v) PEGylated, (vi) zwitterionic, and (vii) modular derivatives thereof; and (viii) charged and (ix) multidentate PEGylated ligands. (D) Amphipol coatings: (i) phospholipid micelles; (ii) hydrophilic polymer backbones grafted with alkyl chains; (iii) triblock copolymers; and (iv) alternating copolymers that hydrolyze to acids or (v) are grafted with PEG chains. (E) Copolymers with pendant PEG oligomers and (i) dithiol or (ii) imidazole groups. Discrete moieties for (a) QD binding, (b) solubility, and (c) bioconjugation are identified where applicable (green). The arrows illustrate a conceptual progression and not synthetic pathways or chronological development. Figure adapted from ref 551. Copyright 2011 American Chemical Society.

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this chemistry. Heterogeneity in Luc8 QD attachment orientation was also not a factor as the large QD absorption could overcome any distance variations during BRET sensitization.

Yong et al. synthesized InP/ZnS QDs as a potential alternative to the use of Cd/Se containing QDs.[575] Following solubilization via cap exchange with mercaptosuccinic acid, the surface-displayed carboxyl groups of the QDs were EDC-coupled to anti-claudin 4 and anti-prostate stem cell antigen antibodies for specific staining and imaging of pancreatic cancer cell lines. Receptor-mediated endocytosis of the QD−antibody bioconjugates by targeted MiaPaCa and XPA3 cells were visualized with confocal microscopy and verified that these covalent bioconjugates could function as a low-toxicity biomedical probe for potential early detection of human pancreatic cancer. Numerous other proteins have been directly attached to QDs with EDC chemistry, including Tf[560] and a range of antibodies that are too numerous to list, which cumulatively attest to the popularity of this approach, despite the lack of finite control over protein orientation in the final QD conjugate.

Thiol groups have also been engineered onto the surface of QDs for subsequent bioconjugation reactions.[576] To accomplish this, a SiO_2 shell was initially grown around thioglycolic acid-capped CdTe core-only QDs using a modified Stöber method, and shell growth was accelerated by precipitating more SiO_2 onto the already formed shell. Mercaptopropyltrimethoxysilane and a PEGylated derivative of the same were used to introduce both PEG, for solubility, and thiols, as a chemical handle, onto the SiO_2 surface. Sulfo-SMCC was subsequently used to join the thiols to the amines present on anti-p53 monoclonal antibodies.[576] Although the final thiolated QDs were quite robust, this particular approach has not seen much recent use due to the synthetically intensive steps required. There have also been numerous examples where sulfo-SMCC, other heterobifunctional linkers, and even amino acids have been used to cross-conjugate QD surface displayed amines to thiols primarily located in antibody hinge regions.[551,577,578]

Jennings and co-workers recently reported two QD bioconjugation reactions that incorporated concepts from both the classical fluorescent labeling of proteins and recently developed bioorthogonal chemistries (section 2.7).[579] The overarching strategy was to provide QDs in a form preactivated for bioconjugation, which would help minimize the processing steps required, in concert with incorporating chemoselectivity to minimize cross-reactivity and heterogeneity. Critically, these chemistries were targeted to the same ubiquitous amines and thiols that most reactive dyes also focus on, see Figure 25. The QDs prepared for these linkages were stabilized in water using a DSPE−PEG lipid (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[carboxy(poly(ethylene glycol)-2000)]). The QD surfaces were further modified with 4-formylbenzamide (4FB) or a reactive maleimide for conjugation with amine or thiol groups, respectively, using proprietary techniques at eBioscience (San Diego, CA). The amine conjugation strategy utilizes a bioorthogonal ligation reaction to link biomolecular amines to preactivated QDs. As shown in Figure 25A, the target biomolecular amines are first modified with a heterobifunctional *N*-hydroxysuccinimidyl ester 6-hydrazinonicotinamide (NHS-HyNic). The HyNic-modified biomolecule is then added to a solution of 4FB-modified QDs along with aniline as a catalyst, and the conjugation reaction rapidly forms a bis-aryl hydrazone bond between the 4FB and HyNic moieties. As discussed in section 2.10.2, aniline-catalyzed hydrazine ligation chemistry originates from Dawson's laboratory and is characterized by enhanced bioconjugation rates (10^1−10^3 M^{-1} s^{-1}) in slightly acidic to neutral aqueous conditions. This stoichiometric chemistry can go to completion in under 30 min using 100 mM aniline catalyst with 10 μM of reactants and yields stable conjugates.[283] Since the hydrazine and aldehyde reactants are orthogonal to almost all other biological functional groups, they do not modify other functional groups already present in the NC (QD)−bioconjugate. The same chemistry was also recently used to attach antiepidermal growth factor receptor (EGFR) antibodies or anti-RNA polymerase to QDs for cellular imaging and for visualizing immobilized DNA, respectively.[580] In contrast, the thiol chemistry approach introduced by Jennings exploits maleimide groups prelabeled onto the QD surface to target free biomolecular thiols, see Figure 25B. Similar to many commercially available maleimide-activated or thiol-reactive dyes, these QDs can be stored lyophilized for extended time periods. In a manner analogous to a protein labeling reaction, the activated QDs are mixed with target protein in the presence of a proprietary reducing agent and allowed to react. Figure 25C compares multicolor immunocytochemical labeling of a MCF-7 epithelial breast cancer cell line where the QD immunoreagents were prepared using either the amine- or the thiol-based chemistries.[579] This example clearly reflects a functional equivalence for both chemistries with the same target antibodies.

3.2.1.2. Peptides. Many bioapplications benefit from having multiple copies of a targeting biological displayed on the QD surface while still maintaining a small hydrodynamic diameter. Because the number of proteins that can be conjugated to QDs is directly related to and limited by their size (*vide infra*), peptides present an attractive alternate form of targeting molecule because they can provide a key functionality without the steric issues originating from protein size and packing constraints. This has been shown to be especially true for *in vivo* cellular delivery where endosomal uptake can be accomplished using the canonical HIV-derived polyArg TAT− or RGD−integrin binding and cell-penetrating peptides (CPPs, <3 kDa). These have been shown to functionally replace use of the iron binding and delivery glycoprotein Tf (~80 kDa).[560,579,581] Further, synthetic peptide chemistry provides facile access to literally thousands of precursors and thereby allows inclusion of non-natural residues and diverse functional groups for further chemistry with control over placement and repetition within a sequence as highlighted in some of the examples below.

Several different CPP motifs have been utilized to achieve cellular delivery with QDs. Cai et al. prepared RGD peptide-labeled QDs for *in vivo* targeting and imaging of integrin $\alpha_v\beta_3$-positive tumor vasculature.[582] The $\alpha_v\beta_3$ cell surface integrin receptor that binds RGD sequences is upregulated during angiogenesis and metastasis of tumors and is used as a marker for detection and treatment. Near-IR (705 nm)-emitting QDs displaying surface amine groups were treated with the heterobifunctional linker *N*-(γ-maleimidobutyryloxy)-succinimide (GMBS) to provide maleimide-functionalized nanocrystals. These were then linked to thiolated RGD peptides (RGD-SH) to yield monodisperse QD705−RGD bioconjugates, and binding affinity was confirmed with fluorescence microscopy by staining cancer cells with no, moderate, and high $\alpha_v\beta_3$ integrin expression levels and challenging them against a peptidyl antagonist. *In vivo* targeting was tested in athymic nude mice bearing implanted tumors with both QD705−RGD conjugates and unconjugated QD705 injected into the tail vein. QD705−RGD successfully imaged the tumor with optimal contrast reached

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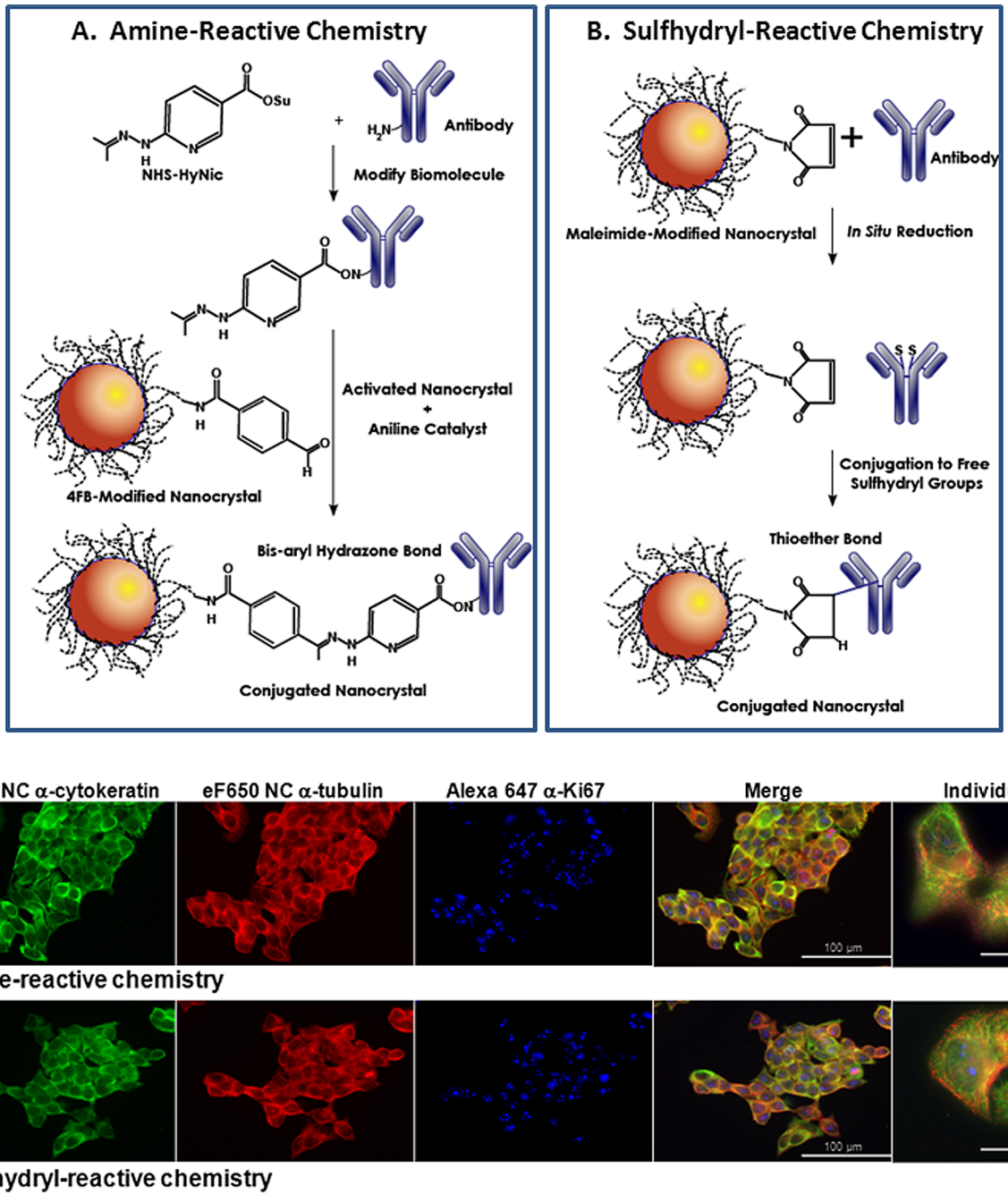

Figure 25. Selective quantum dot modification chemistries. (A) Schematic of the amine-reactive chemistry in which free primary amine(s) on a biomolecule are first modified with succinimidyl 6-hydrazinonicotinate acetone hydrazone (S-HyNic). The resulting hydrazine group on the biomolecule is then specific for a premodified 4-formylbenzamide (4FB) QD nanocrystal (NC), and the conjugation reaction takes place rapidly in the presence of an aniline catalyst (2 h). (B) Schematic of the thiol-reactive chemistry, which consists of a maleimide-functionalized NC and an *in situ* reducing agent. Biomolecules containing either disulfide bonds or available thiol groups are directly reduced in solution with reactive NCs for immediate conjugation. (C) Multicolor immunocytochemical labeling of MCF-7 epithelial breast cancer cells with amine- and thiol-reactive NCs. For each panel, fluorescent micrographs show the eF605 NC emission (false-colored green), eF650 NC emission (false-color red), AlexaFluor 647 emission (false-color blue), and the merged fluorescence. Higher magnification images of individual cells are also shown. Figure adapted from ref 579. Copyright 2011 American Chemical Society.

~6 h postinjection, while the naked QD705 failed to accumulate in the tumor and appeared to be cleared by the reticuloendothelial system. Orndorff and Rosenthal covalently conjugated peptide neurotoxins to QDs for dual labeling of endogenously expressed cellular proteins in glioma cancer cells utilizing a slightly modified chemistry.[583] Chlorotoxin and dendrotoxin-1 peptides, which bind to metalloproteinase II and potassium channels of glioma cells, respectively, were utilized for this investigation. These peptides were treated with Traut's reagent to yield thiolated peptides for reaction with ITK amino(PEG)-coated QDs (Life Technologies) that were premodified with NHS−iodoacetate. Confocal imaging revealed that specific multiplex detection of both neurotoxin targets could be accomplished in live C6 glioma cells, and this was confirmed by further counterstaining with dye-labeled antibodies.

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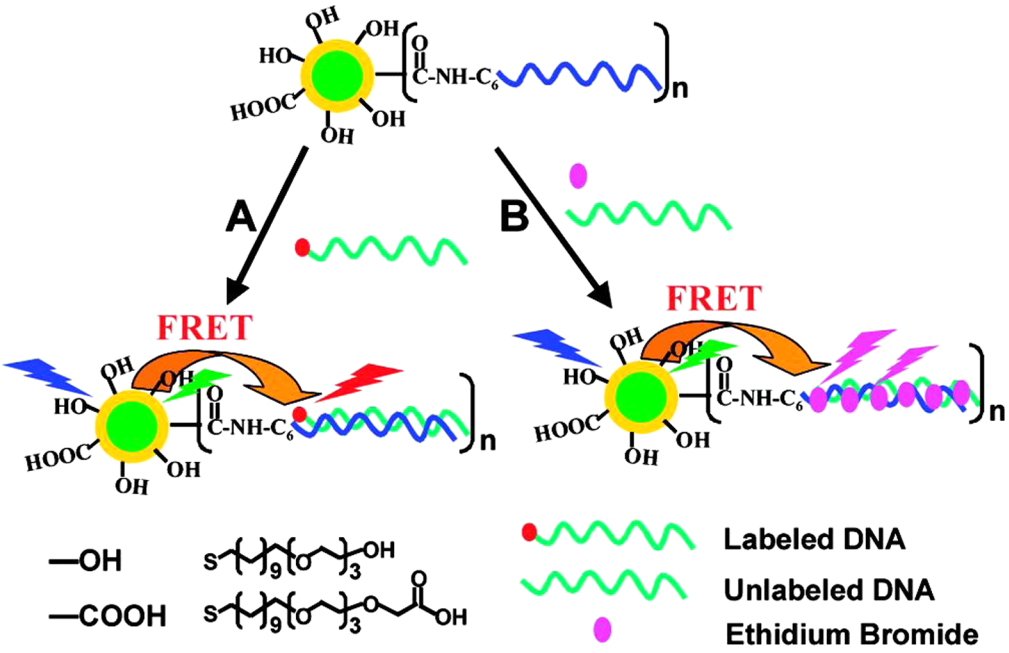

Figure 26. Schematic representation of the principles for hybridization and label-free detection of DNA probes with a covalently coupled QD−DNA-T conjugate via a QD-sensitized FRET signal. Approach A relies on hybridization with an acceptor dye-labeled complementary DNA, while approach B utilizes ethidium bromide to intercalate into the dsDNA structure and function as the acceptor. Figure adapted from ref 587. Copyright 2008 American Chemical Society.

Takayama et al. used a simplified and converse approach to prepare QDs displaying the canonical HIV-derived polyArg TAT sequence, which is perhaps the most common CPP motif used with QDs.[584] ITK amino(PEG)-coated 605 nm QDs were treated with the homobifunctional, amine-reactive BS^3-activated polyArg peptides, Arg_8 or Arg_{12}, to form the bioconjugates. The efficacy of these conjugates was verified by monitoring uptake in HeLa cells versus unconjugated QDs. Direct EDC-based conjugation of oligoArg−CPP or RGD−integrin binding motifs to QDs functionalized with a variety of carboxylate ligands or encapsulating polymers appears to be perhaps the most common approach utilized in the literature.[581,585,586]

3.2.1.3. Nucleic Acids. Zhou et al. developed compact, covalently coupled QD−DNA bioconjugates for specific, label-free detection and quantification of complementary DNA (~1 nM) via FRET.[587] Covalent coupling was strategically used for finite control over the donor−acceptor distance and assembly, which is an important consideration in FRET-based sensing formats. First, hydrophilic QDs capped with a 2:1 mixture of monothiol OH- and COOH-terminated PEG bifunctional ligands were prepared by cap exchange of the native trioctyl phosphine oxide surface on 558 nm emitting CdSe/ZnS QDs. The terminal carboxyl groups were then activated with EDC/NHS and covalently coupled to a 5′-C_6-amine-modified 30-mer target DNA (DNA-T) by amide bond formation (~2.2 DNA-T per QD). Hybridization of the DNA-T on the QD with fluorophore (Alexa 594)-labeled complementary DNA (in ~10 min) results in QD quenching and enhanced emission from Alexa 594 via FRET, see schematic in Figure 26. The specific detection of label-free complementary DNA could also be achieved similarly by addition of complementary DNA and ethidium bromide (a dye that intercalates double-stranded DNA) to the QD−DNA-T bioconjugates. Upon assembly, the ethidium bromide intercalates in the double-stranded DNA and acts as a FRET acceptor with the QDs to signal DNA hybridization.[587]

In a similar format, Wu et al. prepared compact, covalently linked QD−DNA bioconjugates for specific DNA detection with improved FRET.[588] Improved FRET efficiency was achieved by using shorter capping ligands for covalently anchoring the DNA segments, which decreased the distance between the QD donor and acceptor (dye-labeled complementary DNA, dye = TEX red and rhodamine red). Hydrophilic OH/COOH-capped CdSe/ZnS QDs ($\lambda_{emission}$ = 536 and 589 nm) were prepared via cap exchange of trioctyl phosphine oxide-capped QDs with commercially available 2-mercaptoethanol and dihydrolipoic acid (DHLA, 4:1 ratio) ligands. The hydroxyl groups (−OH) were used to control the number of DNA probes that could be coupled to the carboxyl groups (−COOH) by preventing potential steric problems upon DNA hybridization. Amide bond formation was achieved between the amine-modified DNA and the carboxyl groups on the QDs via EDC/sulfo-NHS chemistry to produce the QD−DNA bioconjugates. The improved QD−DNA conjugates detected hybridization of dye-labeled complementary DNA (70% FRET efficiency) within 10 min with a sub-nanomolar detection limit.

Continuing research focused on delivering and monitoring the effects of siRNA has also led to their conjugation with QDs because their intrinsic PL provides a mechanism for visualizing intracellular activity. Bhatia's group prepared QD−siRNA conjugates with both labile and nonlabile linkages. The former relied on dithiol-bridged functional linkers such as SPDP and SMPT, while the latter utilized NHS−PEO_n−maleimide linkers (see Figure 8 section 2.6).[589] Placement of the thiol for QD conjugation on the sense or antisense RNA strand was also evaluated. The authors noted that QD−siRNA constructs with labile linkers lost their knockdown efficiency after ~8 h, whereas constructs assembled with nonlabile cross-linkers maintained efficient knockdown ability due to their superior stability. They also noted that the length of the attachment

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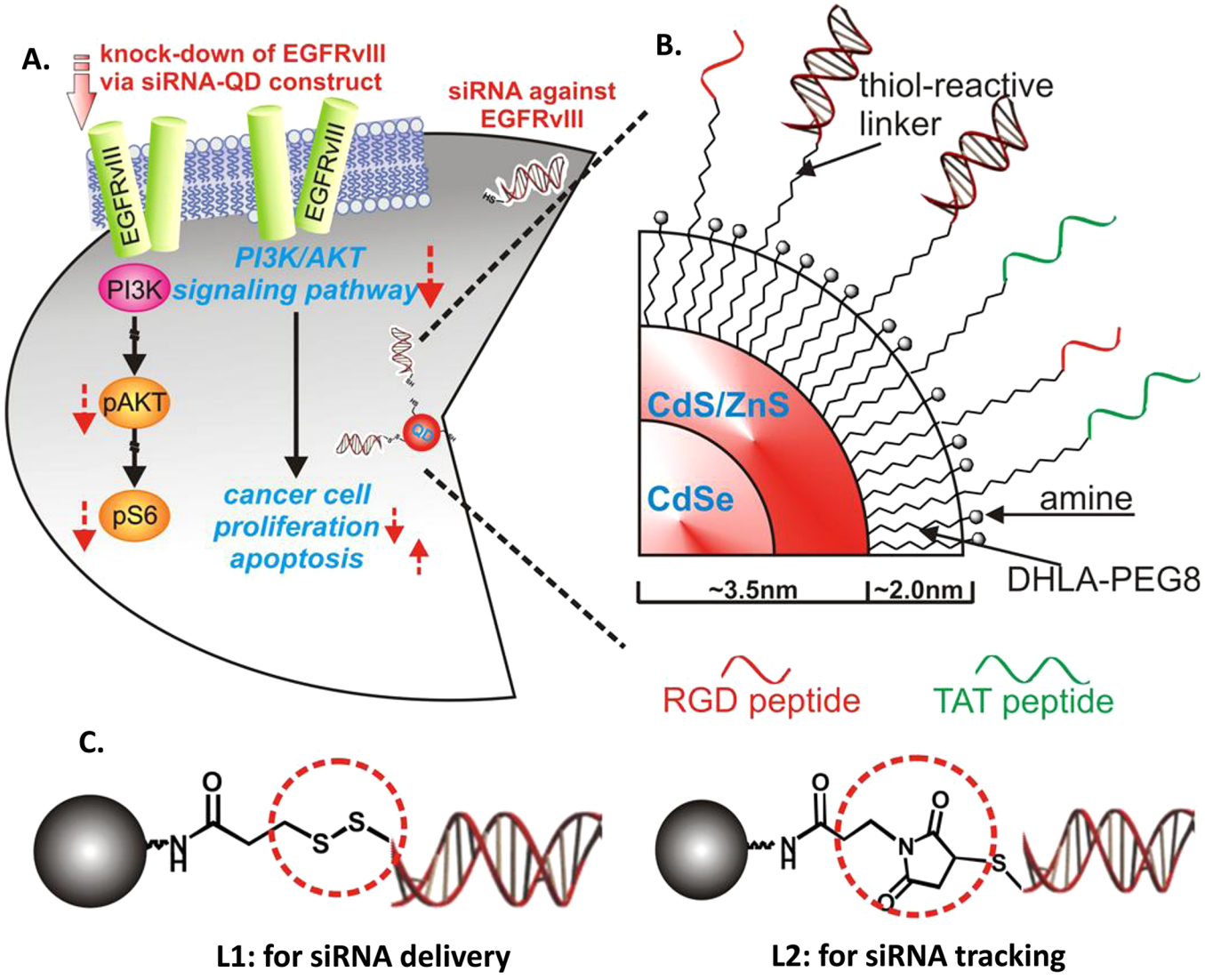

Figure 27. Quantum dot-mediated siRNA delivery. (A) Schematic showing QDs as a multifunctional nanoplatform to deliver siRNA and to elucidate EGFRvIII-knockdown effect of PI3K signaling pathway in U87-EGFRvIII cells. (B) Detailed structural information of the multifunctional siRNA–QDs. (C) Two different strategies for the siRNA–QD conjugate. (L1) Linker for attaching siRNA to QDs through a disulfide linkage, which was easily reduced within the cells to release the siRNA. (L2) Linker for covalently conjugating siRNA to QDs, which enabled tracking of siRNA–QDs within the cells. Figure reproduced from ref 590 with permission of Wiley.

linker was critical and could compromise knockdown efficiency by steric hindrance when too short. Jung and co-workers similarly evaluated labile and nonlabile QD–siRNA conjugation for cellular delivery and tracking applications, respectively, see Figure 27.[590] In this case, the target was the EGFR variant III as expressed in a human U87 glioblastoma brain cancer cell line. QD surfaces were functionalized with a PEGylated dihydrolipoic acid that terminated in an amine (DHLA–PEG–amine). Two custom linkers were synthesized to join the siRNA to the QD–amines. Linker 1 (L1) consisted of 3-(2-pyridyl)-dithiopropionic acid pentafluorophenyl ester and was designed to release siRNA upon entering the cell by disulfide cleavage ligand exchange with endogenous GA. Linker 2 (L2) utilized 3-maleimidopropionic acid pentafluorophenyl ester and was designed to be more robust to enable evaluation of cellular uptake and siRNA construct localization within the cell. In contrast to the previous example, the labile siRNA constructs provided for more efficient target knockdown in this example while also serving to highlight how radically results can vary in different model systems.

3.2.1.4. Sugars. Their principle function as both a carbon source and part of cell surface displayed glycoproteins has also made assembly of QD–sugar conjugates of interest. The simplest route to obtaining these materials has been to synthesize QD-capping ligands that terminate in a sugar moiety. For example, Yu and colleagues synthesized 1-thiol-β-D-lactose and used them to cap CdSe/ZnS QDs for labeling of leukocytes *in vivo*.[591] Similarly, Fields et al. prepared a mannosylated disulfide and used this ligand to functionalize CdS QDs for bacterial detection applications.[592] Kikkeri et al. extended this concept and demonstrated a microfluidic reactor system that was capable of synthesizing QDs from precursors and overcoating the QDs with sugar-appended ligands.[593] For this, they utilized a thiolated PEG surface ligand that terminated in an α-mannose or β-galactose group.

Rather than direct surface modification with a sugar moiety, QDs have also been surface-modified to display phenylboronic

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acid groups due to their capacity to complex to sugars such as glucose or sialic acids. EDC chemistry was used to couple phenylboronic acid to mercaptosuccinic acid ligands displayed on CdTe/ZnTe/ZnS core/shell/shell QDs; this allowed the QDs to assemble into higher order structures in the presence of sugar and function as a glucose sensor.[594] Another approach used similar EDAC chemistry but coupled phenylboronic acids to carboxyls displayed on the outer surface of QDs coated with an amphiphilic copolymer derived from octadecene-modified maleic anhydride.[595] The subsequent QD conjugates were able to bind to sialic acid present on the surface of a PC12 rat adrenal medulla pheochromocytoma cell line and signal the presence of extracellular sialidase activity. In a converse approach, Ohyanagi and colleagues coupled sialic acid residues to phosphorylcholine monolayer-coated QDs.[596] Here, an aminooxy-terminated thiol derivative was coimmobilized within this monolayer coating providing a chemical handle for reactions with a host of different *p*-nitrophenyl glycosides by chemoselective ligation to the sugar's aldehyde or ketone groups, see Figure 28. This approach overcame issues resulting from stereochemistry with the reducing sugar's anomeric carbon. Once appended to the QDs, sugar moieties could function as a substrate and be enzymatically extended via the use of recombinant glycosyltransferases in the presence of appropriate sugar nucleotide reagent. The use of enzymes with different specificities along with control over the order of reactions allowed for specific oligoglycosides to be appended onto the QDs as desired. This approach reflects a fascinating combination of conventional organic chemistry along with carefully controlled enzyme-catalyzed ligation. Subsequent live animal imaging of the distribution of various QD conjugates within mice illuminated how the terminal sialic acid residues have significant effects on *in vivo* lifetimes and organ biodistribution. Using an approach that also relied on a preactivation of the sugar moieties, Hahn's group assembled QD−hyaluronic acid derivatives for imaging the liver in a cirrhotic mouse model.[597] A hyaluronic acid linear polysaccharide moiety was modified with adipic acid dihydrazide, which allowed subsequent EDC coupling to carboxylated QDs. Cycloaddition chemistries have been similarly applied to assembling QD−sugar conjugates. For example, amino-PEG-functionalized 550 nm emitting QDs were EDC-coupled to cyclooct-1-yn-3-glycolic acid to yield a QD−cyclooctyne derivative.[598] The activated QDs were then mixed with azido-tagged mannosamine and allowed to yield the final QD−mannose construct. These were then exposed to Chinese hamster ovary (CHO) cells where they were incorporated into newly synthesized glycoproteins located on the extracellular membrane. This particular approach negated the need for the Cu(I) catalyst, which simplified the coupling chemistry and purification steps required along with preserving high QD PL.

3.2.1.5. Drugs, Dyes, and Imaging Agents. Many drugs, dyes, imaging agents, and other biologically active compounds have also been covalently conjugated to QDs. Similar to some of the conjugations used with sugars, direct utility as a QD capping agent is the most straightforward approach even if the drug's available bioactivity is not directly exploited. For example, de la Fuente utilized tiopronin to cap CdS nanocrystals.[599] Tiopronin, *N*-(2-mercaptopropionyl)glycine, is a non-natural amino acid that has found use as a drug for the treatment of cystinuria and rheumatoid arthritis. In this case, the carboxyl of the QD-conjugated tiopronin was useful as a handle for EDC modification with a TAT CPP, while the thiol provided for QD attachment, allowing for uptake into human fibroblasts.[599] Seeking to put a more bioactive compound on the QDs, Bharali et al. synthesized InP/ZnS core/shell QDs, dispersed them in aqueous solution with mercaptoacetic acid, and then utilized carbodiimide chemistry to attach FA to the QDs.[567] This allowed subsequent QD conjugate binding and multiphoton imaging in a FA receptor overexpressing KB cell line.

Environmentally sensitive dyes have also been attached to QD surfaces to construct ratiometric or FRET-based pH sensors. Here the QD is used in different roles during the sensing where the QD either can contribute PL changes as part of the pH sensor or, alternatively, functions just as the FRET donor. In the case of the first sensor type, Jin et al. coated QDs with GA and used the pendant amine as the site for fluorescein isothiocyanate (FITC) modification.[600] Subsequent pH-induced changes in both the QD PL and the FITC allowed for ratiometric pH monitoring. Snee's group synthesized 40:60 PAA/octylamine amphiphilic polymers using reversible addition−fragmentation transfer (RAFT) for encapsulating native hydrophobic 580 nm emitting QDs and then labeled the thiol of the polymer headgroup (from hydrolysis of the RAFT agent) with a maleimide-activated BODIPY 577/618 FRET acceptor dye, see Figure 29.[601] The development of FRET was used to confirm that the thiol group remained available for reaction with the dye and was not buried under the polymer coating or coordinated to the inorganic surface of the QD. Modification of the same polymer coating with a maleimido-fluorescein yielded a FRET-based ratiometric pH sensor with an ∼450 nm emitting CdS/ZnS QD.[601]

In pursuit of developing multimodal imaging agents, Duconge and colleagues overcoated CdSe/CdZnS QDs with different ratios of 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine−*N*-[methoxy(poly(ethylene glycol)-2000)] (DPPE−PEG2000) and DSPE−PEG2000-NH_2 lipids and then targeted the pendant primary amine for a multistep modification.[602] The first step modified the amine with heterobifunctional sulfo-LC-SPDP, followed by reduction of the pyridine disulfide to yield a free thiol, which was then labeled with a radioactive maleimido-[^{18}F] reagent, see Figure 30. The probe was then injected in mice, and whole body biodistribution and pharmacokinetics were monitored using PET, while the kinetics of cellular uptake was visualized with *in vivo* fibered confocal fluorescence imaging. Similarly, the Bakalova group demonstrated the use of QD−Gd complex bioconjugates for fluorescent and MRI imaging in an experimental animal model.[603] Here, standard Gd-chelating groups such as 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) were coupled to the amines displayed on a silica shell that encapsulated the QDs. The porosity of the silica shell itself allowed for a high density of the complexes to be incorporated into the final probe, which also demonstrated significant photosensitization-driven cytotoxicity in several cancer cell lines.

3.2.2. Electrostatic and Other Interactions. Mattoussi's group pioneered assembling QD−protein bioconjugates via electrostatic interactions.[100] In the first iteration, a chimeric MBP expressing a strongly positively charged leucine zipper attachment domain (MBP-zb) was engineered to bind to the negatively charged surface of DHLA-capped CdSe/ZnS QDs via electrostatic self-assembly. QD−MBP-zb bioconjugates formed with no particle aggregation, and the MBP maintained biological activity as evidenced by binding and maltose-induced release of the bioconjugates from amylose affinity resin. Interestingly, the authors also noted QD PL emission enhancement

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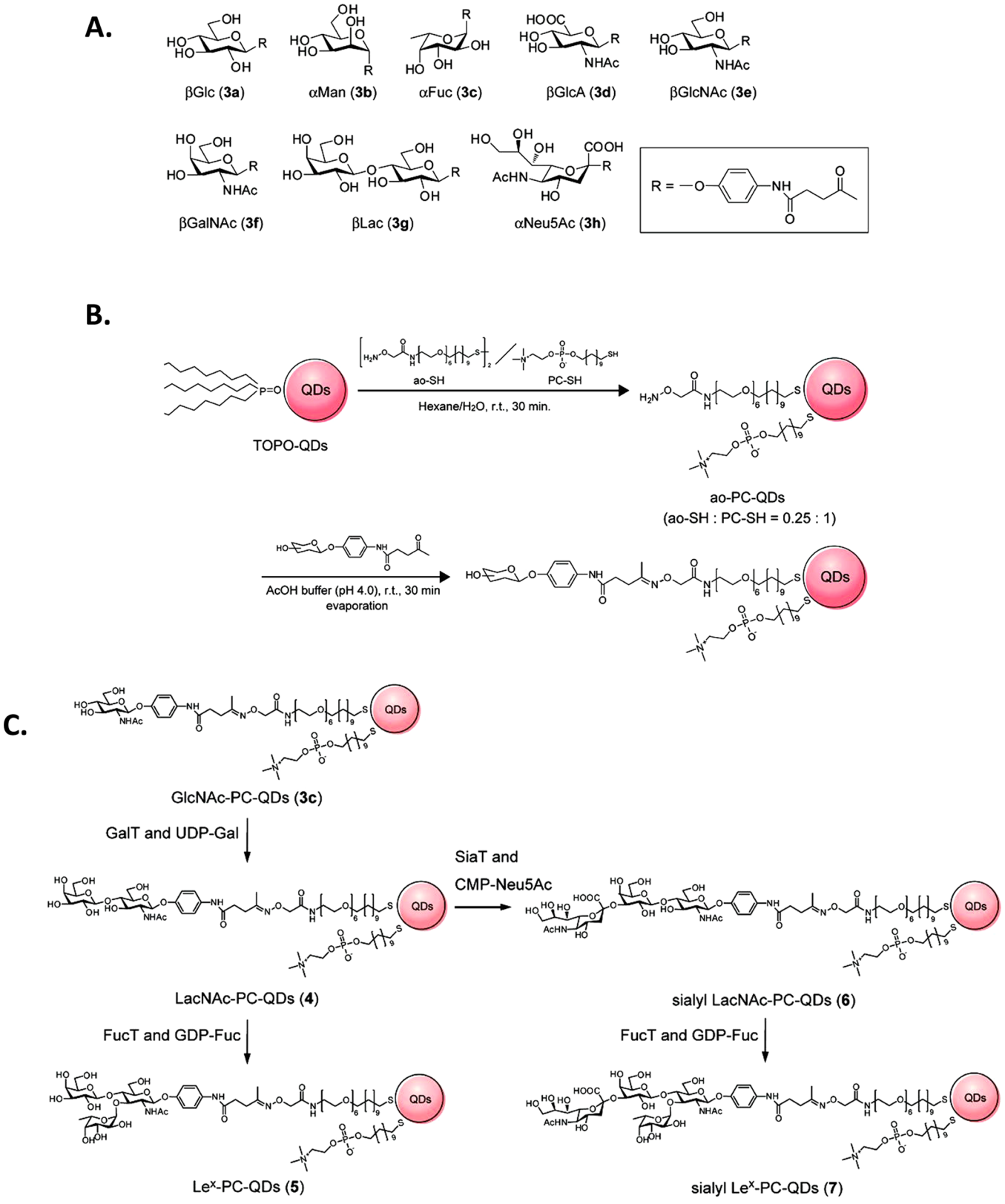

Figure 28. The preparation of glyco-PC–quantum dot bioconjugates. (A) Chemical structures of *p*-(4-oxopentanamido)-phenyl glycosides **3a–h**. (B) A simple protocol for the preparation of glyco-PC–QDs by the reaction of aminooxy-functionalized terminal-thiol-PC–QDs with glycosides **3a–h**. (C) Enzymatic sugar extension of GlcNAc-PC–QDs. Synthetic route of four glyco-PC–QDs by means of three recombinant glycosyltransferases and sugar nucleotides (PC, phosphorylcholine). Figure adapted from ref 596. Copyright 2011 American Chemical Society.

upon protein interaction that reached a plateau corresponding to the saturation of MBP-zb bound around the QD; a phenomena attributed to protein passivation of QD surface states that was subsequently exploited to confirm QD–protein interactions.[604] This electrostatic approach was extended to engineering a two-domain protein G–leucine zipper fusion

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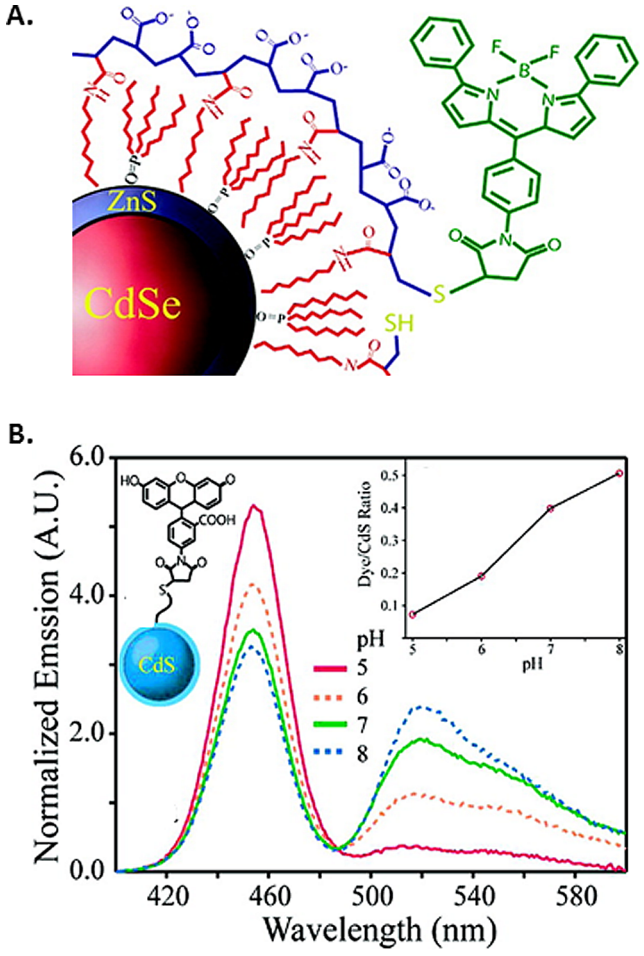

Figure 29. Quantum dot pH sensor. (A) RAFT-synthesized poly(acrylic acid) 40% modified with octylamine to make an amphiphilic polymer. Note that the polymer functionalities are randomly placed. Subsequent encapsulation of a hydrophobic CdSe/ZnS NPs imparts solubilization in water and a free thiol to conjugate to a maleimide functional dye (BODIPY 577/618, as shown in green). (B) Emission of a fluorescein/CdS/ZnS NP results in a ratiometric fluorescence sensor for pH. An isosbestic point is observed at ~490 nm. Inset on the left shows a cartoon of the coupled construct. Inset on the right shows the ratio of the fluorescein (520 nm) to CdS/ZnS (455 nm) emission as a function of pH. Figure adapted from ref 601. Copyright 2008 American Chemical Society.

(PG-zb) expressing the IgG binding domain of streptococcal protein G, which was combined with MBP-zb to create QD−antibody immunoreagents.[605] Here, the PG-zb functioned as an adaptor linking antibodies to the QD, while the MBP-zb was utilized as a purification tool to attach and release the QD-conjugates from amylose resin after washing away excess antibodies. These mixed QD adaptor/purification reagents were demonstrated in immunoassays targeting staphylococcal enterotoxin B and the small molecule explosive TNT. The utility of this approach was then extended to demonstrate a simultaneous four-color QD multiplexed immunoassay jointly targeting cholera toxin, ricin, shiga-like toxin 1, and enterotoxin B.[606]

The adaptor/purification mixed QD−protein approach was later extended with avidin, which can allow QD assembly to almost any biotinylated protein. Although avidin's strong native positive charge (pI ≈ 10) facilitated direct interactions with the DHLA QD's negative surface, the number of avidin assembled per QD had to be maintained at a relatively low ratio (≤6 avidin/QD) to avoid cross-linking the QD−avidin particles between biotinylated proteins. Beyond targeting small molecules in immunoassays, similar QD PG-zb and avidin conjugates have been utilized for cellular labeling and functioned with equal efficacy for labeling transiently expressed membrane proteins or biotin groups attached to the cell periphery.[607] Niemeyer's group also utilized electrostatic interactions between four different positively charged peroxidases and CdS QDs capped with negatively charged mercaptoacetic acid ligands to form protein assemblies.[608] They noted that irradiating the QDs could be used to initiate enzymatic activity suggesting utility as unique light-controlled triggers. Additionally, Vannoy and Leblanc used noncovalent human serum albumin interactions with DHLA-capped QDs as a model system to study protein fibril formation.[609] Although clearly effective at assembling primarily antibodies along with some other proteins to QDs, this electrostatic/multiprotein approach has not been extensively utilized beyond these examples as other QD-bioconjugation chemistries have improved.

Attaching peptides to QDs via electrostatics has not been significantly pursued most likely due to the fear that the peptides would not remain associated with the QDs long enough to complete cellular delivery or other utility. However, Naik showed that this approach could be viable.[610] The 21-residue Pep-1 carrier (KETWWETWWTEWSQPKKKRKV-cysteamine) displaying a hydrophobic tryptophan-rich domain for membrane translocation and a hydrophilic lysine-rich solubility domain was shown to associate with commercial SA conjugated QDs and facilitate their delivery to HeLa cells. A subsequent mixed-surface approach combined biotinylated peptides with the electrostatically associated Pep-1 to achieve organelle targeting and apoptotic triggering. A biotinylated version of the simian virus 40 T-antigen nuclear localization sequence was found to target the QDs to nuclei while a biotinylated proapoptotic Grim protein mitochondrial-mediated cell death GH3 domain was found to induce a significant number of cells to undergo apoptosis. The authors surmised that the Pep-1 carrier initially remained associated with the QD conjugate facilitating the initial crossing of the cell membrane and then dissociated from the complex allowing further intracellular targeting.[610] A similar electrostatic assembly strategy also achieved Pep-1 mediated delivery of CdSe/ZnS QDs to adult human stem cells derived from surgically excised adipose tissue.[611]

The inherently strong charge of nucleic acid species also lends itself to complexation with QD materials. For example, Gao's group encapsulated QDs in polymaleic anhydride-*alt*-tetradecene and then grafted a careful mix of *N*,*N*-dimethylethylenediamine to create a mixed amine/carboxyl QD surface.[612] This surface functioned as both a proton sponge and a site for siRNA adsorption. The QD−siRNA complex was optically tracked during cellular uptake and demonstrated improved gene silencing along with a concomitant reduction in cellular toxicity compared with transfection agent mediated siRNA delivery. In another example, Zhang et al. characterized the dissociation of polymer/DNA polyplexes designed for gene delivery by exploiting changes in QD PL.[613] A pH-responsive pentablock copolymer was designed such that it formed stable complexes with plasmid DNA via tertiary amine segments. Increasing concentrations of the copolymer−DNA mix led to quenching of dithiocarbamate-capped QD fluorescence by complexation. Dissociation of the polyplex was induced using chloroquine and the efficiency of this process was measured through changes in QD PL. This system demonstrated strong

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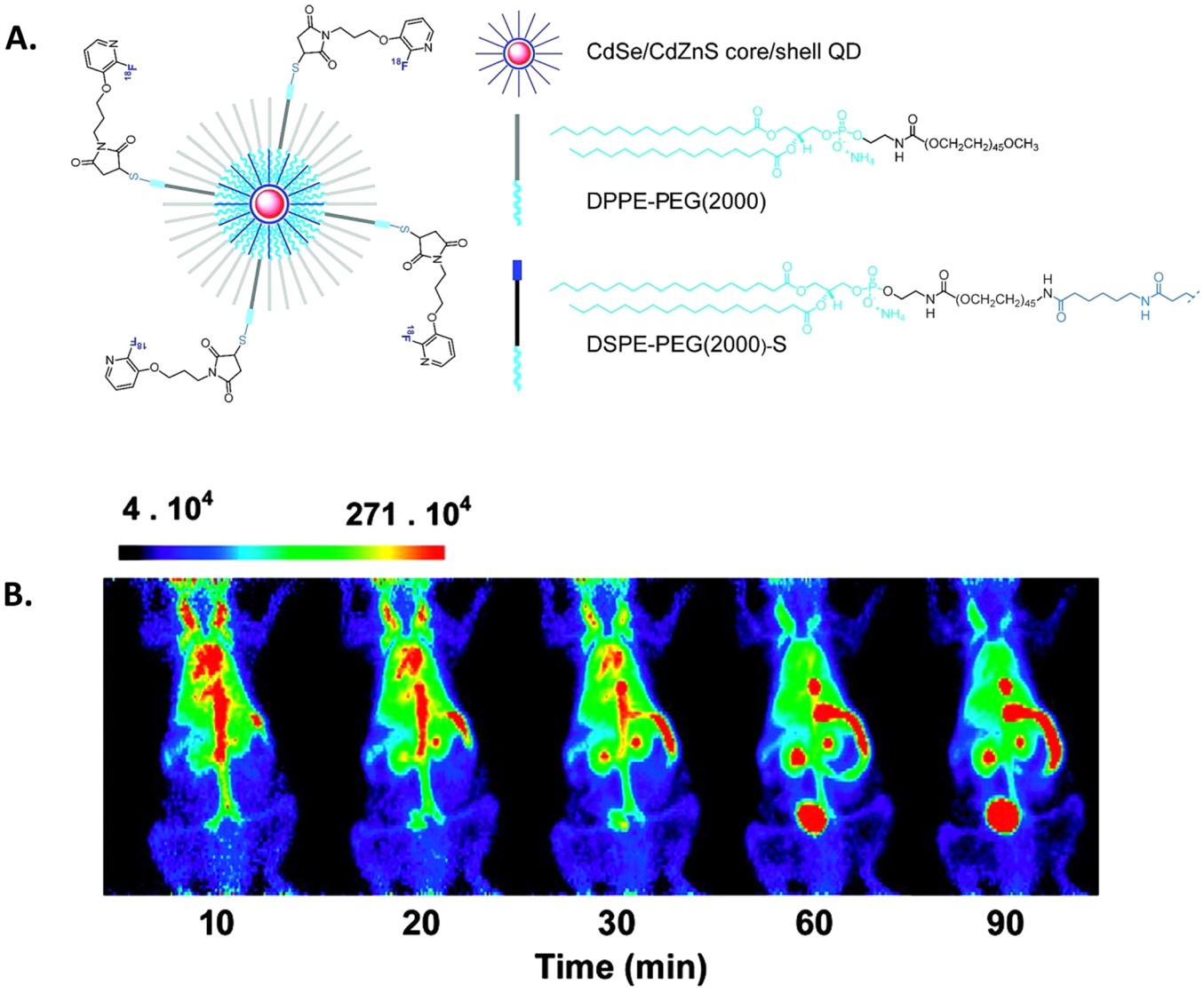

Figure 30. Modified phospholipid quantum dot micelles. (A) Schematic of the [^{18}F]-labeled PEG-phospholipid QD micelle. (B) Whole-body PET projection images acquired after intravenous injection of 200 μCi (7.4 MBq, 1 nmol) of [^{18}F]-QDs in a nude mouse. Each image corresponds to a 10 min acquisition time interval ending at the indicated time. Figure adapted from ref 602. Copyright 2008 American Chemical Society.

potential for studying the release kinetics of DNA delivered to cells.

Noncovalent QD encapsulation with sugars has also been reported. Betanzos and colleagues utilized bacterial endotoxin derived lipopolysaccharide aggregates to capture and encapsulate QDs suspended in an organic media and partition them in water.[614] Cellular labeling by the lipopolysaccharide−QD probes was then shown in mouse monocytes confirming that they could bind directly to the membrane of target cell types.

3.2.2.1. Direct Dative Interactions. 3.2.2.1.1. Proteins. QD−protein bioconjugates have been prepared based primarily on two types of dative interactions: metal-affinity coordination and thiol interactions. The metal affinity in this case is between His_n motifs and divalent transition metals, especially Zn(II) present on the QD surface. This interaction is conceptually quite similar to that of (His_n)−Ni−NTA chelation, see Figure 31 and section 2.5.3. Although His_n−QD surface interactions had been postulated for some time,[615] the first functional example of using this approach for bioconjugation was reported for attaching MBP expressing a C-terminal His_5 sequence to CdSe/ZnS core/shell QDs solubilized with DHLA.[604] The assembled QD−MBP conjugates were subsequently utilized as FRET-based sensors against the disaccharide maltose. This same interaction was later used to attach other His_6-appended proteins to QDs including an anti-TNT specific antibody fragment to create similar FRET sensors targeting explosives,[616] designer variable-repeat β-strand polypeptides to control the extension distance from the QD surface and investigate interactions with AuNPs,[231,617] FRET-based proteolytic substrates,[618] and several different types of fluorescent proteins for either intracellular uptake or enzymatic sensing.[199,304] The same approach has been adopted by other groups to functionalize similarly solubilized QDs with cytochrome P450 enzymes for use as photocatalysts,[619] fluorescent proteins to create long-range FRET assemblies,[620] monovalent avidin to provide cellular staining reagents,[621] and fatty acid binding protein to create a palmitate sensor.[622]

A detailed physical characterization of His_n−QD interactions using a series of model proteins and peptides terminally appended with increasing numbers of contiguous histidine

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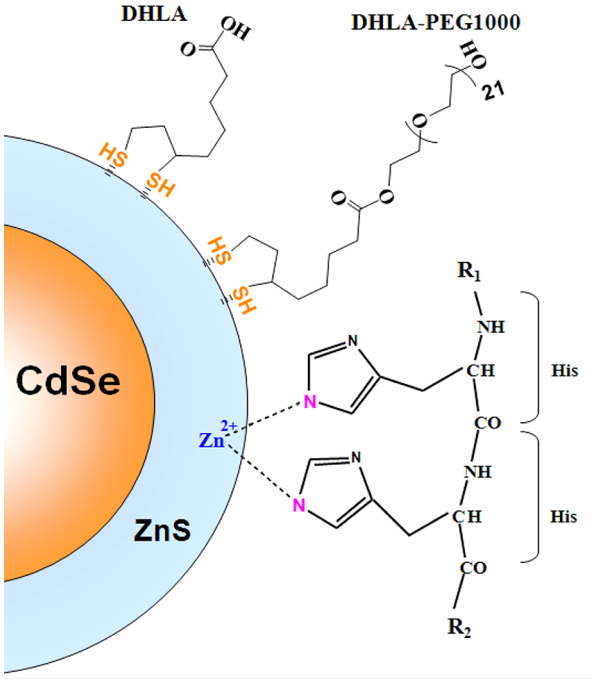

Figure 31. Metal-affinity coordination to quantum dot surfaces. Schematic representation of the metal-affinity interactions and binding with coordination of the histidine molecules to Zn on the QD surface. Structures of the DHLA and DHLA−PEG1000 capping ligands are also shown. R1 and R2 designate peptide sequence and the terminal amino acid, respectively. Only two histidine groups are shown for simplicity. Figure adapted from ref 623. Copyright 2007 American Chemical Society.

residues (His_n where n = 2, 4, 5, 6, 8, or 11) revealed that these multivalent and cooperative interactions manifest potent dissociation constants approaching 1 nM in bulk solution ($K_d^{-1} \approx 10^9\ M^{-1}$) and can reach equilibrium extremely quickly (seconds).[623] His_n−QD interactions also permit control over QD bioconjugation valency by an approximate one-to-one correlation with molar stoichiometries. It is important to note that this correlation is meant to apply to the average valency; the actual distribution within a particular configuration would follow a Poisson distribution process, and this is especially true at lower ratios.[624] The number of proteins that can be self-assembled to QDs via this interaction has also been examined in detail.[625] An analysis comparing experimental gel mobility shift data to ideal fits derived from models constructed using crystallographic structures showed that steric interactions, and not the number of available QD surface binding sites, determined the maximum number of proteins that could attach around the QDs: 12 ± 2 MBP (44 kDa), 20 ± 4 mCherry (27 kDa), and 30 ± 5 myoglobin (17 kDa) proteins per 550 nm emitting QD (diameter ∼6 nm) capped with DHLA. This linear correlation between molecular weight and conjugate valence suggests that for globular proteins, molecular weight can be a good predictor of maximum packing arrangement.[625] In contrast, a maximum number of 50 ± 10 peptides (<3 kDa) was found to assemble around the QD as a function of available QD surface binding sites rather than packing density. Interestingly, His_6-driven metal affinity is also applicable to many different commercial QD preparations despite the fact that they are surface-functionalized with encapsulating polymers. Dennis et al. showed that EviTag QDs solubilized with a lipid/PEG ligand could still permit binding of His_6-tagged fluorescent proteins to the QD surface.[626,627] Analyzing FRET interactions between the central QD donor and the conjugated fluorescent protein acceptors suggested that they penetrated the lipid and were attached to the Zn surface.[627] It should be noted that the QD ligands play a prominent role in determining which molecules can assemble on the nanocrystal in this manner. Large proteins tend to be sterically precluded from the QD surface when the ligands are bulky such as in the case of PEGylated molecules.[628] In this case, it is surmised that the ligand bulk along with that of the protein combine to prevent productive His_n−QD surface interactions. Smaller, far-less bulky peptides and DNA with their more linear structures usually do not encounter this issue. In general, the His_n motif offers several intrinsic advantages for assembly to QDs:[14,551]

- His_n motifs are commonly introduced to recombinant proteins to facilitate purification with Ni−NTA resin.
- Because the His_n motifs provide a single point of attachment to the QD, they eliminate undesirable cross-linking reactions and can, in many cases, even enable control over biomolecular orientation on the QD.
- His_n motifs can be considered bioorthogonal, since they are not normally found in natural proteins.
- The small size of the His_n motif does not usually disrupt native protein function.
- This assembly approach can be extended to many other types of biologicals (*vida infra*).

Rao's group demonstrated that QDs from Life Technologies capped with a carboxylated polymer could also be assembled with active His_6-tagged luciferase enzymes. These assemblies were developed for use as BRET-based protease sensors.[225] Interestingly, the authors noted that adding excess Ni(II) to the QDs significantly increased His_6-luciferase binding and subsequent BRET, strongly suggesting that the QD-surface carboxyl groups chelated the Ni(II) and bound His_6 in a manner analogous to NTA groups. Based upon these findings, Boeneman et al. reasoned that an appropriate fluorescent protein-His_6/QD combination should also be able to assemble *intracellularly* and FRET monitoring could allow *in vivo* confirmation of conjugate formation.[224] To test this, an mCherry-His_6 was engineered and expressed in COS-1 cells, which were subsequently microinjected with 565 nm carboxylated QDs that had been pre-exposed to a small amount of Ni(II), see Figure 32. Intracellular FRET from the central QD to the surface-assembled mCherry did indeed verify conjugate formation along with showing that FRET sensitization could substantially increase the fluorescent protein's intracellular photostability.[224]

Several groups have also developed QD surface ligands displaying accessible NTA groups for direct His_n-based metal-affinity coordination. These have been demonstrated for site-specific QD labeling of expressed, recombinant His_6-cellular membrane proteins *in vivo*,[629] for copurification and QD labeling of His_6-glutathione S-transferase from protein extracts,[630] for intracellular labeling and imaging following microinjection,[217] and as an imaging agent for His_6-CIIA anti-apoptotic protein in Western blot analysis.[223] In a modification of this approach, Dif and co-workers engineered QDs with hybrid PEG−peptide ligands expressing terminal aspartic acids designed to chelate Ni(II) for coordination.[631] This conjugate was elegantly demonstrated for single molecule tracking studies of His_6-end binding protein-1, which specifically binds to microtubules and regulates their dynamics during the formation

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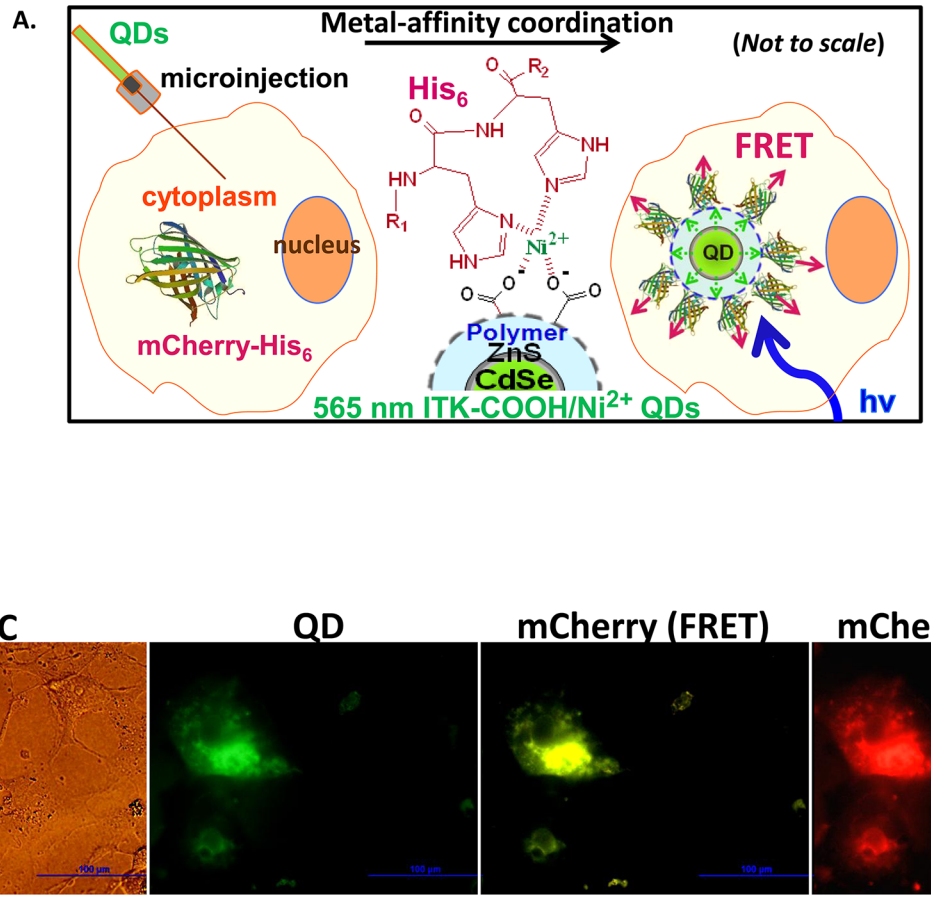

Figure 32. Intracellular metal-affinity coordination to quantum dots. (A) Protein-His_n/QD intracellular assembly. Cells expressing mCherry-His_6 proteins are microinjected with Ni(II)-supplemented 565 nm ITK-COOH QDs (left) resulting in His_6-driven protein coordination to the Ni(II)-COOH QD surfaces (right). Putative interactions of histidine residues with QD-chelated Ni(II) are shown in the center. QD excitation results in FRET-sensitized emission from mCherry confirming the intracellular assembly. (B) Cells expressing mCherry-His_6 microinjected with 565 nm ITK/Ni(II)QDs. Arrow indicates mCherry expressing cell without microinjected QDs. Note the lack of FRET signal from this cell. Figure adapted from ref 224. Copyright 2010 American Chemical Society.

of the mitotic spindle. The ability to monitor a single QD−protein interaction in this example is a direct reflection of the underlying interaction strength.

Thiol interactions can also potentially be used to attach proteins directly to the QD surfaces, although two issues significantly hinder this approach. The protein needs to display available and reduced thiols (nondimerized cysteines) on its periphery and have direct access to the QD surface without hindrance from the capping ligands. In essence, the thiolated proteins could then act as surface solubilizing caps or ligands for the QDs. However, monodentate thiol interactions with QD surfaces are characterized by very dynamic off-rates that can limit long-term conjugate stability.[30,615,632] In an effort to address this, Sandros et al. exploited a polycysteine domain derived from metallothionein to modify MBP for assembly onto CdSe and CdSe/ZnS QDs capped with 16-mercaptohexadecanoate.[474] The authors found that the polycysteine MBP had a higher affinity for the QD than MBP-His_5. A further benefit was provided by the use of a slight excess of Cd(II), which served to protect the metallothionein motif and allow site-specific orthogonal labeling of a cysteine distinct from this site. An alternate approach relied on growing an amorphous silica shell around water-soluble CdTe core-only QDs and then modifying the shell with PEG and thiol-terminated linkers, which could then be conjugated to antibodies.[576] The silica shell also served to increase QD photostability and prevent Cd leaching directly from these core-only nanocrystals.

3.2.2.1.2. Peptides. The initial lessons learned from utilizing metal affinity to coordinate His_n-appended proteins to QDs were similarly extended to peptides. In the first demonstration highlighting the potential inherent to this approach, dye-labeled peptide substrates were noncovalently assembled to QDs to yield bioconjugates of specifically desired valence that were capable of monitoring proteolysis.[633] A modular His_6-terminated peptide design allowed targeting of the proteases caspase-1, thrombin, collagenase, and chymotrypsin without significant changes to the design of each sequence. The ability to quantitatively assemble each dye-labeled peptide to the QDs in a controlled manner was essential to deconvoluting the protease-induced changes in intraconjugate FRET and converting them into units of activity. Peptide-based QD sensors targeting the apoptotic effector caspase-3 and botulinum neurotoxin A protease have been similarly assembled.[634,635] Analogous designs have also been extended to other QD−peptide substrate sensors targeting kinases and histone acetyltransferase activity.[636−638]

Bioconjugation using His_n sequences has also been effective for assembling CPPs onto a variety of QD materials, facilitating their cellular delivery. These peptides typically consist of a polyArg motif that is separated from the His_6-QD binding domain by a short intervening linker.[157,639−641] More importantly,

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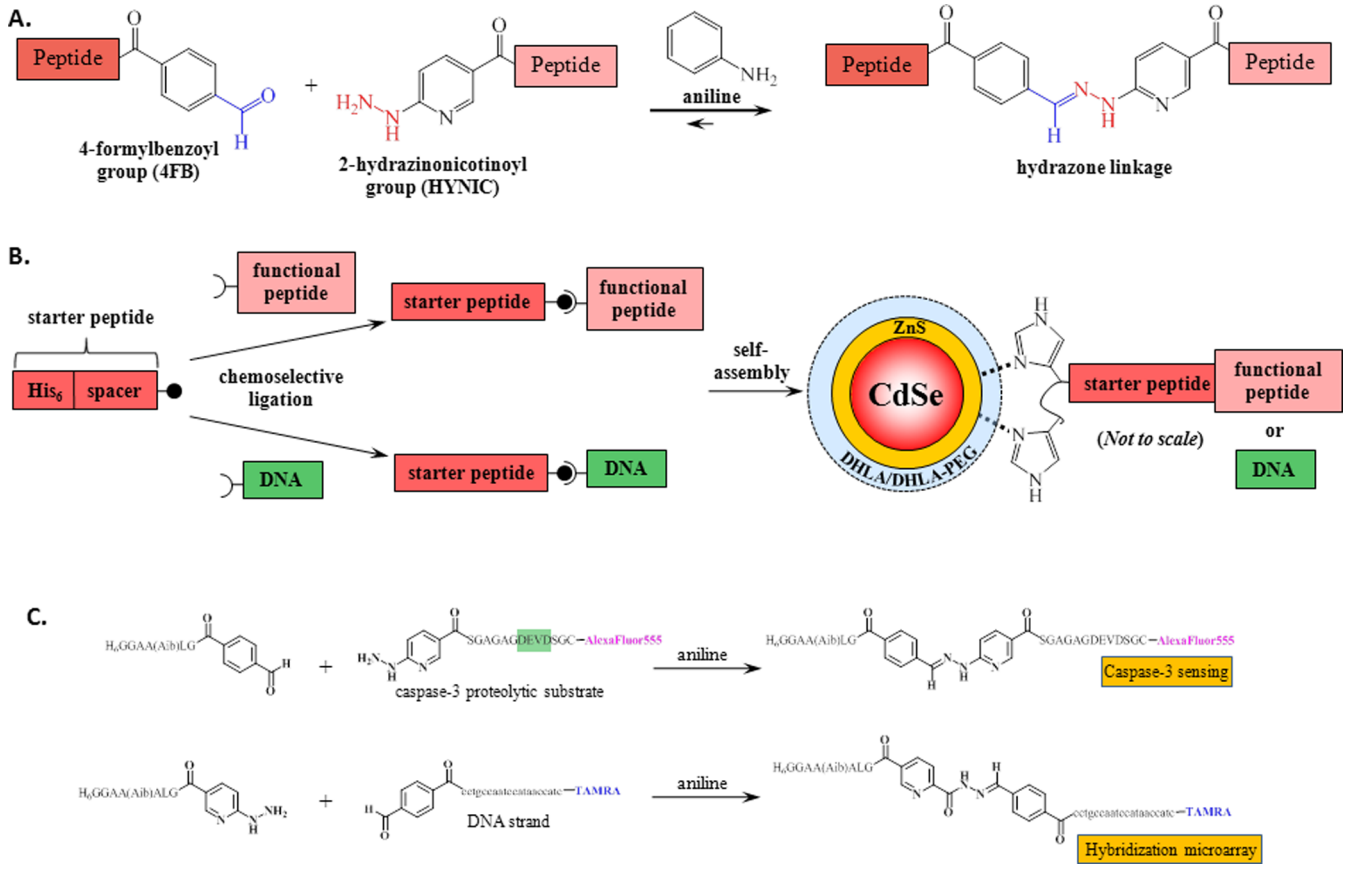

Figure 33. Combining metal affinity coordination with chemoselective ligation. (A) Schematic of the aniline-catalyzed hydrazone ligation between aldehyde and hydrazine peptidyl functionalities color-coded in blue and red, respectively. (B) Schematic of the two-step, modular design for the attachment of biomolecules to the surface of CdSe/ZnS core/shell QDs functionalized with charged DHLA or neutral DHLA–PEG ligands. Initially, a His_6-appended synthetic starter peptide is ligated to the appropriately functionalized target biomolecule of interest using chemoselective ligation. Isolated conjugates are then ratiometrically self-assembled to QDs for use in targeted bioassays. (C) Chemical structures of representative starter and modified peptides, DNA sequence, and resulting chemoselective ligates formed using aniline-catalyzed hydrazone coupling along with the corresponding assays in which they were applied. The location of the proteolytic site present is highlighted in green, and appropriate dye labels are shown at the points of linkage to the peptides or NA. Figure adapted from ref 635. Copyright 2010 American Chemical Society.

His_6-CPPs can be coassembled onto the QDs along with other "cargo", such as fluorescent proteins, highlighting the ability to induce cellular uptake of complex QD–protein conjugates.[198] The fluorescent protein cargos were attached to the QDs in this example with His_6-metal affinity or with biotin–avidin chemistry. The ability to control the number of QD-attached proteins allowed biological cargos exceeding ~10^3 kDa to be codelivered as part of the QD construct. Moreover, intracellular QD–fluorescent protein conjugate integrity could be verified by monitoring intra-assembly FRET. This exercise also highlighted the potential of these composite materials to help elucidate the complex mechanistic details that surround NP-mediated delivery of cargos such as drugs and proteins.[11]

The ease of self-assembly provided by His_n-QD assembly led to the development of a modular approach that could also be combined with chemoselective ligation chemistry to create functional QD bioconjugates.[635] This two-step modular chemistry relied on aniline-catalyzed hydrazone coupling to append "His_6-starter" sequences onto peptides and DNA, providing them with the ability to ratiometrically self-assemble to hydrophilic QDs. The inherent versatility of this labeling approach was highlighted by ligating proteolytic substrate peptides, an oligoArg CPP, or a DNA probe to the cognate His_6-peptidyl sequences, see Figure 33. Subsequent self-assembly to the QDs allowed the full constructs to be engaged as protease substrates, cellular labels, or hybridization probes, respectively, in targeted bioassays. Interestingly, when Chan's group used phage display to select for peptides capable of binding QDs in various biological media, they identified a 7-mer containing five almost contiguous histidines separated by only a lysine residue.[642]

Numerous examples of QD–peptide bioconjugates assembled using thiol-based dative bonding interactions also exist. Such QD–peptide bioconjugates have been successfully used for nanocrystal targeting and imaging *in vivo*. In 2002, Akerman et al. reported the assembly of three thiolated homing peptides on the surface of mercaptoacetic acid-coated CdSe/ZnS QDs for labeling specific vascular sites in mice.[643] Three peptides were used: (1) GFE, which binds to membrane dipeptidase on endothelial cells in lung blood vessels, (2) F3, which binds to blood vessels and tumor cells, and (3) LyP-1, which preferentially binds lymphatic vessels and tumor cells. In some cases, PEGylation of the QD surface was also required to prevent aggregation of the QD–peptide bioconjugates during circulation. *In vitro* epifluorescence microscopy revealed that GFE–QD bioconjugates specifically labeled lung endothelial cells that express membrane dipeptidase. Similarly, F3–QD and LyP-1–QD bioconjugates specifically labeled MDA-MB-435 breast carcinoma cells. The bioconjugates

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were then tested *in vivo* by injection into the tail vein of BALB/c mice. Normal BALB/c mice injected with GFE−QDs showed bright QD fluorescence in the lungs. To test *in vivo* tumor-targeting of F3−QD and LyP-1−QD bioconjugates, tumor xenografts of MDA-MB-435 tumor cells were injected subcutaneously in the chest of BALB/c mice. The tumor-containing BALB/c mice were then injected with F3−QD and LyP-1−QD bioconjugates, which showed targeting of tumor blood vessels and lymphatic vessels.

Thiolated peptides have been applied for direct cap exchange onto the QD, thus providing both solubility and, if needed, intrinsic biological utility. For example, Rosenzweig's group directly cap exchanged CdSe/ZnS QDs with the tetrapeptide RGDC, where the R was subsequently labeled with a rhodamine dye FRET acceptor.[644] The QD-donor/dye-acceptor system was then utilized as a FRET-based proteolytic sensor. Similarly, Pinaud et al. used phytochelatin-derived peptides to biofunctionalize QDs.[102] Analogous to some of the peptides described above, the phytochelatin sequences were also modular in design. They were designed to bind to the nanocrystals via a C-terminal adhesive domain composed of multiple cysteine pair repeats flanked by hydrophobic 3-cyclohexylalanines. This was followed by a flexible hydrophilic linker domain to which various bioaffinity tags such as biotin and a nuclear localization sequence were then attached. The peptide-decorated QDs were subsequently utilized in a variety of direct and indirect cellular labeling applications. The only potential drawback to this elegant approach would be the limitation of synthesizing the requisite peptides in a large scale manner, given the need for excess material during cap exchange.

3.2.2.1.3. DNA. Strategies also exist for creating QD−DNA bioconjugates using self-assembly methods. For example, Medintz et al. created QD−DNA bioconjugates via His_6-mediated self-assembly of DNA onto QDs, a platform that was also functional for sensing DNA hybridization by FRET.[303] The His_6 tag allowed for facile assembly of QD bioconjugates and excellent control over the valence (ratio of biomolecule/QD). A thiol-reactive His_6 peptidic linker was covalently attached to a thiolated, Cy5-labeled molecular beacon and then ratiometrically self-assembled onto CdSe/ZnS QDs capped with DHLA. Assembly of the QD bioconjugates was verified using gel electrophoresis, steady-state fluorescence spectroscopy, and atomic force microscopy. The intrinsic molecular beacon stem−loop structure provided for FRET between the Cy5 and the QD. Addition of complementary target DNA resulted in hybridization and a concomitant opening of the stem feature, which altered FRET efficiency.

Synthetic oligonucleotides can be readily obtained with terminal-thiol modifications, and this has allowed direct conjugation to QDs at their inorganic surface. QDs functionalized in this manner have been utilized in a number of FRET-based hybridization and enzymatic monitoring assays.[645−647] In an alternate strategy that targeted the QD conjugate to bind and illuminate DNA, Zhao et al. capped their CdTe QDs with *N*-acetylCys.[648] The authors used the functionalized QD surface-displayed α-amido-carboxylic moiety to subsequently complex the DNA dye Hoechst 332 and found that the presence of this dye steered the conjugate to the nucleus and illuminated the chromosomal DNA in exposed HK-1 cells.

3.2.3. High-Affinity Secondary Binding. The strong biotin−avidin interaction coupled to the wide availability of biotinylation reagents makes them an attractive initial bioconjugation strategy for attaching proteins and antibodies to QDs. Thus, the first commercially available bioconjugates consisted of QDs functionalized with SA (presumably assembled with EDC chemistry). These materials are still available and loosely characterized as displaying 5−15 SA molecules, each with three nominally available binding sites, yielding an estimated maximum of 15−45 conjugation sites per nanocrystal.[81] Similarly, biotin displaying QDs are also commercially available from several sources, see Supporting Table 1, Supporting Information. The number of proteins, peptides, and DNA conjugated with these materials and their uses are too numerous to list beyond the following representative examples, which focus on the applicability of this chemistry in sensor development.

Gill et al. utilized biotin−SA interactions to assemble CdSe/ZnS−GOx bioconjugates that served as ratiometric fluorescent glucose nanosensors.[649] The sensors were designed to transduce signal as QD PL quenching induced by H_2O_2, a by-product of GOx-catalyzed glucose oxidation. GA-capped CdSe/ZnS QDs were conjugated with FITC-labeled avidin and then linked to biotin-modified GOx (B-GOx) to yield the final B-GOx−FITC-avidin−QDs bioconjugates. Glucose was oxidized by the GOx to generate H_2O_2, which quenched QD PL, while the reference FITC dye remained unaffected. In an example of peptide application, Biju et al. explored the use of an insect neuropeptide, allatostatin, to prepare QD−peptide bioconjugates for transfecting, targeting, and imaging living mammalian cells.[650] First, allatostatin 1 was treated with biotin-*N*-hydroxysuccinimidyl ester to yield biotinylated allatostatin. SA-coated 605 nm emitting CdSe/ZnS QDs were mixed 1:1 with the biotinylated allatostatin to afford the allatostatin−QD bioconjugates. The allatostatin−QD bioconjugates were shown to efficiently transfect NIH 3T3 and A431 mammalian cells. After 1 h, bright fluorescence was observed inside the cell nuclei, which indicated that allatostatin facilitated QD delivery to the nucleus. By implementing a modified hybridization approach, Zhang et al. utilized SA−biotin interactions to assemble QD−DNA bioconjugates capable of sensitive single-molecule FRET-based detection of complementary DNA.[651] The QD nanosensor provided better sensitivity and 100-fold improved responsivity (i.e., signal when target is added) for DNA hybridization than commonly used dye-based molecular beacons.

The combination of QDs with biotin−SA chemistry can be a very potent one giving rise to "deep" multiplexed optical analysis and diagnostics.[652,653] However, it is important to note that the heterogeneous attachment of SA to the QDs in conjunction with its tetravalency can lead to highly heterogeneous QD−DNA structures. A detailed photophysical characterization of the architectures resulting from His_6−DNA assembly to QDs and assembly of biotinylated-DNA to SA-functionalized QDs revealed how starkly the resulting conjugates can differ.[81] A series of complementary dye-labeled DNA acceptors were positioned at different points along a double-stranded DNA sequence attached to the QDs with each chemistry, and the resulting FRET interactions were monitored and used to probe and model the underlying structures. His_n-self-assembly yielded QD−DNA bioconjugates in which predicted and experimental separation distances matched reasonably well. Although displaying some rotational freedom, the DNA in this case assembled to the QD with its structure extended out from the QD surface as predicted. In contrast, although displaying extremely efficient FRET, measurements from the QD−DNA bioconjugates assembled using biotin−SA chemistry did not match any predicted separation distances. Modeling revealed that the random orientation of SA on the QD surface resulted in DNA with a wide variety of possible

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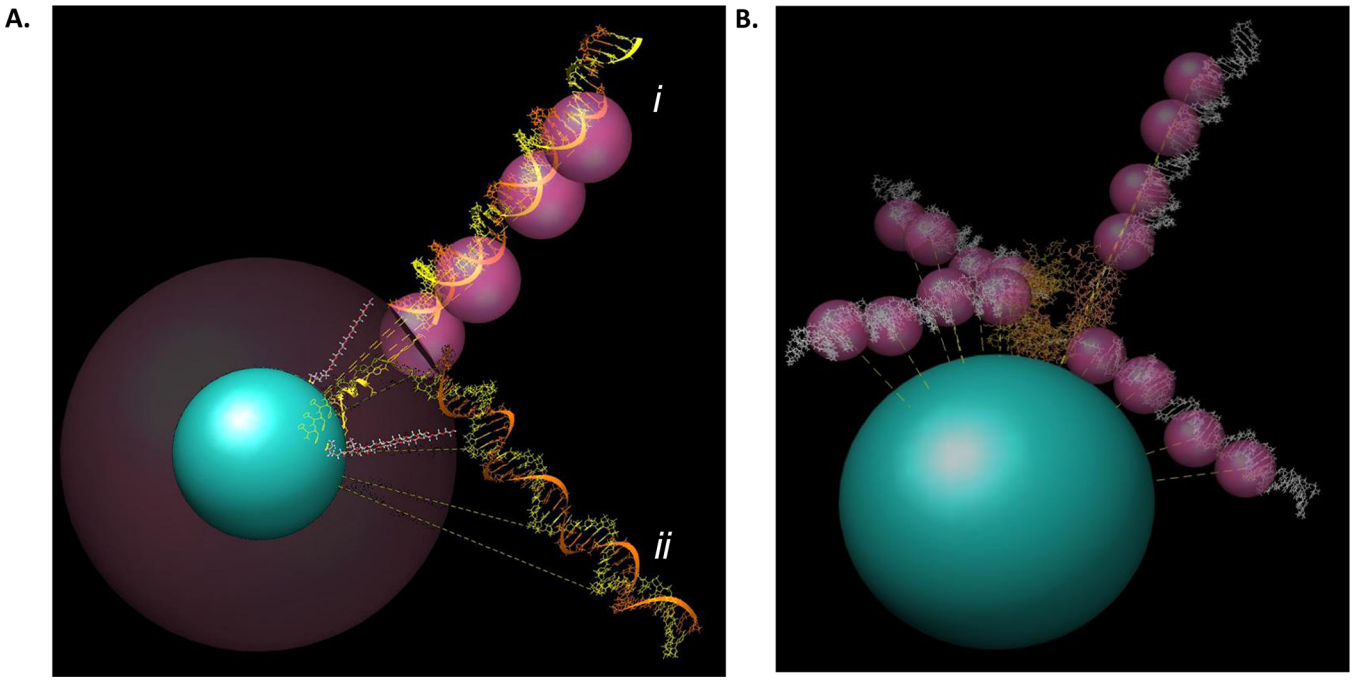

Figure 34. Models of peptido-DNA structure as assembled to quantum dots. (A) His_6-peptide−DNA bound to 530 nm QDs. The QD is shown as the central blue sphere, the DHLA−PEG ligand is indicated by the crimson halo with an estimated extension of 30 Å utilized here for modeling purposes. DHLA−PEG ligands in an energy-minimized conformation are shown within the crimson sphere. The His_6 portion of the peptide is shown with a yellow ribbon attached to the HYNIC linker. Individual DNA strands within the dsDNA structure are shown in orange and yellow. The rotational extension of the dye molecules is shown by the magenta spheres. Two possible orientations of the DNA relative to the QDs are shown, (i) DNA extending linearly outward from the QD surface and (ii) DNA adjusted for actual FRET-measured values. Dashed lines represent expected or measured distances for each configuration. (B) Biotinylated-DNA bound to the 605 nm streptavidin QDs. The QD core/shell polymer is simulated by a blue sphere of ~75 Å radius according to manufacturer specifications. The SA is shown in orange with DNAs (white) attached at all four binding sites. Fluorescent extensions of the dye molecules are shown by the magenta spheres. Note that regardless of orientation, several dyes at all possible acceptor sites are always in close proximity to the QD surface. Figure adapted from ref 81. Copyright 2010 American Chemical Society.

orientations relative to the QD, the position of which cannot be controlled during the self-assembly process, see Figure 34.[81] Clearly, if a particular QD−DNA biocomposite structure is desired, the type of bioconjugation chemistry utilized will be an important factor and should be carefully considered beforehand. For example, random orientations may suffice for hybridization applications, while controlled orientations may be needed for assembly of functionalized 3-D or origami structures. Indeed, there are already reported examples where such differences would have had a significant impact on the QD−DNA construct's final function such as in a recently assembled QD−DNA multistep-FRET photonic wire.[654]

Beyond biotin−avidin chemistry, a variety of antibody and other molecularly functionalized QDs are also available that provide direct QD conjugation to other common haptens/target molecules. These include QDs that recognize glutathione-*S*-transferase (GST) or dinitrophenol, along with wheat germ agglutinin conjugates that bind to *N*-acetylglucosamine and *N*-acetylneuraminic acid (sialic acid) residues on glycoproteins and glycolipids (www.Invitrogen.com). QDs functionalized with secondary antibodies or $F(ab')_2$ antibody derivatives targeting the conserved IgG heavy and light chains on primary antibodies derived from mouse, rabbit, goat, rat, chicken, and humans are also available and are designed for use in fluorescence microscopy, binding assays, and flow cytometry (www.Invitrogen.com). These are further summarized in Supporting Table 1, Supporting Information.

3.2.4. Biological Templating. As mentioned, biotemplated synthesis of NPs is an alternate strategy to directly attach biomolecules to nascent NP materials. While the main focus has been on the synthesis and properties of the resulting NPs, this approach can also potentially provide bioconjugated NPs that are readily available for further applications; this may be especially true for the DNA-programmed growth of QDs. The Kelly group has previously shown that the use of chimeric or modular DNA sequences can accomplish both by providing one sequence that controllably programs nanocrystal growth by serving as a ligand and providing passivation, while another portion of the same sequence contributes to further biorecognition.[374] The DNA portion that serves as a ligand is constituted from a phosphorothioate backbone for QD interactions while the recognition domain is backboned by standard phosphates, see Figure 35. Synthesis is carried out in ambient atmosphere at 100 °C with water as the solvent. CdTe QDs obtained in this manner and displaying complementary sequences, or alternatively thrombin and cell-binding aptamers, were shown to bind DNA, thrombin, and cells displaying the requisite markers.[374] The same group extended this approach to create higher order programmed structures consisting of controlled arrangements of different sized QDs.[370] Within these structures, finite control over the valence of DNA per QD was absolutely critical because it directly contributed to the order and position of a particular QD (see Figure 35). The assembled composites were found to act as a coupled entity under the right pH conditions and manifested what appeared to be a very efficient FRET process from the smaller green to larger red QDs. The polydispersity and spectral properties of QDs synthesized in this manner are not yet equal to those synthesized

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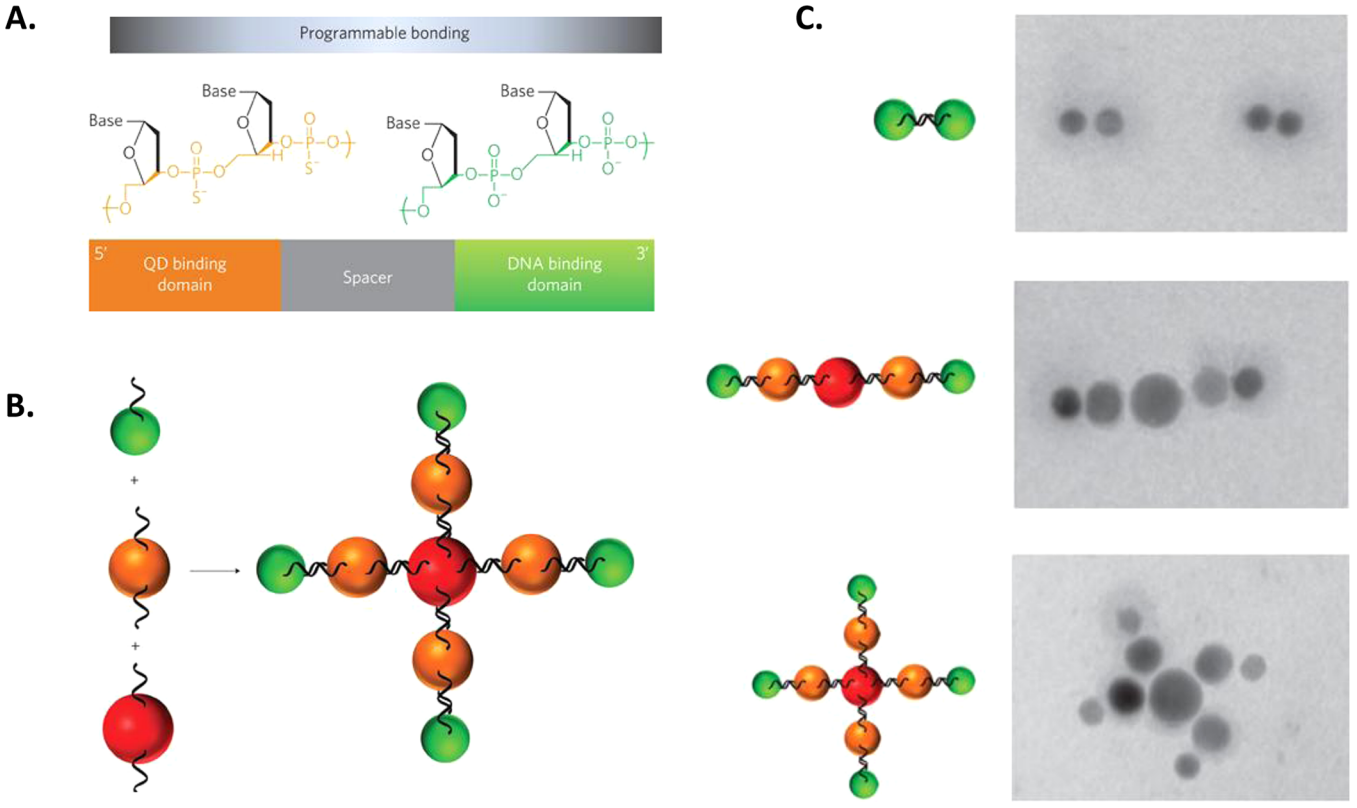

Figure 35. DNA-mediated synthesis and assembly of quantum dot structures. (A) Chemical structure of phosphate and phosphorothioate fragments and the schematic design of DNA strands. (B) Programmable bonding to allow selective interactions between specific different QDs to generate desired assemblies. (C) Representative high-resolution TEM images of DNA-programmed QD complexes. Reprinted from ref 370. Copyright 2006 Macmillan Publishers Ltd.

using pyrophoric conditions in organic media. Nevertheless, the potential of this approach is clearly evident from just this initial demonstration. It will be particularly interesting to see whether His_n or thiolated peptides can be added into this synthetic mix to yield mixed surface QDs derived in the same manner but also capable of other utility such as cellular uptake.

Overall, the undiminished interest in applying QD properties in a biological environment has led to the design, prototyping, and availability of many different bioconjugation chemistries with demonstration of a myriad of functional conjugates. With perhaps the exception of polymeric NP bioconjugate use in drug delivery (see below), this NM may have already accumulated the widest variety of bioconjugation descriptions to date. We also note that much of the chemistry utilized for peptide and DNA assembly to QDs mirrors that utilized for proteins but with the constraint of the smaller size factored in. Interestingly, peptide and DNA attachment may not require complex chemistry because it can be efficiently accomplished in a facile manner using EDC chemistry. Issues of heterogeneity can be minimized by limiting the number of reactive sites per biomolecular target to one. For example, coupling QDs displaying carboxyl groups to peptides displaying a single primary amine only allows for one reaction outcome (a Poisson distribution of conjugate valence notwithstanding). In terms of controlling QD−peptide conjugate valence, judicious choice in reactant stoichiometry and empirical optimization can provide for excellent control over the final ratios.[655]

3.3. Metal Oxide Nanoparticles

3.3.1. Iron Oxide. IONPs are among the most extensively studied types of NMs. This intense interest is justified by the underlying characteristics that make IONPs very attractive for a variety of applications in materials science and biomedical engineering.[656−659] The synthesis of IONPs is facile and cost-effective, yielding biocompatible colloidal suspensions that display unique, size-dependent magnetic properties generally referred to as superparamagnetism. This phenomenon appears at the nanoscale when the dimensions of a ferromagnetic material are reduced below the typical size of magnetic domains.[660,661] Superparamagnetic (SP) materials consist of only a single magnetic domain, and the kinetic energy transferred to the system from background thermal motion is sufficient to cause reversal of magnetization. SP materials are therefore characterized by paramagnetic behavior; however, they also display a very large magnetic susceptibility. In practical terms, SP materials are prone to elevated magnetization when subjected to a magnetic field yet revert to their nonmagnetized state upon field removal. Suspensions of SP-IONPs are generally referred to as *ferrofluids* and are essentially paramagnetic liquids with a magnetic behavior that is useful in several biomedical applications.[658,659,662−664] Biocompatible ferrofluids can be obtained by synthesizing a colloidal suspension of IONPs in the presence of a dispersant, with polysaccharides such as dextran or CHI extensively utilized for such purposes. Ferrofluids have been clinically employed as MRI contrast agents, where the IONP size and coating defines the biodistribution, and consequently, the organs that become magnetically highlighted.[665] More recently, IONPs have been investigated as energy transducers for the hyperthermic treatment of cancer[666] and also as drug delivery vehicles.[667] Briefly, when IONPs are exposed to an alternating magnetic field, their magnetic dipole rapidly inverts as it aligns with

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the external field. The magnetization curves for this process indicate that some of the transferred energy is also dispersed by alternative mechanisms (Néel relaxation and thermal motion), resulting in a very rapid and efficient heating of the environment surrounding the NPs.[668] Heating cancer tissue above 46 °C usually results in tissue death, and this thermal ablation approach to treatment has already been clinically employed.[669] Adding to biomedical interest, IONPs are biocompatible, are readily metabolized by the liver, become part of the body's iron pool, and are entirely cleared in less than 48 h.[670]

IONPs consist of a crystalline core of either Fe(II) or Fe(III) oxide with either magnetite (Fe_3O_4) or maghemite (γFe_2O_3) crystal structure. Often, the initial IONP synthesis generates magnetite, which is then slowly oxidized to maghemite.[671] The relative magnetite or maghemite content, as well as the average size distribution of the NP population, is defined by the synthetic conditions; these have been amply reviewed.[663,672] The most common and cost-effective synthetic method to obtain IONPs is the Massart method, where a defined stoichiometric ratio of Fe(II) and Fe(III) salts is coprecipitated in aqueous basic conditions to give γFe_2O_3 NPs.[673] Albeit rapid and able to generate a large amount of IONPs, Massart's method allows very limited control over the size distribution of the population. Better methods more suited to yield relatively monodispersed IONPs are usually based on synthesis in constrained environments, where the crystal growth is confined within a preformed templating structure such as reverse micelles,[674] liposomes,[675] dendrimers,[676] phospholipid vesicles,[677] or apoferritin protein cages.[678] The choice of templating environment paired with additional synthetic parameters (pH, temperature, concentration, etc.) permits the synthesis of highly monodispersed and water-dispersible IONPs. Thermal decomposition of organometallic Fe precursors is also a very simple and rapid way to obtain highly monodispersed and crystalline IONP populations, usually with superior magnetic properties.[679] Thermal syntheses, however, are mostly conducted in organic solvents, and this must be considered if the resulting IONPs have to be used in an aqueous environment. Other less common synthetic methods are also available to access IONPs, as reviewed in refs 663, 672, and 680.

Generally speaking, biofunctionalization of IONPs can be attained either directly by conjugation to the native oxide surface or indirectly by conjugation to the surrounding stabilization layer. Similar to most colloidal NP suspensions, a key aspect in the preparation of IONPs is the introduction of surface stabilizers or ligands that prevent the colloidal suspension from aggregating and precipitating. These stabilizers can be introduced directly during the synthesis or immediately after formation of the initial IONP suspension. A wide variety of stabilizers have been introduced on the surface of IONPs including carboxylates, phosphates, polymers, and inorganic materials such as silica and gold, all reviewed in refs 663, 672, and 680. Colloidal stabilization provided by such molecular layers can be electrostatic[681] or steric in nature.[682] Although providing an extensive list of colloidal stabilizers is beyond the purpose of the present review, it is again essential to point out that these coatings play an important role in the further biofunctionalization of the IONP itself. In fact, due to the limited chemical reactivity of the IONP surface, it is often the coating and not the NP surface that is chosen as the site of chemical/biological functionalization. Because of the importance and versatility of IONPs, it is only natural that multiple routes for their conjugation to biological and organic entities have been extensively investigated.[680,683,684]

3.3.1.1. Direct Conjugation. Direct conjugation of molecules to the IONP surface is not a versatile procedure because it relies on the very limited reactivity of the native surface. This conjugation route relies on the ability of the incoming molecule to reach the native oxide surface and displace the stabilization layer. This can usually be achieved by selecting functionalizing moieties with high affinity for the oxide surface and by "driving" the displacement reaction stoichiometrically when increasing the concentration of the incoming reagent, that is, ligand exchange by mass action. Direct conjugation of organic small molecules or biomolecules to IONPs is therefore not very common, although there are examples where certain proteins and enzymes have been immobilized directly to the surface of freshly precipitated IONPs through a carbodiimide-mediated process.[685−690] In one example, the antibiotic vancomycin was directly immobilized to the IONP surface via an EDC-mediated coupling.[691,692] Although not much detail is provided on the reaction mechanism(s), it seems as though pH is important to ensure the availability of protonated surface hydroxy groups and that the reaction proceeds by forming ester or amide bonds with these groups. Simple electrostatic interaction has also been exploited to bind enzymes[693] and proteins.[694] These approaches are, however, rarely utilized as a single-step procedure for introducing biomolecules because they require freshly prepared IONPs and are most likely to yield surfaces with variable stability and heterogeneously immobilized conjugate structures.

More commonly, the surface of the IONP is functionalized with an intermediate molecular layer that is subsequently exploited for further conjugation to a wide array of molecular partners. A variety of nucleophiles, such as hydroxy groups,[695,696] diols,[697,698] carboxylates,[699−702] phosphates,[699,701] and thiols[703,704] have a relatively good affinity for IO surfaces and have been extensively investigated as functional intermediaries for attaining direct conjugation. The nucleophilic character of these moieties is responsible for binding to the electrophilic IO surface through interaction with iron's empty orbitals, forming a combination of covalent and physisorptive bonds.[705] The simplest method for direct conjugation onto a native IO surface is through binding with conjugated bases, such as phosphates,[706−709] sulfonates,[706] and carboxylates.[699−702] In the most common procedure, a freshly synthesized suspension of IONPs is immediately functionalized with an excess of nucleophile. Alternatively, the synthesis of IONPs can be conducted directly in the presence of the nucleophile, which in some cases has been shown to play a role in directing the synthesis.[710,711]

Carboxylates are a very common surface functionalization group for IONPs,[712] and are an important component in their synthesis when utilizing the thermal decomposition route in organic solvents, because alkyl carboxylates readily adsorb on the surface of the growing NP.[713−717] The resulting hydrophobic IONPs can be brought into aqueous suspension by exploiting a bipolar surfactant that forms a bilayer-type structure with the IONP coating[679,718] or other coating-exchange methods.[719−721] In a representative example, 11-bromoundecanoic acid was introduced on IONPs to yield bromoalkyl-substituted IONPs.[722] These IONPs were then conjugated to a lipase enzyme via nucleophilic substitution facilitated by the presence of Br as a leaving group. The covalently immobilized enzyme was found to be stable for 1 month following this attachment. While alkyl carboxylates are mostly utilized under nonaqueous conditions, other carboxylic acids permit the

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functionalization of IONPs in an aqueous environment. For example, citric acid, commonly used to stabilize AuNPs, has been successfully employed for IONP derivatization as well.[723−727] Two of the three available carboxyl groups are involved in bonding with the oxide surface. The remaining free carboxyl group acts as an electrostatic stabilizer and also as a chemical appendage for further conjugation. This permits, for instance, the utilization of standard EDC-mediated amide bond formation for attachment to primary amines.[728] Dimercaptosuccinic acid is also a common reagent for direct IONP surface functionalization.[729] This reagent binds to the IONP surface through a carboxyl and a thiol group, leaving the remaining free carboxyl group and thiol exposed on the surface.[720,730] While the surface carboxyl group is mainly responsible for electrostatic stabilization, the thiol group is available for conjugation to thiol-reactive molecules.[731] Conjugation to dyes,[732] peptides,[733] and antibodies[734] in this manner has been reported. Although dimercaptosuccinic acid-coated IONPs are mainly functionalized via thiol chemistry, with most of the remaining free surface thiols becoming partially bridged,[729,735] it has also been reported that the free carboxyl groups are available for conjugation.[736] Other, less common carboxylic acids such as aspartic, glutamic,[712] and gluconic acid[737] have also been utilized in a similar fashion.

Phosphates (PO_4^{3-}) and phosphonates (PO_2^{2-}) bind to IONP surfaces[706,738,739] in a manner similar to carboxylates although experimental evidence with carboxy-alkylphosphonic acids indicates that they form stronger bonds.[738−740] This reagent yields suspensions where the phosphonate moiety is bound to the IONP via a tridentate interaction, while the carboxylic acid is still available for conjugation.[709,741−743] Similar to alkyl carboxylates, it is believed that functionalization of IONPs with alkyl phosphates or phosphonates results in the formation of bilayer-type coatings, stemming from hydrophobic interactions between aliphatic chains, and this can somewhat limit the dispersibility of the resulting suspension in organic solvents.[739] Many phosphate or phosphonate derivatives have been successfully employed for the direct introduction of a variety of molecules.[744−746] In one example, IONPs were first functionalized with *N*-(phosphonomethyl)iminodiacetic acid, and after attachment to the surface, the iminodiacetic moiety was complexed with Ni(II) ions. The resulting IONPs−Ni(II) complexes were then used for the separation of His-tagged proteins including green fluorescent protein (GFP) and the enzyme chloramphenicol acetyltransferase.[747,748] With the same functionalizing reagent, urease was also conjugated to the acid moiety by carbodiimide-mediated coupling.[707] In another representative coupling, IONPs were initially grafted with 2-aminoethylphosphonate, leading to reactive surface amines that were used as appendages for the introduction of azides via diazotransfer or for direct reaction with isothiocyanate derivatized dyes.[749] The azide moieties were further reacted through CuAAC. Similar procedures have allowed immobilization of enzymes on IONPs[707,750] or the introduction of azides for click chemistry.[751] The photoactive group perfluorophenylazide has been introduced on IONPs using a phosphate derivative.[752] The subsequent photoinitiated reaction permitted the coupling to carbohydrates. Phosphorylcholine has also been utilized as a coating agent, binding to the IONP surface by the phosphate head.[753] This reagent, however, yields positively charged IONPs that are not easily amenable to further chemical conjugation.

Bis-phosphonate derivatives have been demonstrated as very good binders for IONP surface modification.[754] The two phosphonate moieties of this reagent chelate the oxide surface forming a very stable complex,[738,755] permitting the facile formation of a stable coating. As already pointed out for monophosphonates, when the bisphosphonate also carries a carboxylic moiety, only the phosphonate groups are involved in the complexation of the IONP surface.[756] The remaining free carboxylate can then be reacted with free amines with the usual EDC-mediated coupling or interconverted into another functional group using a heterobifunctional cross-linker. Using this bisphosphonate conjugation strategy, ^{99m}Tc and ^{64}Cu radionuclide-modified IONPs have been obtained.[757] Primary amines have also been directly introduced on IONP surfaces using a 3,3′-bis-(phosphonate) propionic acid linker.[758] Although the final product was a peptidomimetic−IONP conjugate, the reactive amine can conceivably be functionalized with a variety of molecular partners or interconverted into a different functional group through standard bioconjugation methods. In addition to carboxylates and phosphates/phosphonates, there is some limited evidence that sulfates[759,760] and sulfonates[706] can form bonds with the IONP surface, but they are not as commonly exploited.

3.3.1.2. Alcohol, Diol, and Catechol Coordination. The functionalization of IONPs with hydroxy groups is common and also widely used.[761] Simple alcohols bind weakly to IO surfaces[695,705,761] limiting the applicability of this functionalization approach. Poly(vinyl alcohol) binds more strongly,[762,763] although its limited reactivity hinders any further conjugation. In contrast, diols bind quite strongly to IONP surfaces and have been utilized successfully in a range of conjugation protocols. Diols can be readily prepared by OsO_4 oxidation of terminal alkenes, giving access to an array of functionalized diols that can be promptly introduced onto the IONP surface during ligand exchange.[698,761] Among the diols, catechols, in particular, form very strong bidentate complexes with metal oxides.[764] Catechol-derivatives display varying degrees of affinity for IONP surfaces and can generate remarkably stable colloidal suspensions.[765,766] Because the electron density of the hydroxyl groups is strictly related to the electron delocalization into the benzene ring, the effect of substituents has been investigated, indicating that their electronegativity also plays an important role in regulating the affinity for the surface.[767] Among catechol derivatives, dopamine (4-(2-aminoethyl)benzene-1,2-diol) is arguably the most extensively utilized linker for direct conjugation to IONP surfaces.[768−770] While the bidentate catechol portion of the molecule binds the IO surface through coordination bonds, the primary amine remains exposed to the surrounding environment, imparting water dispersibility and acting as a potential handle for introducing biomolecules or for functional group interconversion. For example, boronic acid was attached to the surface of a magnetite NP through 3,4-dihydroxybenzaldehyde and used for subsequent binding to glucose, see Figure 36A.[741] Dopamine and its nitro analog have been used to coordinate to the IONP surface in this manner, while the amine portion of the molecule permitted conjugation to peptides,[771,772] antibodies,[773] polymers,[774,775] chelating agents,[776] and polysaccharides.[777] In one representative example, Zhu and colleagues synthesized dopamine−peptide dendritic ligands for functionalizing IONPs, see Figure 36B, where an L-glutamic acid-based G3 dendritic ligand is shown with dopamine at the focal point.[772] The functionalized materials demonstrated saturation magnetization of 55 emu g^{-1}, which was significantly higher than that for materials coated with an L-lysine-based dendritic ligand. Kim et al. synthesized a catechol bis-NTA chelate and showed that when immobilized on an IONP, it could bind

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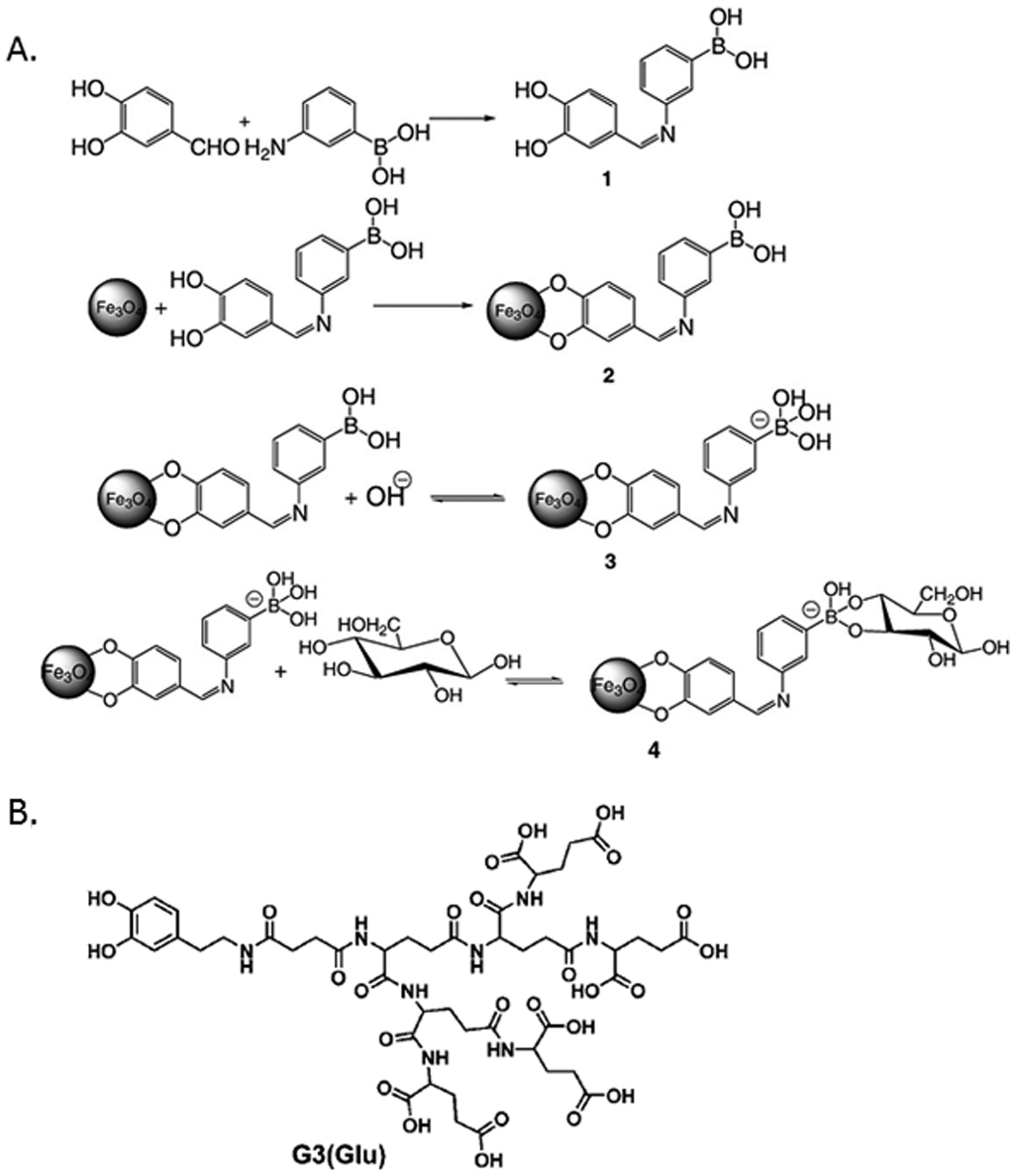

Figure 36. Iron oxide nanoparticle functionalization. (A) Synthetic route and glucose binding mechanism of boronic acid functionalized magnetite NPs. Figure reproduced from ref 741 with permission from Elsevier. (B) Structure of L-glutamic acid dendritic ligand. Adapted from ref 772.

native, folded proteins with higher efficiency than commercial materials, highlighting a strategy where polyvalency can increase the amount of purified protein in a much shorter time frame.[778] The use of dopamine as a conjugation linker has, however, been somewhat questioned as the catechol−IO complexes have a tendency to dissociate under physiological pH and in many cases are only stable in a basic environment. Further, dopamine is prone to oxidation and degradation, leading to deterioration of the interface and colloidal instability.[779] One promising avenue that may address this problem has been the recent synthesis of multidentate dopamine/PEG ligands where each ligand has multiple catecholic points of interaction with the IONP surface.[780] A closely related linker to dopamine, 3,4-dihydroxybenzylamine, has been similarly utilized to immobilize a lanthanide fluorophore following PEGylation; the latter was itself used to increase solubility.[781]

3.3.1.3. Silanes. The formation of silicon−oxygen bonds using organosilanes has been extensively exploited for the functionalization of a variety of metal oxides, including IO.[782−784] In what is probably the most common procedure, the IONP surface can be directly derivatized with bifunctional organosilanes. For instance, treatment with widely available aminopropyl-[783,785] or mercaptopropyl-trimethoxysilanes[742,783,786−788] permits the direct introduction of, respectively, an amine or a thiol moiety that can be then utilized for further conjugation or functional group interconversion. Note that coupling of the silane to the IO surface is in contrast to the growth of silica coatings on, for example, QDs, where the mercapto or amino group coordinates to the inorganic surface and the displayed silane group "primes" silica growth. The introduction of various hydrophilic organosilanes has also been employed as a strategy to disperse hydrophobic IONPs with concomitant surface functionalization in aqueous environments.[789] Other types of organosilanes have also been used for IONP functionalization[790] and have allowed the subsequent conjugation of enzymes,[791,792] polysaccharides,[793] proteins and peptides,[789,794,795] polymers,[796] and antibodies using standard bioconjugation methods.[797,798] Cyanoethyltrimethoxysilane has been utilized as a bifunctional silane for the introduction of nitrile

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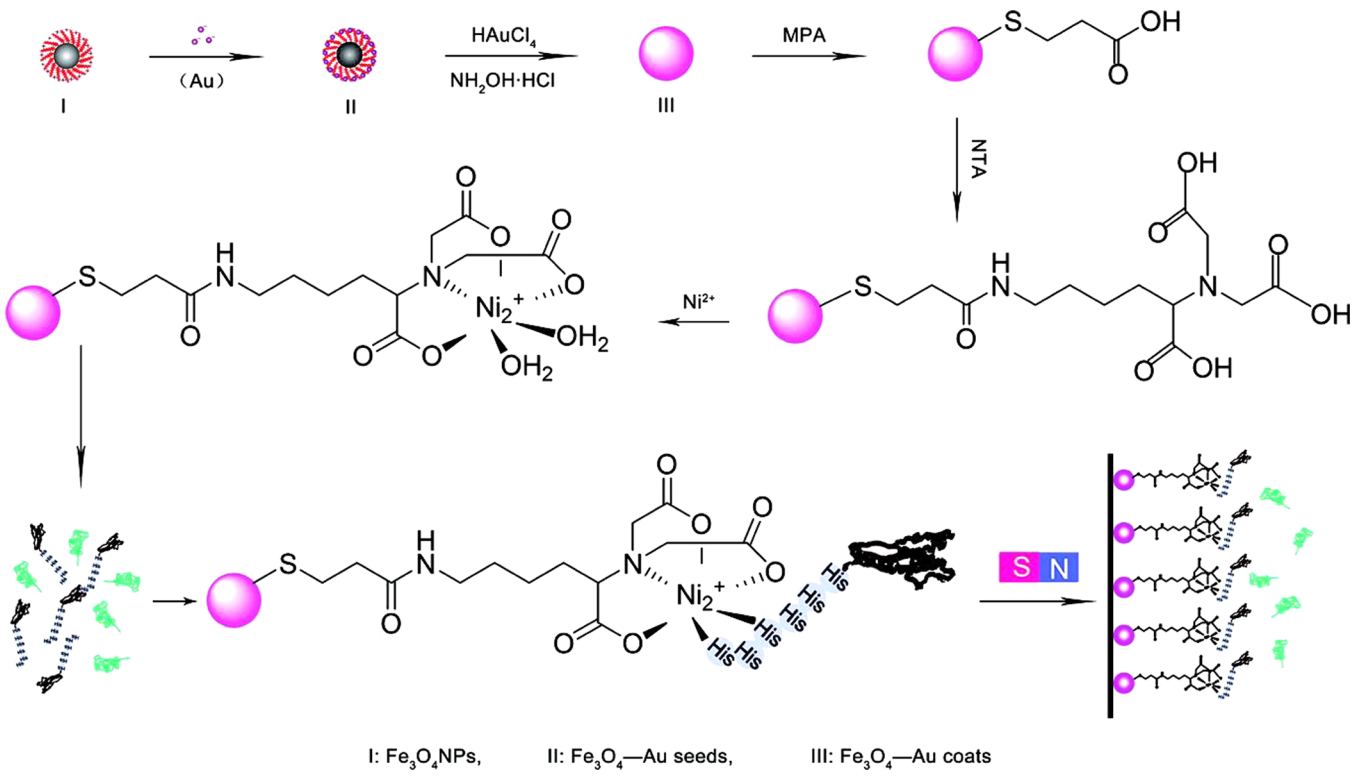

Figure 37. Schematic representation of the preparation procedure for biofunctionalized magnetic Fe_3O_4/Au–NTA–Ni(II) NPs and their use in enrichment and separation of proteins. Figure reproduced from ref 216. Copyright 2010 American Chemical Society.

groups.[799] Silanization with hydrophilic silanes has been similarly employed as a procedure to transfer hydrophobic IONPs into an aqueous environment.[800]

An alternative approach to IONP silanization involves preliminary formation of an intermediate silica layer through deposition of triethoxysilane (TEOS) on the IONP surface.[801−803] The silica coating can then be further silanized with bifunctional organosilanes as described earlier. Although preliminary TEOS deposition is more time-consuming and difficult to control,[804] there is some indication that the formation of the intermediate silica shell yields more stable surface functionalization and better magnetic properties in hybrid materials.[803,805,806] While silanization can be an important and efficient method, it is worth reiterating that it is also a procedure that can be difficult to control. Hydrolysis of silanes and polymerization are competing mechanisms that can reduce the yields of surface functionalization and result in NP aggregation and colloidal instability.[807−809] It is therefore important to perform silanization under controlled conditions, using fresh reagents, and with the knowledge that optimization is an important part of the process.

3.3.1.4. Gold Coatings. An interesting alternative method for functionalizing IONPs relies on precoating the surface with a thin layer of gold. The formation of these IO-core Au-shell NPs (IONP@Au) can be achieved by a variety of methods.[810−819] Most rely on the preliminary formation of Au seeds on an IO nanocrystal followed by electroless Au deposition. The Au layer is very stable, possibly even more than polymeric or monomolecular ligand layers directly attached to the IONP surface. Additionally, the gold shell imparts interesting optical properties[820−822] and changes in magnetic properties.[823−825] Upon formation of the Au layer, the resulting IONP@Au can be functionalized using standard conjugation methods available for Au, mainly relying on the rapid formation of Au–S bonds as described in section 3.1.1.3.1.[826−828] For example, antibodies have been immobilized on IONP@Au after an initial functionalization step with the heterobifunctional linker dithiobis(succinimidylpropionate) which introduced amine-reactive succinimidyl moieties via thiolation of the Au surface.[813] Alternatively, IONP@Au were first reacted with mercaptopropionic acid to introduce carboxyl groups, followed by standard EDC/NHS coupling to conjugate an antibody[815] or a NTA–Ni(II) complex for capturing His_n-tagged proteins;[216] see Figure 37 for a schematic of the latter chemistry. Cysteine-containing peptides have also been immobilized directly onto the Au shell.[829] In another interesting example, a layer of boronic acid terminal groups was introduced on the IONP@Au by conjugation to an intermediate mercaptopropionic acid linker. The exposed boronic acid groups were then exploited to readily conjugate diols under slightly basic conditions.[815]

3.3.1.5. Click and Other Chemistries. CuAAC has been extensively utilized to conjugate a number of different molecules to appropriately modified IONPs.[830,831] This approach necessitates prefunctionalizing the IONP surface to display the appropriate azide or alkyne moiety prior to the actual conjugation. An example, already mentioned, is given by the introduction of azides via diazotransfer on an amino-substituted IONP.[749,832] The azide–IONPs were further reacted with propargyl FA by click chemistry to yield FA-conjugated IONPs, which were demonstrated for FA receptor mediated delivery in several cancer cell lines. Azide–IONPs have also been prepared via conjugation of succinimidyl 4-azidobutyrate to amino-derivatized IONPs. The resulting azido-IONPs were reacted with alkyne-derivatized oligonucleotides in the presence of Cu(I), formed *in situ* by reduction of Cu(II) with ascorbic acid, to yield oligo-IONPs. White and co-workers have shown that IONPs synthesized in the organic phase and coated with oleic acid can be readily functionalized with either a

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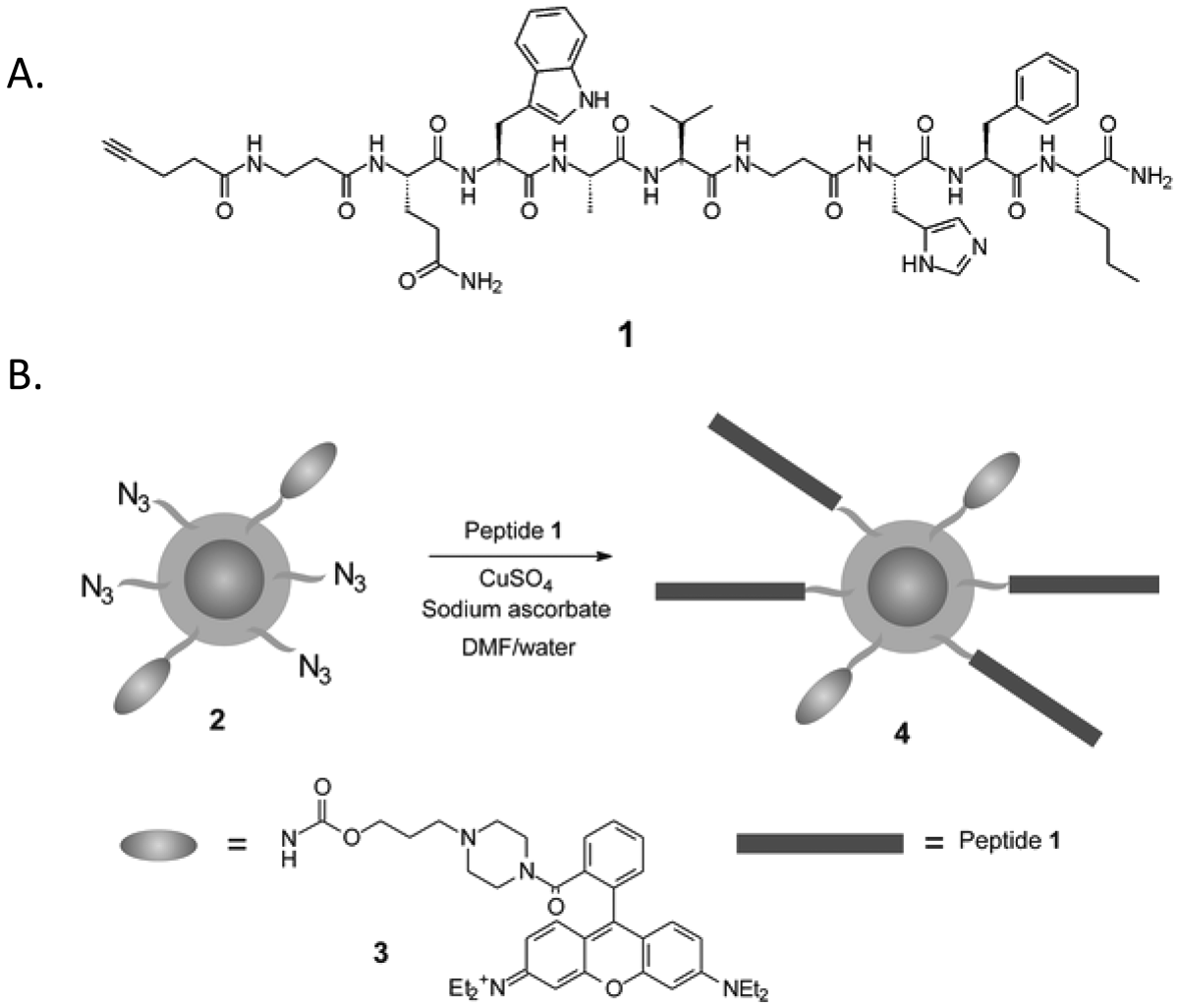

Figure 38. IONP modification by multiple chemistries. (A) Chemical structure of the alkyne functionalized pan-bombesin peptide (1) and (B) schematic of its attachment to an IONP (2) along with a dye (3) to yield the final functionalized NP (4). Figure reproduced from ref 837 with permission.

phosphate−azide or carboxyalkyne via ligand exchange.[833] The substituted IONPs were then reacted with an appropriate alkyne or azide molecular partner to yield functionalized IONPs. Similarly, phosphate-bearing azides were introduced directly onto the IONP surface and reacted with alkyne−ferrocenes.[751] Direct ligand exchange reactions to obtain an azide-substituted IONP can also be accomplished with diol-bearing azido linkers or by nucleophilic displacement of an existing bromine group by the azide.[697] Here, cycloaddition was accomplished by thermal reaction rather than by Cu(I) catalysis. This ligand exchange reaction approach was similarly utilized for the introduction of riboflavin.[834] In a somewhat more laborious procedure, azido-functionalized IONPs were obtained by thermal synthesis of allyl-IONPs followed by reaction with sodium azide as mediated by bromohydrin.[835] Alkyne-bound FA was then used as a molecular partner for the Cu(I)-catalyzed reaction. Synthetic peptides, such as an alkyne-functionalized pan-bombesin sequence, have been successfully conjugated to azido-IONPs, yielding constructs with enhanced affinity for cancer cells both *in vitro* and *in vivo*.[836,837] Figure 38 shows the structure of the alkyne functionalized pan-bombesin peptide and a schematic of its conjugation to the NPs along with a fluorescent rhodamine derivative, which was successfully targeted to a PC-3 human prostate cancer cell line.[837] PAA-coated IONPs have also been utilized as a multimodal platform where click chemistry introduced FA, taxol, and fluorescent dyes.[838] Strain-promoted alkyne−nitrone cycloaddition (see section 5.3.6) has been recently reported for the functionalization of IONPs with anti-HER2 antibodies.[839]

There are other, more unusual methods for the conjugation of molecules onto IONPs. For instance, boronic acid-functionalized NPs have been prepared and shown to be effective substrates for the attachment of sugars.[741] Lastly, and as with many other NP materials, numerous biotinylated IONPs have also been produced and used for conjugation with streptavidinylated partners such as dyes; and, of course, the opposite configuration is equally viable and amply represented.[840,841]

3.3.1.6. Indirect Conjugation via Polymer Layers. Functionalization of IONPs can also be achieved by chemical attachment to an intermediate polymer layer coating the surface of the NPs, which is itself usually introduced directly during synthesis or postsynthetically. The use of polymer layers as coating agents for IONPs is a very active field that has been amply reviewed.[666,842] Colloidal suspensions of polymer-coated IONPs usually display very high stability, which largely stems from the physicochemical properties of the polymer itself. Thus, the presence of reactive chemical residues on the polymer, and their interconversion or functionalization, usually induces

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minimal variation on the stability profile of the NPs. As a result, indirect conjugation often, though not always, permits the preparation of more stable IONP conjugates, along with the further introduction of functional groups or molecules that would otherwise make the native NP colloidally unstable. The polymer−IONP interface, however, is not very well characterized, and the polymeric coating can often be shed over time resulting in IONP conjugates with a limited shelf life. Further, unbound polymers might lower the yield of conjugates or result in reaction mixtures that are difficult to purify. Such considerations notwithstanding, conjugation to a polymer layer is an important procedure for functionalizing IONPs and is especially desirable for laboratories where the direct synthesis of IONPs or "in house" functionalization is technically challenging. Indirect conjugation is also the method of choice in bioapplications where the pharmacodynamic characteristics have to be well-defined and are directly related to the type of polymeric coating employed. Conjugation to IONPs coated with stabilizing polymers follows essentially common conjugation methods (see polymer section 4.3), although there are some more specific or chemoselective chemistries available depending on the particular type of coating polymer.

One of the most ubiquitous coating polymers for IONPs is the branched polysaccharide dextran.[843] Dextran-coated IONPs are easily formed using the method originally developed by Molday and Mackenzie.[844] Briefly, IONPs are formed by co-precipitation in the presence of an excess of dextran. The molecular weight of dextran as well as the ratio of dextran to Fe(II) and Fe(III) defines the size distribution and stability of the resulting colloidal suspension.[845] Dextran complexes the IONP surface through weak interactions between the hydroxy groups of dextran and Fe on the IONP surface,[846] resulting in a relatively stable coating that can, however, be displaced over time, through dilution or in the presence of better surface binders. An improved synthetic method, yielding a more stable dextran coating, was pioneered by Weissleder[847,848] and consists of synthesizing dextran-coated superparamagnetic iron oxide (SPIO) NPs followed by cross-linking with epichlorohydrin. These materials are usually referred to as cross-linked IONPs and the cross-linked coating further contains pendant reactive groups (chlorides or epoxides) that can be used for additional functionalization by nucleophilic substitution. For instance, the cross-linked dextran coating can be reacted with ammonia to yield aminated-cross-linked IO.[849,850] The primary amine can then be directly conjugated or interconverted to a variety of other functional groups. This two-step approach has been used to synthesize IONP conjugates displaying peptides,[850−852] antibodies,[853] and proteins.[854]

Dextran can be partially oxidized with $NaIO_4$ to yield reactive aldehydes that can then be conjugated by reductive amination.[855−857] This method has also been adapted to stabilize dextran coatings. In this case, the stabilization consisted of a preliminary silanization of the native IONPs followed by the introduction of partially oxidized dextran,[857] leading to the intermediate formation of imines, which were then reduced to stable secondary amines.[847,858−863] An alternative method for functionalizing dextran coatings is through cyanogen bromide (CNBr) activation.[864] In this procedure, the hydroxyl groups of dextran are activated, forming cyanate esters that are then further reacted with a primary amine to produce isourea linkages.[865,866] Note that CNBr is an extremely toxic reagent and requires careful handling. The dextran coating can also be introduced with functional groups already present to facilitate further functionalization. For example, carboxymethyl dextran and aminomethyl dextran coated IONPs have been successfully coupled to, respectively, primary amines and carboxylic groups by EDC-mediated amide formation.[690,789,867−869]

Beyond dextran, other polymers have been utilized to stabilize IONPs and in some cases conjugation can be successfully accomplished with these alternative polymers as well. For this, EDC-mediated conjugation is again the preferred conjugation route. For example, IONPs were prepared by coprecipitation and coated with a silane copolymer that was then thermally cross-linked to yield stabilized conjugates.[870] The copolymer introduced a carboxyl group that was modified to an amine with 2,2′-(ethylenedioxy) bis-(ethylamine) and then labeled with Cy5.5 dye using EDC-mediated conjugation in each step. The final conjugate allowed for simultaneous magnetic resonance/optical imaging of tumors in a mouse model. In other examples, CHI and carboxymethyl-CHI were reacted using both the free amines and carboxylic moiety via carbodiimide-mediated reaction, reductive amination or reaction with isothiocyanates.[871] Functionalized PEG polymers have also been successfully employed,[872] although these procedures require specific polymers not readily available. PMAA has also been explored as a potentially functionalizable polymeric coating, presenting a carboxylic moiety that can be used directly or interconverted.[700]

Overall, bioconjugation to IONPs can be accomplished through a variety of direct or indirect methods. The most common remains carbodiimide-mediated reactions with carboxy- or amino-modified polymeric coatings or by surface complexing ligands such as phosphates, diols, and carboxylates. Depending on the nature of the specific conjugate and on the application, certain methods are more suited than others and the many alternatives require a judicious choice in selecting the most appropriate synthetic and bioconjugation route. Some issues still need improvement. For instance, bioorthogonal chemistries to access conjugates displaying multiple moieties would significantly advance this field (see section 2.7).

3.3.2. Silicon Dioxide. Silica NPs (SiO_2 NPs) were among the earliest synthetic nanoparticulate materials to be studied in great detail,[873] although the early attention focused mainly on the preparation of colloidal suspensions, rather than exploiting their physicochemical properties in specific applications. More recently, SiO_2 NPs have elicited growing interest in the fields of drug delivery[874,875] and diagnostics,[876] driven by their biocompatibility and ease of surface functionalization compared with other NMs. Consequently, much effort has been directed toward the preparation of fluorescent SiO_2 NPs where a fluorophore is either supported on the surface or enclosed in the NP itself.[29,877] The latter format offers the possibility of using SiO_2 NPs as a matrix for carrying other less biocompatible and harder to functionalize NPs.[878−881] Although the number of SiO_2 NP bioconjugates is expanding quite rapidly in parallel with their utility, the chemistry for producing such hybrids is actually quite limited beyond initial silane chemistry and is often strongly tied to their synthesis.[882]

The most common synthetic route to access SiO_2 NPs is the Stöber method where tetralkyl silyl esters (i.e., alkyl silanes) are hydrolyzed in an alcoholic solution in the presence of ammonia.[873,883] While the silanes act as a source of oxidized silicon, the ammonia acts as a capping agent for polymerizing −O−Si−O− silanes, stabilizing the resulting nanoaggregates and defining their size and morphology. To date, most SiO_2 NPs are still almost exclusively synthesized using this procedure with TEOS, although many variations are also known.[884−888]

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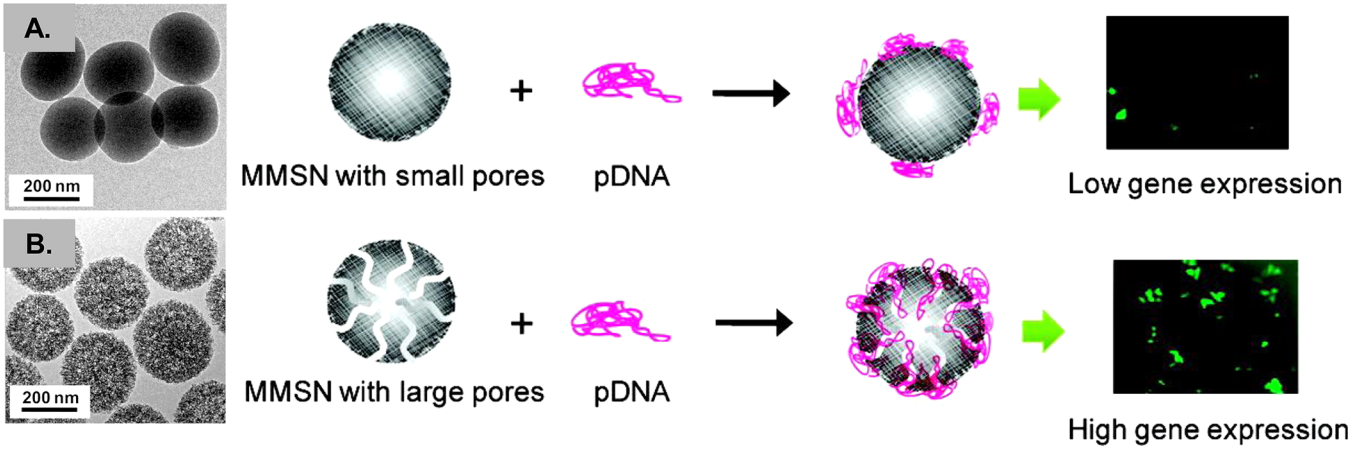

Figure 39. TEM micrographs and use of monodispersed mesoporous SiNPs for gene delivery. Panel (A) highlights materials with pores ~2 nm, while (B) shows that of materials with pores >15 nm. Reprinted with permission from ref 905. Copyright 2011 American Chemical Society.

A particularly well-utilized SiO_2 NP derivative material is that characterized by a mesoporous structure.[28,889−892] These are typically prepared via the liquid crystal template-assisted polymerization of orthosilicic acid.[875] Mesoporous SiO_2 NPs retain the biocompatibility and ease of surface functionalization of nonporous SiO_2 NPs, while also providing an internal porous structure where the size of pores can be synthetically defined. The porosity and facile loading of the composite, which usually manifests a large cargo carrying capacity, provides for a very versatile nanocarrier or drug delivery system.[28,890,893] These can be loaded with a variety of molecules including, for example, drugs such as camptothecin, DOX, sulfasalazine, and ibuprofen, along with a wide range of fluorophores.[890,894−898] Mesoporous SiO_2 NPs can be further covalently functionalized on the exterior surface[899−901] or internally inside the pores,[902,903] allowing for much chemical versatility and many design options. Differential functionalization of the outer surface and within the pores can be accomplished by adopting postsynthetic grafting with alkoxysilanes and co-condensation with organic silanes during NP synthesis, respectively. In the latter method, both the surface and interior of the mesoporous structure are equally functionalized, although the degree of functionalization can be defined by controlling the ratio of reagents. Various synthetic procedures and orthogonal functionalization strategies are extensively reviewed in ref 904. Mesoporous structures also display size selectivity and some level of loading control over the target molecule.[28,890,897,905] Min's group elegantly demonstrated this concept by controlling the pore size of monodispersed mesoporous SiO_2 NPs.[905] They showed that monodispersed mesoporous SiO_2 NPs with pores >15 nm allowed loading of large amounts of plasmid DNA and further protected them from endogenous nucleases during cellular delivery. This increased the level of GFP expression in a model cellular system compared with the same sized control materials with ~2 nm pores treated in the same manner, Figure 39. Modifications to synthetic procedures can also imbue the final mesoporous SiO_2 NP with multifunctional properties (i.e., magnetism and fluorescence).[906,907] Hollow SiO_2 NP structures are another interesting NM that can be synthesized by polymerizing alkyl orthosilicates over an organic or inorganic sacrificial template that is subsequently removed.[908−910]

Independent of the physical NP structure, functionalization of these materials is almost exclusively dominated by the silanization of surface sites, relying on the formation of strong siloxane bonds.[911−913] Both chloroalkylsilanes and alkoxysilanes can be utilized for this purpose and rely on controlled silane hydrolysis and polymerization on the NP surface.[914,915] A large variety of functionalized silanes are commercially available, providing access to an array of reactive chemical moieties. Arguably, the most common silanizing agent is 3-aminopropyltriethoxysilane (APTES), which readily introduces a reactive primary amine onto the SiO_2 NPs. APTES can be used to functionalize the SiO_2 NP surface postsynthetically,[905,916−920] or even used directly as a silicon precursor to introduce amines and other linked molecules directly during NP synthesis.[921,922] For instance, a photochromic diarylethene and rhodamine dye pair was preconjugated to APTES before SiO_2 NP synthesis, and this reagent was incorporated during NP synthesis, resulting in fluorescent SiO_2 NPs that were capable of photoswitching.[923] In any case, the result is a dense covering of the SiO_2 NP surface with reactive amines that can be used directly as an anchoring point (e.g., EDC-mediated amide coupling) or exploited for functional group interconversion (e.g., SMCC, iminothiolane).[921,924] Peptides[925] and other small molecules, including FA and dyes,[926] have been successfully immobilized on SiO_2 NPs by APTES-mediated routes.

An APTES monolayer also imparts a net positive charge to the SiO_2 NPs, which can be used to conjugate negatively charged species. Electrostatic immobilization of DNA on APTES-modified SiO_2 NPs has been extensively investigated for development of nonviral transfection vectors[905,918,919,922,927] and electrochemical DNA sensors.[928] Proteins can also be immobilized by electrostatic[142] or covalent[929] methods. For instance, SA has been introduced on APTES-modified SiO_2 NPs and subsequently conjugated to biotinylated oligonucleotides.[929] Methacryloxypropyltrimethoxysilane (MPS) is another rather common silanization reagent for direct introduction of free reactive thiols onto the NP surface.[886,930−932] Thiol-modified oligonucleotides have been successfully introduced on SiO_2 NPs postsynthetically modified with MPS by using a disulfide exchange reaction,[933] and the resulting DNA−SiO_2 NPs conjugates were then utilized for hybridization studies. MPS-functionalized SiO_2 NPs have been further utilized as reactive substrates for synthesizing Au-shell/SiO_2-core NPs[934] and for the orthogonal attachment of peptides onto a drug encapsulating carrier.[894] As shown in Figure 40, which highlights the latter example, encapsulation is used to load Camptothecin and an MPS coating is then used as a handle to attach either Tf or a

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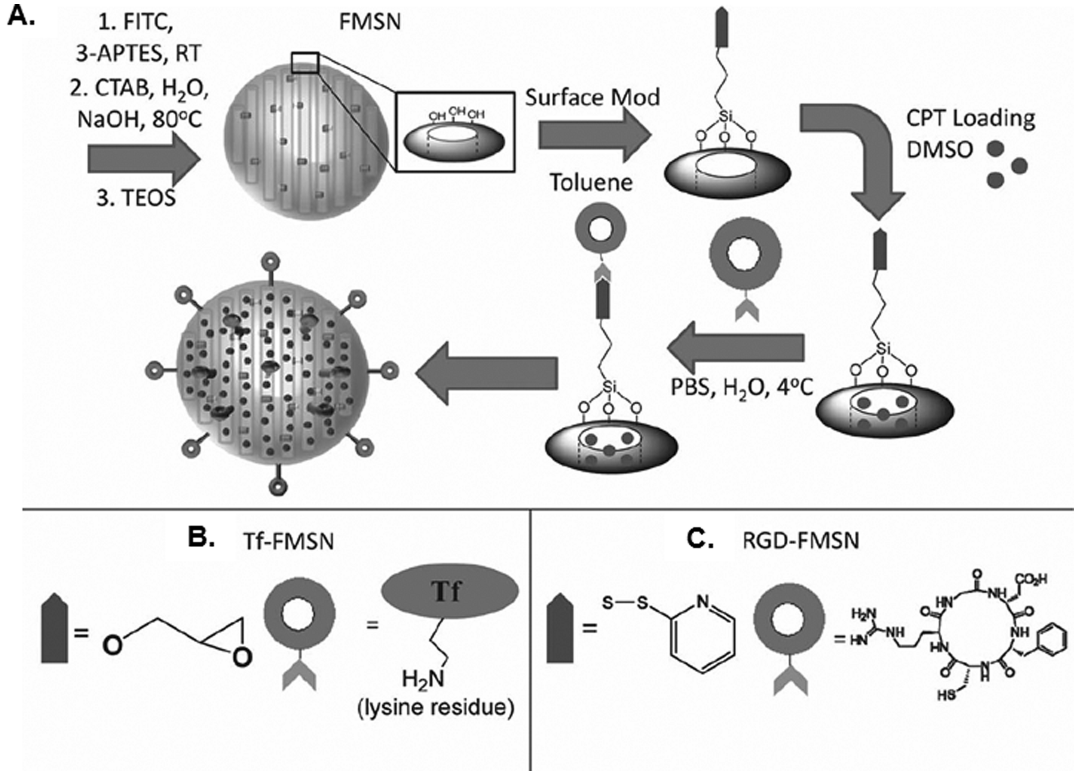

Figure 40. Methods for SiNP drug loading and attachment of the protein or peptide. (A) General overview for each major step in the synthetic scheme is displayed. Specifically, to attach the Tf (B), the mesoporous silica particle is first modified with 3-GPTMS, loaded with camptothecin (CPT) in dimethylsulfoxide (DMSO), and then reacted with the Tf to provide the particle−cell signaling and uptake enhancement. To attach the RGD cyclic peptide (C), the surface was thiol-modified with 3-mercaptopropyltrimethoxysilane, reacted with 2,2′-dithiopyridine (2,2′-DTP), CPT loaded in DMF, and then allowed to react with the peptide to bind it covalently. FMSN = fluorescently modified mesoporous silica NPs. Reprinted with permission from ref 894. Copyright 2011 Wiley.

cyclic RGD peptide on the NP surface. Maleimide-modified SA can also be conjugated to obtain SA−SiO_2 NPs, which should bind to any biotinylated partner.[935] Free surface thiols, however, must be reacted promptly to avoid oxidation, disulfide bridge formation, and concomitant colloidal instability,[936] and this is a potential reason why MPS is not as popular as APTES.

3-Glycidoxypropyltrimethoxysilane (GOPS) has been successfully exploited for the introduction of reactive epoxides on the SiO_2 NP surface.[894,937,938] The epoxides can then be reacted with nucleophilic species such as hydroxy, amino, and mercapto derivatives, and this method has been demonstrated for the attachment of proteins[894] and enzymes.[937,938] Carboxylated SiO_2 NP surfaces can be similarly obtained by reaction with carboxyalkylsilanes,[939] and this strategy has been commonly implemented for introducing SA using EDC-mediated coupling.[920,940] Biotinylated aptamers or fluorophores can then be introduced to impart recognition of cancer cells and optical tracking, respectively. Alternatively, carboxylates can be introduced in a two-step reaction by reacting APTES-modified SiO_2 NPs with succinic anhydride.[917,941,942] Other silanes and surface modifications can be introduced in a similar manner,[911−914] extending the repertoire of available surface chemistries.

An interesting, although less exploited, synthetic procedure for SiO_2 NP surface functionalization is preliminary reduction of the SiO_2 surface to Si−H bonds, followed by photocatalyzed or thermal catalyzed hydrosylilation with terminal alkenes.[943−945] This approach has been successfully exploited for the generation of MRI active SiO_2 NPs.[946] CNBr has also been explored as a conjugation reagent for the immobilization of biomolecules on SiO_2 NPs.[947,948] CNBr reacts with surface silanols, yielding a brominated surface that can be directly derivatized with nucleophiles.

CuAAC has been adapted to the functionalization of SiO_2 NP surfaces. The alkyne or azide functionality needed for this coupling has been introduced on the surface by several methods. In one approach, the SiO_2 NP surface is initially chlorinated by treatment with 3-chloropropyltrimethoxysilane, which is followed by nucleophilic substitution with sodium azide.[949] The azide-substituted SiO_2 NPs were then reacted with hexynyl-DNA to yield the desired oligonucleotide conjugates. The azide moiety can be similarly introduced using other reagents. For example, Patel et al. treated the surfaces of mesoporous SiO_2 NPs with APTES and then alkylated the amine-functionalized material with a tri(ethylene glycol) monoazide monotosylate unit to give an azide-terminated surface.[950] The SiO_2 NPs were then used to create enzyme-responsive snap-top covered silica nanocontainers. Alternatively, alkyne-modified SiO_2 NPs can be obtained by reacting the APTES-modified surface with propargyl bromide.[951,952] Similar click reactions have been also adapted to immobilize polymers such as PEI and poly(*N*-isopropylacrylamide) on SiO_2 NPs.[949,953]

His_n-tagged proteins can be readily conjugated to SiO_2 NPs by modification with the requisite metal chelates.[954] A chelating agent, such as iminodiacetic acid or dipicolinic acid, can be easily introduced onto APTES or chlorinated surfaces, complexed to Cu(II), then exposed to a His_n-appended polypeptide to yield stable conjugates. This approach should be easily

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extended to other biomolecules by introducing a His_n-tail postsynthetically using ligation methods.[212,303] In contrast to using covalent chemistry for conjugation at the SiNP surface, Rocco et al. demonstrated that genetically engineered peptides with affinity for SiO_2 could be site-specifically introduced at the termini or within loops of cellular proteins.[955] This allowed for efficient fluorescent labeling of *E. coli* proteins with 30 nm diameter SiO_2 NPs.

Lastly, the synthesis of multifunctional SiO_2 NPs is growing in popularity and these can now be produced through a variety of protocols, although again almost all the chemistry builds off of the initial silane chemistry.[896,906,956,957] Moreover, many of these constructs exploit mesoporous SiO_2 NPs to achieve multifunctionality. For example, the Lin group assembled AuNP-capped mesoporous SiO_2 NPs, which they used as a controlled-release, intracellular drug delivery system.[958] Here, a photolabile linker was first coupled to the surface of AuNPs, which were then used to cap paclitaxel guest-loaded SiO_2 NPs. UV irradiation functioned as the trigger to allow intracellular release of the drug and decreased viability in model cellular systems.

3.3.3. Titanium Dioxide. Titanium dioxide NPs (TiO_2 NPs) are an important class of NMs that have found extensive use in a variety of common household products such as pigments,[959] sunscreens,[960,961] and a multitude of other cosmetics.[962] More recently, TiO_2 NPs have become important as photoactive elements in the development of solar cells[963−965] and as UV-activated photocatalysts.[966,967] In contrast to the popularity of TiO_2 NPs in the above-mentioned applications, their use in the biomedical field is still not very widespread, due to concerns about their safety[968] and, especially, potential long-term effects following chronic exposure.[969,970] The photocatalytic properties of TiO_2 NPs have been utilized for the development of bactericidal composites[971] or for the exploration of photoactivated cancer treatment.[27,972] Additionally, unlike other NPs with more prominent optical or magnetic properties, TiO_2 NPs lack physicochemical features that can be easily exploited for sensing applications, although a few examples of photoelectrochemical sensors for gases have also been reported.[973,974] For these reasons, reports on the conjugation of biomolecules are somewhat limited compared with other NMs.

TiO_2 NPs can be synthesized by a variety of routes as amply reviewed by Chen.[975,976] The most common route is the sol−gel method,[977] where an inorganic Ti precursor such as titanium tetraisopropoxide or a titanium alkoxide, $Ti(OR)_4$ (R = alkyl), is hydrolyzed resulting in polymerization and formation of Ti−O−Ti chains. Nonhydrolytic synthesis (sol method) in the presence of capping agents is another common method.[978] Other viable, although less common, synthetic routes are hydrothermal, solvothermal, direct oxidation, chemical vapor deposition, physical vapor deposition, electrodeposition, sonochemical, and microwave-assisted synthesis.[975,976]

Several reports have described protocols for the conjugation of biomolecules onto their surfaces and these have been partially reviewed in ref 979. As with most metal oxides, the simplest way to functionalize the surface is by reaction with silanes.[980−982] In the case of Ti, this reaction is especially efficient because the Ti−O bond is extremely strong.[981] TiO_2 NPs have been, for example, functionalized with APTES in anhydrous dimethyl sulfoxide to yield amino-functionalized NPs.[983] The intermediates were then biotinylated with NHS−biotin to provide substrates for binding SA.[983] Displacement of a hydrophobic oleic acid layer with carboxy-silane yielded COOH-functionalized TiO_2 NPs with concomitant transfer from an organic to an aqueous phase. These materials were then used for the electrostatic or covalent immobilization of DOX.[984] Silanization of TiO_2 NPs also has an effect on their photocatalytic activity.[985] Although reports on the silanization of TiO_2 NPs are somewhat scarce, it is reasonable to assume that any silanizing agent will result in successful functionalization of the surface and that silanization strategies and reagents utilized for other metal oxides could be easily adapted.

Complexing to under-coordinated surface sites has also been accomplished with dopamine and 3,4-dihydrophenylacetic acid to yield, respectively, amino- and COOH-functionalized TiO_2 NPs.[986] Catecol-type ligands, in general, coordinate the TiO_2 surface forming stable bonds while altering their optical and catalytic properties.[764,987−989] For instance, the dihydrophenylacetic acid route, followed by EDC/NHS amide formation, has been exploited for the synthesis of antibody-coated TiO_2 NPs that were then utilized for producing specific photoinduced cytotoxicity after binding to A172 and U87 brain cancer cell lines.[990] Dopamine has also been demonstrated as a viable generic linker for the introduction of amines on TiO_2 surfaces.[991] In this role, dopamine was utilized as a heterobifunctional linker for conjugation to oligonucleotides[992−994] and biotin.[995]

Analogous to the functionalization of other metal oxides, TiO_2 NPs surfaces can form stable complexes with phosphates and phosphonates,[996−998] and this conjugation approach has been exploited for the introduction of Gd chelates as MRI probes.[999,1000] There is some evidence that phosphonates and bisphosphonates yield strong interactions resulting in coordination polymers of the −P−O−Ti−O−P− type, which also permits an increase in loading levels.[1001] This affinity also applies to phosphates, which form bidentate bonds[1002] as witnessed by the facile attachment of a flavin mononucleotide.[1003] Carboxylic acid groups can be similarly introduced by selective binding of a phosphate linker terminating in a COOH, which is then available for additional functionalization or interconversion.[1004,1005] Although not applied to TiO_2 NPs yet, a CuAAC approach has been implemented on TiO_2 surfaces.[1006] There, an azide-functionalized catechol was introduced on the oxide surface and further reacted with an electroactive alkyne. It should be possible to extend a similar procedure to TiO_2 NPs as well. Another underexplored conjugation approach still remains the coating of TiO_2 NPs with an intermediate polymer layer. In a preliminary report, PAA-coated TiO_2 NPs were prepared, yielding stable suspensions at basic pH that could be functionalized with antibodies by standard EDC chemistry.[1007]

3.3.4. Other Oxides. Because many metals can be both oxidized and synthesized as nanocrystals, there are a vast number of metal oxide-based NMs with interesting physicochemical properties that could, in principle, be conjugated to organic molecules and biomolecules for the purpose of developing assays, drug-delivery vehicles, and other novel NMs.[1008] Most of these NMs are still not widely exploited for bioconjugation purposes and are usually employed in niche applications. A comprehensive review of metal oxide NMs is beyond the current scope, and the reader is referred to more informative sources.[1009−1013] The conjugation chemistries available are essentially the same as those described for other more-commonly employed metal oxide NPs above. For instance, the well-known interaction between phosphates or phosphonates and metal oxides[1014] has been exploited for the functionalization of $BaTiO_3$,[1015] ZnO,[1014] SnO_2,[1016] zirconia,[1017] Gd_2O_3,[1018] and alumina NPs.[1019] Carboxylates are also

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Table 9. Chemical Functionalization and Modification of Transition and Post-Transition Metals

Covalent Interactions					
metal NPs	surface modification	cross-linker	biomolecule	use	ref
Cu	carboxylation by mercaptoacetic acid	EDC	amine-capped DNA	DNA *in vitro* biosensing	37, 1045
CoPt−Au	carboxylation by lipoic acid−PEG-COOH	EDC	NeutrAvidin	MRI imaging of biotin labeled amyloid β	26
FeCo	amine functionalization by APTES	EDC	IL-6 antibody	IL-6 immunoassay	1043
GMR[a]	amine functionalization by APTES	EDC	IL-6 antibody	IL-6 immunoassay	1043
Pb	carboxylation by mercaptoacetic acid	EDC	amine-capped DNA	DNA electrochemical sensor	1040, 1041, 1044
Ti	collagen/hydroxyapatite spin-coating	EDC	collagen/hydroxyapatite	orthopedic implants	1052

Noncovalent Interactions				
metal NPs	surface modification	biomolecule	use	ref
Al	electrostatic interaction	BSA	demonstrative	1053
Al	electrostatic interaction	DNA	nucleic acid quantification by RLS	1054
Cu	self-assembly	thrombolytic peptides	treatment of thrombolysis	1055, 1056
Ti, Co, Cr, Mo	cellular adhesion	endothelial and vascular smooth muscle cells	biocompatibility of vascular stents	1057
Zn	wet-milling	insulin	drug delivery	1058
Zr	adsorption	myoglobin, lipases, and DNA	demonstrative	1059−1061
Zr	Lewis acid−base interaction	BSA/streptavidin	fluoroimmunoassay of PSA	1062

[a]Giant magnetoresistive NPs.

viable ligands for direct functionalization of metal oxide NPs, albeit forming bonds that are less stable than phosphates. For instance Cu_2O,[1020] Gd_2O_3,[1021] and ZnO NPs[1022] have been successfully passivated with self-assembled monolayers of carboxylic acids. The formation of strong Si−O bonds is arguably the most popular method for the formation of functionalized metal oxides and examples utilizing this method have been extensively described above. Alkoxy and chlorosilanes are extensively exploited for this purpose and are commercially available with a variety of reactive chemical moieties for imparting additional chemical reactivity. Essentially all metal oxides react with silanes, making these reagents very useful cross-linkers for conjugation. ZnO NPs have been successfully silanized,[1023,1024] as well as Nd_2O_3,[1025,1026] alumina,[1027,1028] ceria,[1029] and zirconia.[1030] Although there are some limited examples of bioconjugation to these more exotic oxide NPs (see below),[1015,1031−1034] viable conjugation procedures should be accessible by simply adopting the well-established procedures described in other sections and optimizing them to the reactivity of the specific NM under consideration.

3.4. Transition and Post-Transition Metal Nanoparticles

Many of the transition metals and some selected post-transition metals, such as lead and aluminum, have intrinsic magnetic, optical, and catalytic properties that make them potentially useful in the biomedical realm as nanoscale bioconjugates.[55,1035] A few isotopes of the transition metals are also functional as radionuclides (e.g., ^{64}Cu, ^{188}Re, and ^{99m}Tc) and these have long been conjugated to numerous biomolecules for specific targeting in radionuclide therapy. There is significant literature on this topic, and applications have been extensively reviewed for targeting of melanoma,[1036] breast,[1037] and ovarian cancer.[1038] A number of these radionuclide elements are now being synthesized on a NP scale and are being further investigated for modification using conjugation strategies similar to those described here with the goal of making clinical advancements in the treatment of cancer and other diseases.[1039] Despite these and other promising characteristics, many of these metals are considered quite toxic and are therefore not being pursued for *in vivo* utility but are rather undergoing preparation and bioconjugation for application as *in vitro* electrochemical biosensors.[37,1040−1045] Because many of these metals have been bioconjugated within the form of oxides and mixed oxide NPs,[1046−1050] or as dopants within semiconductor QDs,[1051] the applicable chemistries for these types of NPs are primarily reviewed in previous sections. Assembly of many transition metal NP bioconjugates utilizes similar covalent and noncovalent strategies as described for other metal NPs along with those summarized in Table 9. However, of particular interest are the roles of Ni and Zn in the binding of His_n-modified proteins and the attraction of Zr for phosphorylated proteins and peptides, as discussed below.

3.4.1. Covalent Chemistry. Numerous transition metal and post-transition metal bioconjugates have been assembled based on standard covalent conjugation strategies. A selection of some recent bioconjugates is summarized in Table 9. As shown in this table, EDC coupling is again quite often used, though there are examples of several other types of cross-linkers being applied. One of the earlier and more prominent constructs consisting of an aluminum/peptide bioconjugate was reported in 1997.[1046] Aluminum has long been utilized as a vaccine adjuvant because of its ability to stimulate an innate immune response, though Al-based bioconjugates are often not useful for oral delivery due to their pH lability. Frey and colleagues described a potential HIV vaccine that utilized Al-NPs functionalized with a peptomer derived from the HIV gp120 protein with the goal of mucosal delivery.[1046] Peptomers are peptides cross-linked in a specific head-to-tail manner to form a polymer. They began with calcinated aluminum oxide NPs and utilized APTES to modify the NP surface hydroxyl groups to provide the display of 16 mM of primary amine per gram of NP solid. *N*-Acetylhomocysteine lactone was then used to place a reactive thiol on the NP surface, and this allowed conjugation to the bromoacetylated peptomer (derived by using *N*-succinimidyl bromoacetate) via formation of a thioether linkage (Figure 41). The final product was estimated to have around 16 mg of peptomer per gram of NP (~55% yield), which corresponded to the display of 5.3×10^4 peptomer epitopes per NP.[1046] Although the testing of subsequent immunogenic capacity was not described in the same report, this approach clearly highlights the capacity of NPs to display an extremely high localized concentration of epitopes.

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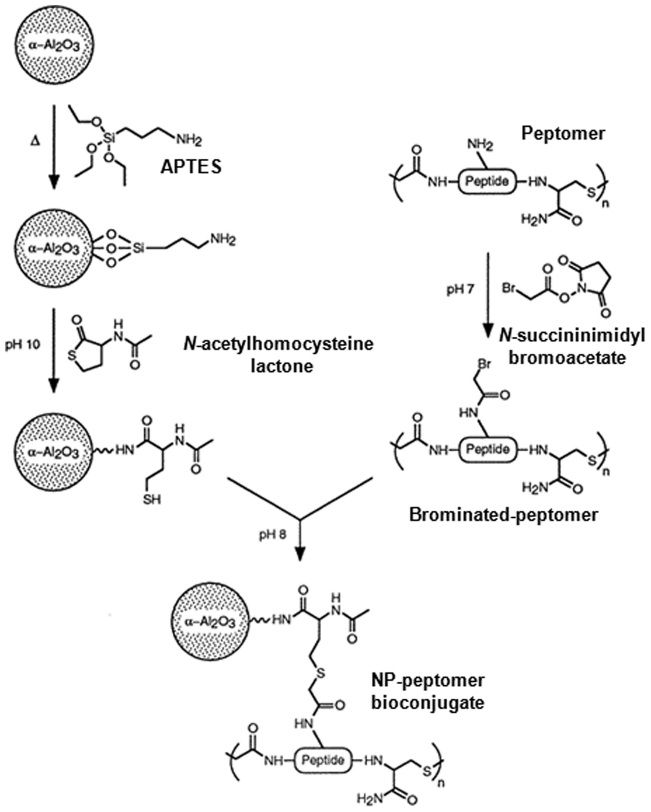

Figure 41. Synthetic steps used to derive the Al–peptomer bioconjugate. Figure adapted from ref 1046. Copyright 1997 American Chemical Society.

Another potential application is the use of Ti in orthopedics. Similar to calcium phosphate, nanoparticulate Ti is often used in bone resins and cements in orthopedic implants and has been shown to have improved capabilities on the nanoscale, especially when combined with hydroxyapatite.[1063] Nanoparticulate Ti was recently biofunctionalized with the osteoblast adhesion peptide KRSR by applying APTES to modify the surface hydroxyl groups.[1063] Nanophase titanium has also been spin-coated with collagen and hydroxyapatite and then cross-linked with EDC for potential orthopedic use. In both cases, the coated nanosurfaces showed increased osteogenic properties that may be useful in improving bone cement and resin.[1052] Other covalently bound transition metal bioconjugates take advantage of their large magnetic moments both within *in vitro* biosensors and for *in vivo* diagnostic imaging. Metal-based giant magnetoresistive (GMR) immunosensors detect stray magnetic fields from the binding of magnetic labels and are being pursued as potential biosensors of protein biomarkers.[1064] In one demonstration, Srinivasan et al. bioconjugated a GMR surface with biotin and functionalized cubic FeCo NPs with SA using APTES and EDC coupling, see Figure 42, which highlights the chemical assembly and signal transduction mechanism of the sensor assembly.[1043] Initial detection limits of the biotin–avidin interaction on this sensor were in the zeptomolar range. EDC coupling was again used to attach the magnetic FeCoNPs to an anti-interleukin 6 antibody and application of the modified GMR sensor in a sandwich immunoassay format demonstrated a dose-dependent sensitivity in the micromolar range. This method was 13 times more sensitive than existing assays.[1043]

The high magnetic moments of Co, Fe, and Ni are also of use in MRI applications; however, their application is still limited due to their high reactivity in aqueous environments. Choi and colleagues described an approach where CoPt/Au core/shell NPs were synthesized to negate this reactivity.[26] The Au-coated NPs appeared dumbbell shaped in structure with a 9 nm AuNP grown next to, and slightly overcoating, the 6 nm CoPt NP. These were subsequently functionalized with a modular lipoic acid–PEG ligand displaying either an OH or COOH end group; these were previously described for use with QDs.[99] The dithiolane headgroup on the lipoic acid interacted with the Au via chemisorption, the PEG provided aqueous solubility and the COOH provided a handle for subsequent chemical modification. NeutrAvidin was EDC-coupled to the NP surface-displayed COOH groups allowing a biotinylated Aβ peptide, which is known to form aggregates in Alzheimer's disease, to bind the complex and provide MRI contrast.[26]

Covalently bioconjugated transition metal NPs have also found common use within DNA biosensors. For example,

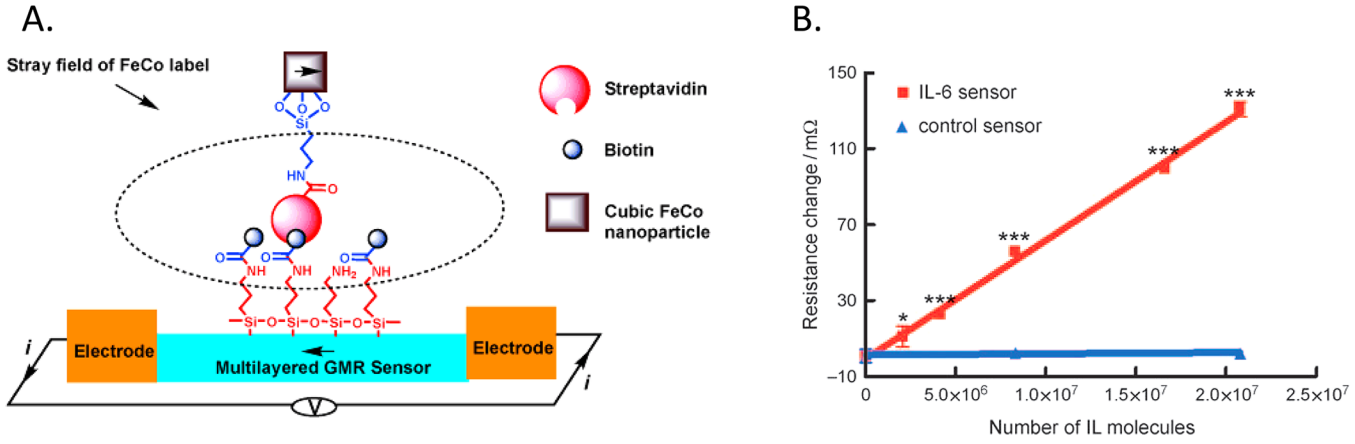

Figure 42. Giant magnetoresistive (GMR) immunosensor modification. (A) GMR sensor schematic for detection of molecular recognition between SA and biotin and in immunoassays. The GMR multilayer structure consists of Ta (5 nm)/$Ir_{0.8}Mn_{0.2}$ (10 nm)/$Co_{0.9}Fe_{0.1}$ (2.5 nm)/Cu (3.3 nm)/$Co_{0.9}Fe_{0.1}$ (1 nm)/$Ni_{0.82}Fe_{0.12}$ (2 nm)/Ta (5 nm). (B) Dose–response curves of resistance change detected by sensor and IL-6 molecules. The same amount of capture antibody and detection antibody-modified magnetic nanoparticles were applied to each sensor with varied numbers of IL-6 molecules. Data points, mean; bars, standard deviation (n = 3); *, P < 0.10; **, P < 0.01; ***, P < 0.001. Figure reproduced from ref 1043 with permission of Wiley.

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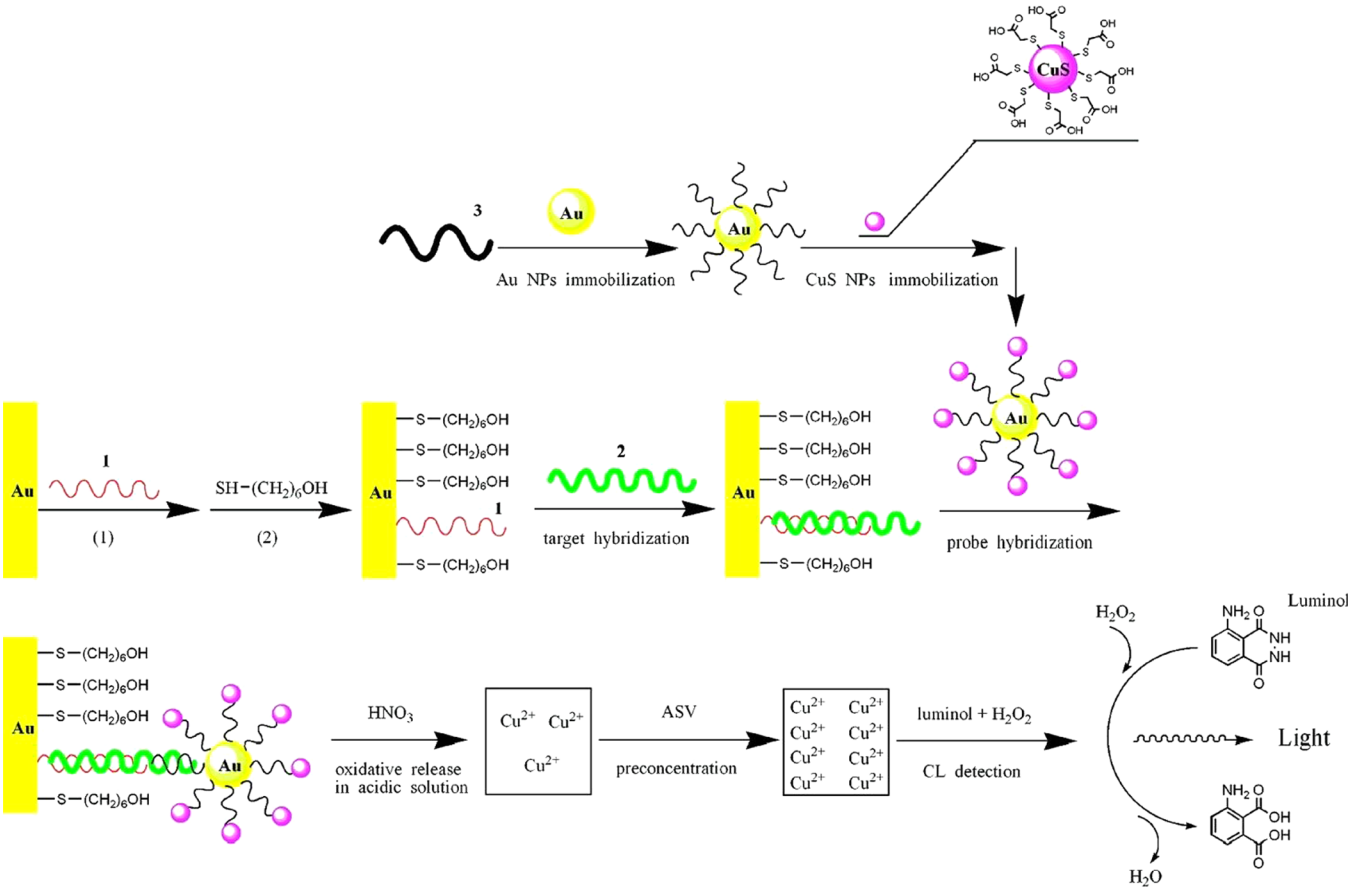

Figure 43. Synthetic and sensing strategy highlighting how CuSNPs functionalized with thiolated DNA (1) displayed around a AuNP along with other thiolated alkanes (2) can be incorporated into a chemiluminescence detection scheme. Labels 1, 2, and 3 without parentheses represent probe and target DNAs. Sensitivity is further amplified by concentration steps. Figure adapted from ref 1045. Copyright 2008 American Chemical Society.

Ding and colleagues synthesized cupric sulfide NPs (CuSNPs) using mercaptoacetic acid as the stabilizer and then coupled aminolated DNA sequences to the NPs with EDC chemistry.[37] Following hybridization on glassy carbon electrodes, the CuSNPs were dissolved by addition of nitric acid, and the resulting solution was injected into a flow-chemiluminescent system where luminol–H_2O_2 chemiluminescence generated in the presence of Cu^{2+} catalysts was monitored and demonstrated 10^{-13} M detection limits. Subsequent improvements to this sensor design used AuNPs displaying the smaller CuSNP–DNA constructs on their surfaces and significantly increased the sensitivity as schematically shown in Figure 43.[1045] The AuNPs did not augment the Cu^{2+} catalytic activity; rather they served to display high localized concentrations of the CuSNP–DNA during hybridization, which, along with some preconcentration steps, dramatically increased the amount of Cu^{2+} acting as catalysts. Similar electrochemically based DNA sensors, where Au- and PbSNPs were DNA-labeled by EDC coupling, have also been reported.[1040,1041,1044]

3.4.2. Noncovalent Interactions. Examples of electrostatic/noncovalent bioconjugation methods with transition and post-transition metals are relatively few. One report describes the electrostatic interactions of $Al(OH)_3$ suspensions consisting of NP sizes ranging from 26 to 82 nm with BSA in pursuit of developing improved vaccines and understanding Alzheimer's disease etiology.[1053] In this example, a variety of complex gelation and aggregation behaviors were noted and suggested the formation of protein–$Al(OH)_3$ along with the converse $Al(OH)_3$–protein core/shell nanostructures.

3.4.2.1. Metal Affinity. It is somewhat ironic that although many transition metal NPs are synthesized from elements that can coordinate NTA and bind His_n sequences by metal affinity (i.e., Ni, Zn, Co, Cr, Fe, Mn), they are almost never utilized in this role when synthetically incorporated into the NP. The principle reason is that the available NP surfaces are in oxide form and so the requisite cations are not available in the 2+-divalent state. Rather, it is the hydroxyl groups that the NPs commonly present on their surfaces, which undergo chemical modification to present the NTA coordinating groups. Following loading with typically Ni(II), Zn(II), or Co(II), this then allows for self-assembly driven binding to His_n-modified peptides and proteins. This chemistry is usually accomplished by initial NP surface activation to display an amine, using APTES, for example, and then cross-linking to a terminal NTA precursor. As an alternative to this, Bele et al. coated maghemite NPs with a thin layer of silica using TEOS, incubated the NPs with zinc acetate, and then demonstrated that BSA could coordinate to the Zn-treated NPs and be competitively released by adding imidazole.[1065] In this example, it is not a contiguous polyhistidine sequence that interacts with the Zn-coated NPs, but rather several histidine residues displayed around the protein surface. In a somewhat converse assembly, histidine-functionalized AuNPs have been shown to aggregate in a pH-dependent manner upon addition of Fe(III) ions.[1066] The Matsui group has taken a different approach and

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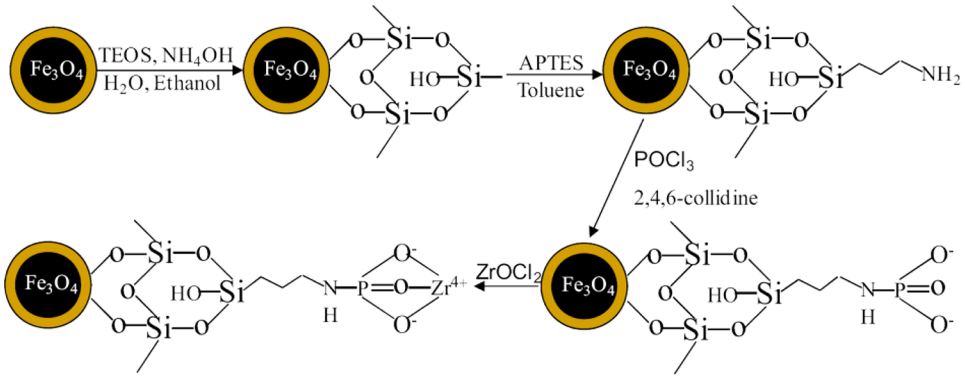

Figure 44. Synthetic scheme to prepare Zr(IV)−PO_3-modified magnetic NPs. (TEOS, tetraethoxysilane; APTES, 3-aminopropyl-triethoxysilane) Figure reproduced from ref [1073] with permission of Springer.

utilized histidine-rich peptides to fabricate CuNP-coated nanotubes.[1067] Amine group displaying carbon nanotubes were functionalized with the histidine-rich peptides, which coordinated Cu(II) ions, which then became nucleation sites for the CuNP growth during a subsequent reductive reaction. Similar strategies have also yielded Ni, Zn, and magnetic NPs on the nanotube scaffold.[1068−1070]

3.5. Zirconium Nanoparticles

Zirconium NP bioconjugates are also being developed for select bioapplications due to their interesting noncovalent or adsorptive properties especially when displaying different phosphate species. Liu et al. used hydrolysis to synthesize 100 nm ZrO_2 NPs and showed that they could reversibly adsorb and desorb DNA by simple addition of a basic solution.[1061] Bellezza and colleagues grafted phosphate onto 60 nm ZrO_2 NPs by heating in the presence of phosphoric acid to enhance the adsorption of horse heart myoglobin.[1059] Subsequent myoglobin interactions with the ZrO_2−P NPs resulted in reduced protein activity and induction of what appeared to be prefibrillar-like aggregates. In contrast to this loss of activity, Chen et al. found enhanced lipase performance when it was immobilized onto zirconia NPs.[1060] The NPs were first premodified with the surfactant erucic acid, and then lipases from either *Candida rugosa* or *Pseudomonas cepacia* were directly adsorbed to their surfaces. When assayed in isooctane organic solvent, both lipases demonstrated significantly higher esterification and acylation activity along with improved enantioselectivity. In this case, the authors suggest that conformational rearrangement of the proteins upon NP adsorption produces a more stable and active lipase form.

When displayed on NP surfaces, zirconium phosphonate, Zr(IV)−PO_3, has shown a unique propensity to selectively bind phosphorylated peptides making this material of particular utility for mass spectral and proteomic analysis.[1071−1073] Representative linkage chemistry to assemble Zr(IV)−PO_3 around NPs is shown in Figure 44. In this and other examples, Fe_3O_4 magnetic NPs were coated with SiO_2 using TEOS and APTES and then reacted with $POCl_3$ and 2,4,6-collidine before incubating with $ZrOCl_2$ to yield Zr(IV)−PO_3.[1072,1073] In application, the Zr(IV)−PO_3 NPs demonstrated the ability to selectively bind to phosphopeptides and undergo magnetic separation and enrichment yielding femtomole detection limits.

3.6. Rare Earth Nanoparticles

These metals, which include the 15 lanthanoids (La, Ce, Pr, Nd, Pm, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, and Lu) plus scandium and yttrium, have unique optical, magnetic, and catalytic properties that arise from their 4f shell electrons being shielded and often unpaired. They are desirable for use in biological fluorescence and MRI imaging, as free radical scavengers against neurodegenerative diseases, and potentially as radionuclide cancer therapies. Four rare earth ions [Dy(III), Eu(III), Tb(III), and Sm(III)] are luminescent and have distinct spectral properties including extremely long, excited-state lifetimes that are in the micro- to millisecond range,[1074,1075] large Stokes shifts, and multiple sharp emission peaks as shown in Figure 45.[78,1076] They also have the unique ability to undergo optical upconversion as nanophosphors, meaning they can convert very low intensity excitation in the near-IR range into high-energy emission in the visible spectrum through a sequential multiphoton process that is accessible at much lower excitation intensities than two-photon fluorescence.[1077] Cumulatively these properties contribute to a strong interest in utilizing these NMs for *in vivo* imaging because they facilitate deep tissue observation while minimizing background.[1078−1080] While their luminescence is not size-dependent like QDs, doping of NPs with different combinations of rare earth metals can tune their relative emission and allow for a multitude of discrete emission wavelengths suitable for multiplexing.[1081,1082] Their luminescent properties have been extensively exploited in time-resolved bioanalytical assays,[1074] and recent advancements allowing monitoring of upconversion FRET have led to development of more complex biological assays.[1077]

Rare earth metals are commonly used as MRI contrast agents because they are able to alter the relaxation times of water protons *in vivo*. They alter either the longitudinal relaxation, the time taken for the protons to realign with the external magnetic field (T_1), or the transverse relaxation, the time taken for the protons to exchange energy with other nuclei (T_2). Gd(III) is ideal for MRI usage because of its large magnetic moment, long electron spin relaxation time, and symmetric electronic spin rate. Its use as an MRI contrast agent has been extensively reviewed.[1083] All of the current Gd contrast agents consist of discrete atoms attached to nine-coordinate chelates as exemplified by the family of polyamino carboxylate ligands.[1083] Agents such as Magnevist and Dotarem are anionic with the Gd coordinated to diethylene triamine pentaacetic acid (DTPA) and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) chelators, respectively, while agents such as Omniscan with DTPA−1,3-dimethylalloxazine and Prohance with 10-(2-hydroxypropyl)-l,4,7,10-tetraazacyclododecane-l,4,7-triacetic acid, also known as HPD03A, are neutral. Other rare earth metals can also function as contrast agents. Eu has been

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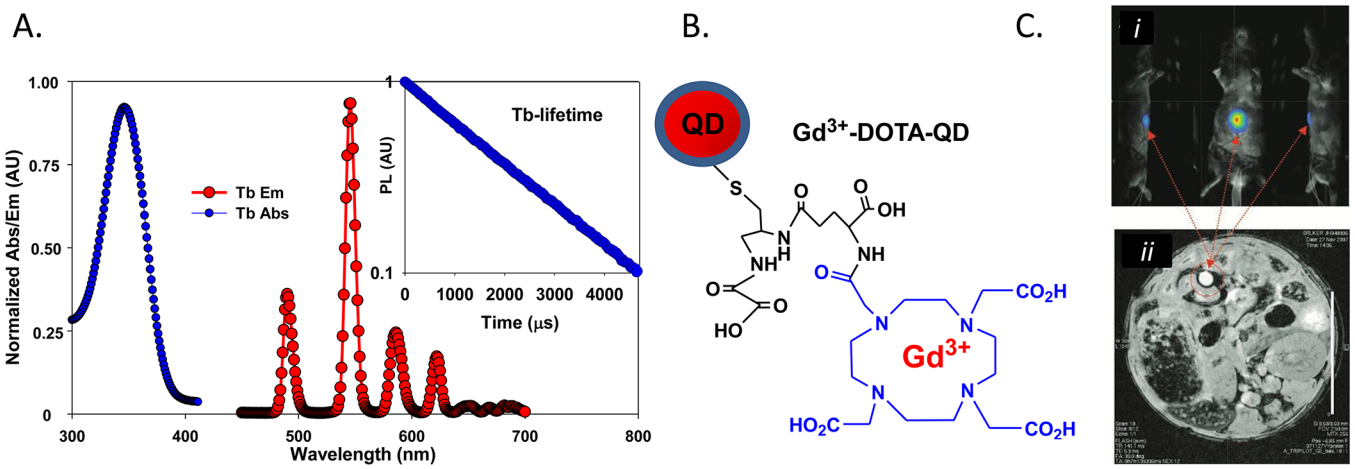

Figure 45. Rare earth NP probes. (A) Emission profile of a Lanthascreen Tb fluorescent probe ($\lambda_{ex} \approx 343$ nm) highlighting the unique sharp emission bands. Inset shows the extremely long monoexponential excited-state lifetime (τ) of several milliseconds measured at ~550 nm. (B) Schematic of Gd(III)−DOTA-functionalized CdSeTe/CdS QDs with a GA coating (not to scale). Following synthesis the core/shell QDs were made hydrophilic by cap exchange with the GA tripeptide. The primary amine on this was modified with a NHS ester of DOTA, and then the final construct was allowed to chelate $GdCl_3$. (C) (i) NIR-fluorescence image and (ii) T_1 weighted MR axial image of a mouse. A phantom containing 10 μM of Gd(III)−DOTA−QDs was embedded into the mouse abdomen. Arrows represent the location of the phantom visualized by both fluorescence imaging and MRI. White bar in the MRI image is 1 cm in length. Panels B and C reproduced from ref 1088 with permission of the RSC.

demonstrated as a redox-sensitive contrast agent, Dy is classified as a negative contrast agent and has higher relaxivity than Gd at high magnetic fields,[1084] while La has potential as a PARACEST (paramagnetic chemical exchange dependent saturation transfer) contrast agent, which can be switched on or off using a saturating irradiation pulse at NH or OH exchangeable sites in the vicinity of the bulk water resonance.[1083] Newer generation contrast agents are being pursued with better tissue specificity, higher relaxivity, better pharmacokinetic properties, and better thermodynamic stability. Incorporating these agents into nanoscale structures such as micelles and dendrimers and the creation of nanoparticulate-based contrast agents are deemed critical to long-term improvement in MRI imaging.

NPs are ideal as carriers of rare earth contrast agents because they increase "payload" delivery to the site of interest and therefore increase localized relaxivity and contrast. They are also amenable to incorporation as part of multimodal or theranostics assemblies. However, a continuing issue is that their intrinsic properties can change considerably when formulated on the nanoscale, thus making the NP formulation less desirable than the bulk as, for example, in the case of manifesting decreased fluorescent emission.[1085] Several Gd-based NP agents are currently in clinical development including polymer-coated, CHI or lipid-encapsulated constructs, shell-cross-linked knedel-like NP, and aluminosilicate zeolites, as discussed in refs 78 and 1086. Gd contrast properties have also been pursued for multimodal imaging by using Gd−DTPA−bis(stearylamide) along with CdSe/ZnS core/shell QDs co-encapsulated within CHI NPs,[45] coating QDs with the same Gd functionality attached to a lipid inserted into a surrounding micellar layer,[1087] or as Gd−DOTA chelates attached to GA-functionalized CdSeTe/CdS QDs, see Figure 45C.[1088] The latter examples highlight the growing interest in doping these materials within other NP constructs in pursuit of multimodal or multifunctional "smart" imaging agents.[78,1086]

Besides imaging and contrast applications, Ce, for example, can function as a potent free radical scavenger, and this has sparked interest for potential therapeutic applications against neurodegenerative diseases where their increased S/V can greatly enhance this capability; extensively reviewed in ref 79, CeO_2 NPs have been shown to increase the lifespan of neural cells and protect against free radical damage from UV, peroxides, γ radiation, and excitotoxicity.[79,1089,1090] They are also protective against radiation induced pneumonitis in murine models[1091] and normal human breast cells but did not protect the MCF-7 breast cancer line from oxidative damage.[1092] This latter finding suggests the exciting possibility of further use for inhibiting the toxicity of radiation therapy to normal tissue. Lastly, some rare earth metals also exist as multiple isotopes, including in radioactive states, and this has been recently examined for radionuclide cancer therapy. ^{177}Lu-labeled hydroxyapatite has been studied for use in liver cancer,[1093] and a NP version of ^{153}Sa has also been synthesized for potential use in cancer treatment.[1094]

Clearly, the advancement of these materials for all biological applications, especially in NP form, will be directly dependent upon the ability to biofunctionalize them. For example, specific types of bioconjugation will be needed to develop CeO_2 as a clinical free-radical scavenger. Antioxidant therapy for neurodegenerative diseases requires the use of NP materials that can freely cross the blood−brain barrier to cellular sites experiencing oxidative stress. Of particular interest, for example, would be CeO_2 NPs with a bioresponsive coating sensitive to oxidative stress that could undergo self-regulation of radical scavenging by the redox status of the environment.[79,1089,1090]

3.6.1. Covalent Bioconjugation Chemistries. Rare earth NPs come in three generalized structures: (1) inorganic NP matrices or doped inorganic matrices, (2) polymer NPs that are impregnated with rare earth ions, and (3) NPs displaying chelates of rare earth ions. The vast majority of rare earth NPs are synthesized as oxides,[994,1084,1089,1091,1092,1095−1100] though there are examples of hydroxides, fluorides,[1078,1101−1104] and phosphates.[1105] Numerous methods to synthesize them as nanotubes and nanowires have also been developed including microemulsion,[1091] hydrothermal methods,[1102] and spray pyrolysis.[1099] The NPs are primarily insoluble in aqueous solution as synthesized and often require further treatment

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Table 10. Examples of Lanthanoid−Nanoparticle Bioconjugation through Surface Modification and Cross-Linking

lanthanoid NPs	surface treatment chemistry	functionalization surface handle	cross-linker utilized	linkage	biological attached	refs
BPTA−Tb(III)	AEPS	amine	glutaraldehyde	amine to amine	streptavidin	1109
Eu(III) and Tb(III) chelates	6-maleimidocaproic acid	maleimide			caspase-3	1110
Eu(III) chelate/doped	commercial "Fluoro-Max" particles	carboxyl	EDC	carboxyl to amine	streptavidin	1111
Fe_2O_3 core/$NaYF_4$:Yb,Er shell	TEOS/APS	amine	glutaraldehyde	amine to amine	streptavidin	1112
Gd_2O_3	APTES	amine	DITC	amine to amine	streptavidin, fibronectin	1095
LaF_3	2-aminoethyl phosphate	amine	PEG	amine to amine	biotin	1101
LaF_3	poly(St-*co*-MAA)	carboxyl	EDC	carboxyl to amine	DNA	1102
$LaPO_4$	AHA	carboxyl	EDC	carboxyl to amine	streptavidin	1105
$NaYF_4$:Yb,Er	Lumieux−von Rudloff	carboxyl	EDC	carboxyl to amine	streptavidin	1106
Pr_6O_{11}	hydrolysis/aminopropylsilane	amine	SMCC	amine to thiol	DNA	1097
SiO_2-coated Eu	APTES/succinic anhydride	carboxyl	EDC	carboxyl to amine	streptavidin	1108
Tb(III)-doped Gd_2O_3	APTES/TEOS	amine	NHS-biotin/NHS-DNA	amine to biomolecule	DNA/streptavidin	1113

with surface stabilizing ligands or polymers to impart water solubility.

For rare earth NPs to be used with covalent bioconjugation, these surface treatments should also ideally impart some functional groups or handles onto the surface. The latter strategy has already been applied to NPs using a variety of techniques. For example, amide surfaces have been added to Gd_2O_3 using APTES and TEOS[1095] or, alternatively, to LaF_3 via 2-aminoethyl phosphate.[1101] Carboxylation of rare earth metals has been demonstrated with poly(St-*co*-MAA) (St = styrene) treatment of LaF_3[1102] and 6-aminohexanoic acid treatment of $LaPO_4$.[1105] Huang's group synthesized various rare earth NPs stabilized with hydrophobic oleic acid ligands and utilized the Lemieux−von Rudloff reagent ($NaIO_4$−$KMnO_4$) to oxidize the oleic acid into azelaic acids, which resulted in the generation of free carboxylic acids for bioconjugation.[1106] A summary of relevant surface treatments resulting in the display of some common functional or reactive groups (amine, carboxyl, or maleimide) that are commonly utilized for downstream (bio)modification of rare earth NPs is presented in Table 10.

Once appropriately surface-functionalized, the NPs then usually undergo bioconjugation by either direct chemical modification of the available groups or handles or alternatively through a multitude of intermediary or secondary cross-linkers, see Table 10. EDC coupling is again the most commonly used chemistry for these purposes and usually binds primary amine containing biomolecules to the carboxylated NPs. For lanthanoids, it has been used, for example, to attach antibodies for prostate-specific antigen (PSA) immunoassays[1085,1107] and SA to attach biotinylated DNA for assembling nanoscale DNA sensors, see Figure 46.[1106,1108] Other cross-linkers that bridge two amine surfaces have also been used, mostly as proof of principle in SA−biotin binding demonstrations.[1095,1101] A derivative method utilizing glutaraldehyde has also been applied to attach an α-fetoprotein antibody by amine cross-linking for use in a Tb-based time-resolved fluoroimmunoassay.[1109] As an alternative to bulky SA/biotin conjugations, bifunctional sulfo-SMCC has allowed linkage of the amine groups functionalized on a Pr_6O_{11} NP with a thiolated single-stranded DNA, which was subsequently used in a DNA diagnostic based on electrochemical impedance spectroscopy sensing, see Figure 47.[1097]

Similar types of cross-linking strategies can also yield quite complex doped rare-earth NP structures for bioapplications. Louis and co-workers synthesized doubly luminescent rare earth core/shell NP structures.[1113] Here, oxide NPs consisting of Gd_2O_3 cores doped with luminescent Tb(III) ions were directly precipitated in polyalcohol using a polyol route. Because water sensitivity can affect Tb emission, the cores were further embedded in a protective shell of aminated-polysiloxane, which also improved emission intensity. Fluorescein dye was also incorporated into the shell providing a further fluorescent tag. The amine groups were then modified with NHS-ester-activated biotin allowing further SA immobilization, which was confirmed in a biotin-binding chip assay. NHS-ester-activated DNA was also used to functionalize the NPs for subsequent use in a hybridization assay. This double luminescence format provided access to both the strong fluorescein emission and a much longer Tb emission, which could be monitored with time-gated fluorescence measurements.[1113] Cheng's group synthesized 5−15 nm diameter Fe_2O_3 NP magnetic cores, which were then coated with a Yb and Er codoped $NaYF_4$ shell by coprecipitation in the presence of fluoride and ethylenediaminetetraacetic acid; the rare-earth shell layer functioned as an efficient IR-to-visible up-conversion phosphor.[1112] This assembly was then coated with silica using TEOS hydrolysis followed by aminosilane modification and reaction with glutaraldehyde to allow cross-linking to SA. The final composite demonstrated both a strong magnetic response and up-conversion fluorescence along with binding to biotinylated IgG in proof-of-concept assays.[1112]

Rare earth NPs can also function as a central nanoscaffold for polymeric modification yielding multifunctional materials that provide targeted imaging and drug delivery to cancer cells. Rowe et al. synthesized Gd metal−organic framework NPs via reverse microemulsion using CTAB as the surfactant and 1,4-benzenedicarboxylic acid as the chelating agent.[1114] Copolymers of acrylamide, acryloxy-NHS, and fluorescein *O*-methacrylate were prepared by RAFT polymerization, and the NHS functionality was utilized to attach therapeutics such as the drug methotrexate along with the RGD integrin-binding cell-targeting peptide. Further use of a trithiocarbonate RAFT allowed polymer end group reduction to thiolates, which attached to vacant Gd(III) orbitals on the surface of the metal−organic framework NPs. Using a FITZ-HAS canine endothelial sarcoma cell line, the authors demonstrated use of the NPs for specific cellular targeting, bimodal imaging (fluorescence and MRI),

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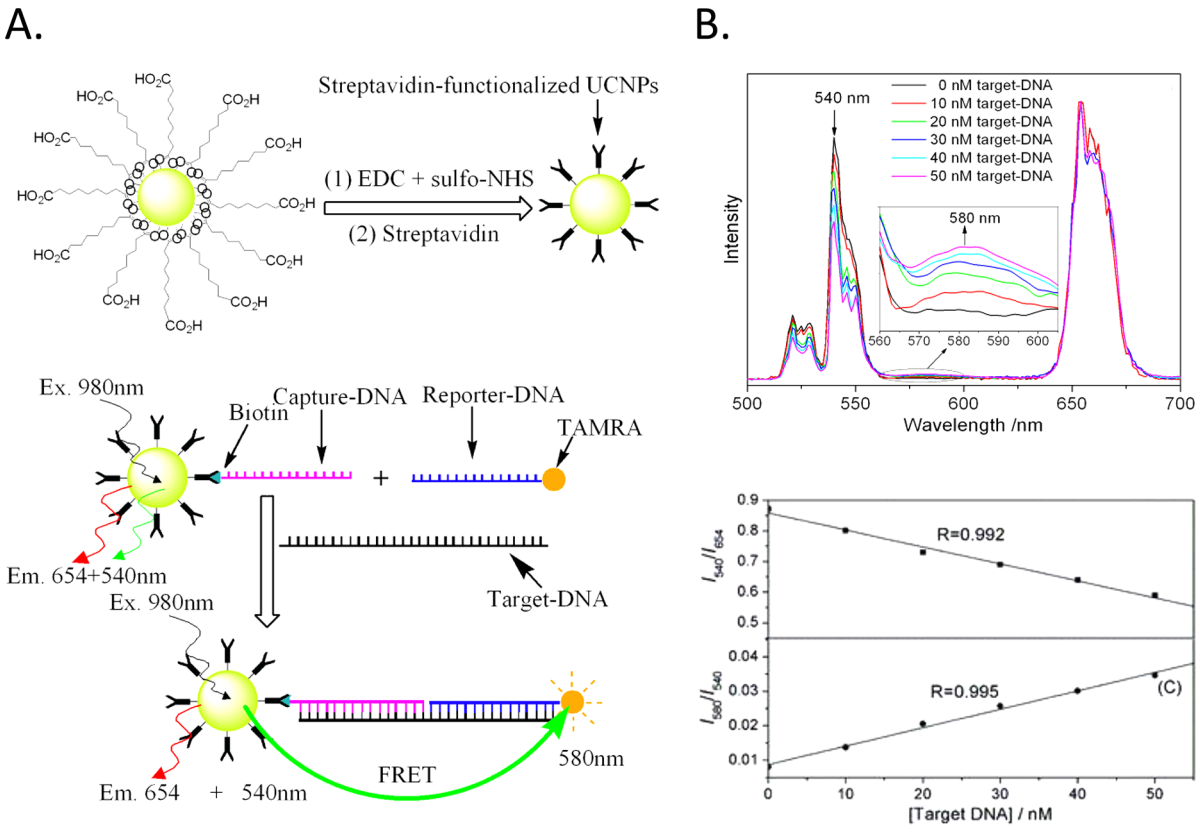

Figure 46. Modification of upconversion NPs. (A) Synthesis of SA-functionalized upconversion NPs (UCNPs) and schematic of DNA sensing. Carboxylated UCNPs are functionalized with SA using EDC chemistry. Biotinylated capture and TAMRA-labeled reporter DNAs are only joined in the presence of the target DNA, which is signaled by FRET. (B) Luminescence spectra of a mixture of SA-functionalized $NaYF_4$:Yb,Er NPs, capture DNA, and reporter DNA in the presence of different concentrations of target DNA under continuous-wave excitation at 980 nm and the linear relationships between target DNA concentration and the intensity ratios I_{540} (donor)/I_{654} or I_{580} (acceptor)/I_{540} (donor). I_{654} is used for comparison purposes because it remains essentially unchanged. Figure reproduced from ref 1106. Copyright 2008 American Chemical Society.

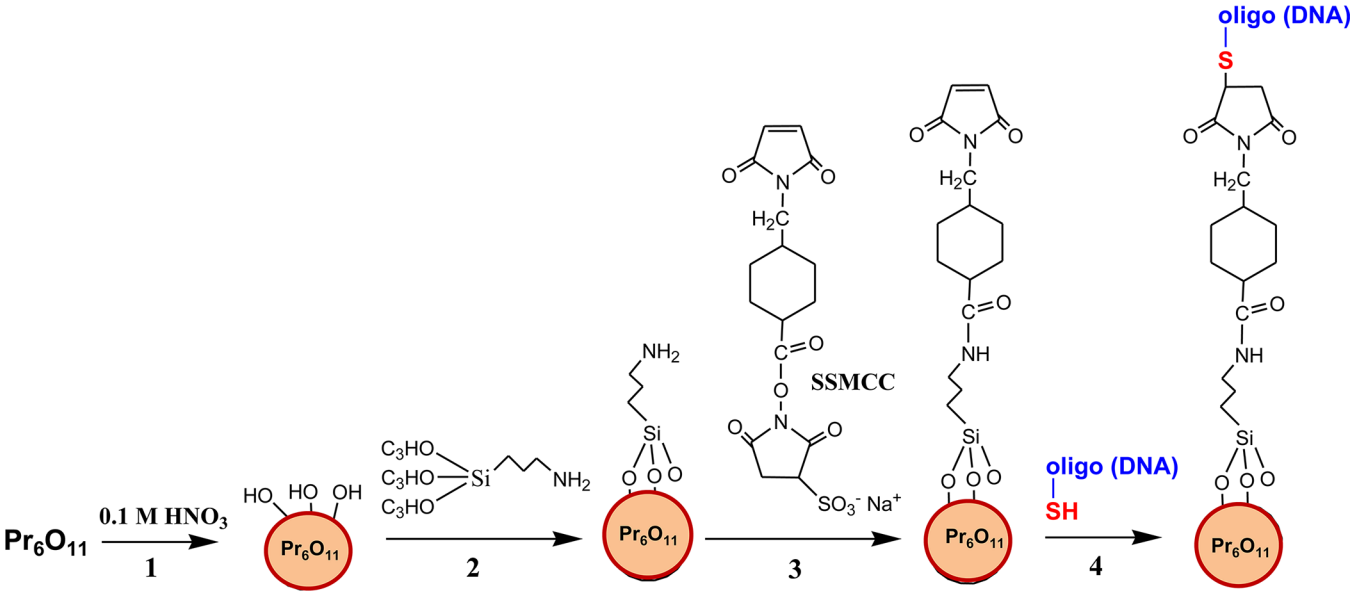

Figure 47. Schematic of the chemical synthesis of Pr_6O_{11} NPs (**1**), modification with a primary amine after hydrolysis (**2**), coupling of the amine and subsequent display of a maleimide using the SMCC cross-linker (**3**), and specific attachment of thiolated DNA via the maleimide (**4**). Adapted from ref 1097.

and delivery of a potential treatment agent, which inhibited cell proliferation.[1114] In a converse approach, polymer NPs derived from PAA starter NPs incorporating $LaPO_4$:Ce,Tb were synthesized for luminescence resonance energy transfer (LRET) assays.[1115] EDC was used to modify the NPs with biotin hydrazide, allowing binding to avidin-coated AuNPs. The AuNP

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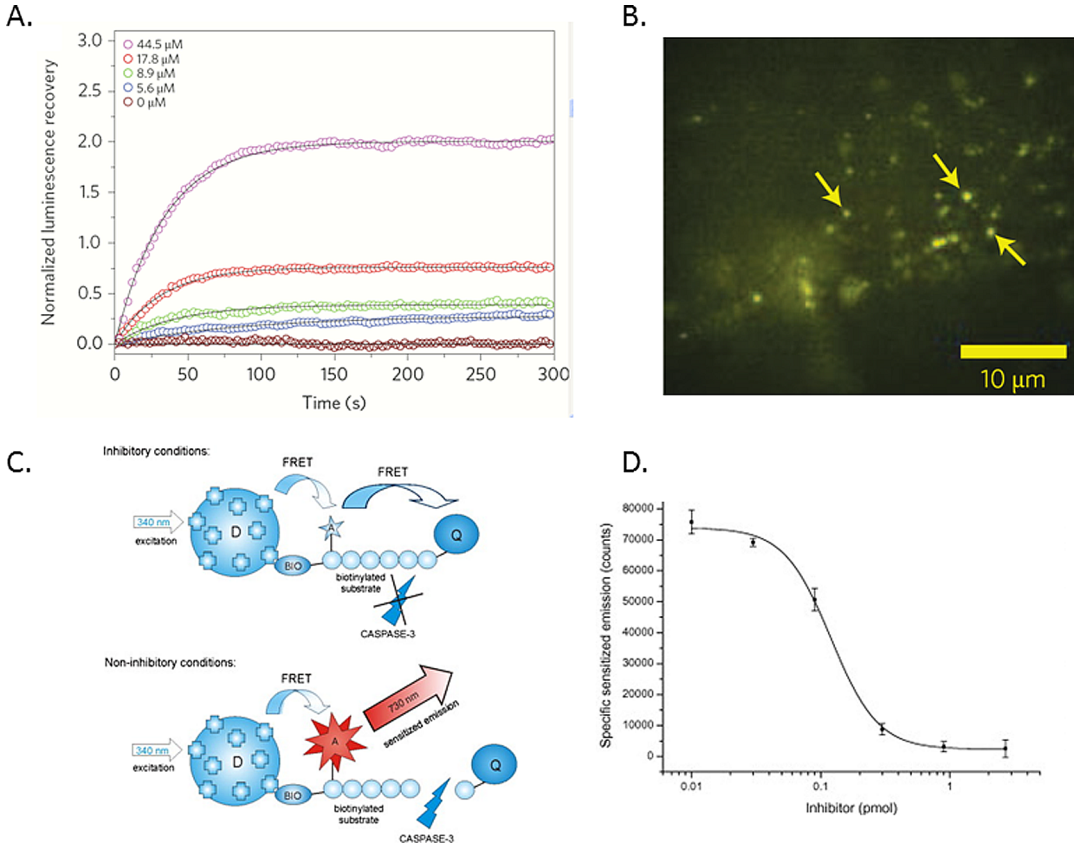

Figure 48. Rare earth NP sensors. (A) Luminescence recovery of silica-coated $Y_{0.6}Eu_{0.4}VO_4$ NPs after photobleaching and following the addition of the indicated H_2O_2 concentrations at $t = 0$. (B) Fluorescence image of silica-coated $Y_{0.6}Eu_{0.4}VO_4$ NPs internalized in vascular smooth muscle cells (excitation at 466 nm, detection centered at 617 nm) following photobleaching and at 30 min after endothelin stimulation, which produces H_2O_2. Arrows highlight the NP presence in the cell. Figure reproduced from ref 1117. Copyright 2009 Macmillan Publishers Ltd. (C) Dual-step FRET quenching assay. SA-coated Eu(III)-chelate-doped NP donors, D, derived from carboxylated Fluoro-Max particles are excited, and energy is transferred to the proximal Alexa Fluor 680 acceptor, A, on the attached biotinylated peptide substrate. In inhibitory conditions, the biotinylated peptidyl substrate remains intact, and the sensitized acceptor emission is efficiently attenuated by the terminal BlackBerry Quencher 650, Q. In noninhibitory conditions, the enzyme cleaves the substrate, releasing quencher and leading to emission of the fluorescent acceptor on excitation. (D) Quenching assay response to varying doses of the inhibitor Z-DEVD-FMK. Panels C and D reproduced from ref 1111. Copyright 2008 Elsevier Publishing.

driven quenching of the NP emission was monitored with both steady state and luminescent lifetime analysis.

NP structures displaying multiple chelated rare earth atoms have been developed and this is also usually in the context of creating multifunctional materials. For example, Paunesku et al. functionalized TiO_2 NPs with glycerol-modified DNA and Gd atoms immobilized into a dopamine-modified DOTA ligand; the glycerol hydroxyls and dopamine diols bound the TiO_2 surface.[1116] The final composite NP materials were estimated to average almost 2000 Gd atoms per NP. Transfection of cultured MCF-7 breast cancer cells resulted in intracellular Gd concentrations 1000-fold higher than in cells treated with Gd-ligand alone. A follow-up study showed that the labeled NPs were retained at specific sites in rat PC12 adrenal medulla pheochromocytoma cells by the glycerol-modified DNA affinity to intracellular targets.[994] The authors suggest that this type of NP conjugate might provide increased Gd accumulation in targeted cells for Gd neutron-capture cancer therapy.[1116] The chemical structures of chelating groups available for incorporation into these types of materials are too numerous to list here and can be found in reviews covering fluorescent[1076,1077,1107,1110] and MRI imaging applications.[1083,1086]

It should also be noted that in certain cases, wholly inorganic chemical coatings can be critical to the biological utility of rare-earth NPs. Silica-coated $Y_{0.6}Eu_{0.4}VO_4$ NPs of 20−40 nm were recently utilized to measure the temporal pattern of reactive oxygen species (ROS) produced within cells.[1117] The initial $Y_{0.6}Eu_{0.4}VO_4$ NPs were synthesized by salt coprecipitation with NaOH and then overcoated with a thin silica layer, which stabilized the NPs and provided permeability to small molecules due to its amorphous nature. When introduced into vascular smooth muscle cells, these NPs became photoreduced under laser irradiation but were reoxidized by intracellular H_2O_2, which was transiently generated by intracellular signaling processes. A precalibrated recovery in luminescence could thus be used for intracellular signal transduction at single NP resolution, see Figure 48. A similar synthetic strategy allowed incorporation of a zinc phthalocyanine photodynamic therapy agent (PDT) in the mesoporous silica coating of $NaYF_4$ NPs.[1118] This approach protected the PDT agent from degradation while still allowing upconversion excitation from the rare earth core to sensitize ROS production.

3.6.2. Noncovalent Attachment. Rare earth NP bioconjugates have also been assembled using noncovalent chemical strategies. For example, uncharged NeutrAvidin was passively

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adsorbed onto Fe_3O_4 NP cores with Eu- and Tb-doped Gd_2O_3 shells and further assembled with biotinylated DNA to yield functional DNA sensors. These sensors detected bacterial 16S rDNA and single nucleotide polymorphisms indicative of a feline autosomal dominant polycystic kidney disease, which has an analogous human disease counterpart.[1098,1099] Kamimura et al. also reported that negatively charged SA could be coimmobilized on rare earth metal doped Y_2O_3 NPs and bind biotinylated antibodies, although further biosensing with these conjugates was not shown.[1096] Passive binding of Tf to CeO_2 NPs has been used to facilitate subsequent cellular uptake of the conjugates.[1100] In this case, binding of the positively charged Tf was shown to be dependent on the ζ potential of the particle. Overall, these noncovalent methods are much less common and are thought to be less stable, making covalent attachment the preferred conjugation chemistry for these NMs.[1077]

3.6.3. Commercially Available Rare Earth Nanoparticles. Rare-earth NPs are also commercially available in both hydrophobic precursor and hydrophilic bioconjugable form. Kanto Reagents offers almost all the rare earths as oxide NPs with average diameters less than 15 nm, although these appear to be as powder and may not be directly water-soluble. Nanomaterials Company, Five Star Technologies, NanoAmor, and PlasmaChem also offer a selection of rare earth metal NPs as summarized in Supporting Table 1, Supporting Information. Seradyn offers carboxylated polystyrene Eu-loaded NPs under the trade name Fluor-Max. These NPs contain approximately 30 000 tris(naphthyltrifluorobutanedione) chelates of Eu on a single NP, imparting an intense and long-lived fluorescence.[1119] Carboxylated Fluoro-Max particles have been successfully used in a number of bioconjugates. For example, Soukka bound an antibody specific for PSA to these NPs using EDAC and sulfo-NHS coupling chemistry.[1120] The construct was used to detect PSA on solid phase and in solution with high sensitivity and specificity.[1119−1121] Valanne has also attached SA to similar NPs by EDC allowing binding to a biotinylated peptide sequence with specificity for the enzyme caspase-3, which is an effector in the apoptotic cascade.[1111] This peptide contains both a fluorophore that acts as a FRET donor (Alexa Fluor 680) and a terminal quencher (BlackBerry Quencher 650) that acts as an acceptor. The resulting NP construct functioned as a two-step FRET-based sensor capable of detecting dose-dependent caspase-3 activity by proteolytic release of the quencher (see Figure 48C,D). This sensor was shown to be useful in high-throughput screening of caspase-3 inhibitors.[1111]

3.7. Alkaline Earth Metal Nanoparticles

Of the six alkaline earth metals, Be, Mg, Ca, Sr, Ba, and Ra, only Ca and Mg have been pursued as NP formulations for biological use, and this is mostly due to their favorable biocompatibility. The two metals are among the most abundant minerals found naturally in the body, alleviating some potential toxicity concerns associated with biomedical usage. Interestingly, Ca and Mg minerals are also on the U.S. FDA generally recognized as safe (GRAS) list of substances for food additives, although it is important to note that this has no predictive capacity for use in NP formulations. They have found extensive use as NP formulations in orthopedic and dental applications and are currently undergoing intensive research for use as drug delivery and gene therapy vectors. The most common alkaline earth metals used in NP formulations is Ca in the form of $Ca(H_2PO_4)_2$, $CaHPO_4$, and $Ca_3(PO_4)_2$,[1122] which are collectively referred to as calcium phosphate NPs (CaP NPs), and also naturally occurring hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$.[1123−1127] Other alkaline earth NP formulations include $CaCO_3$,[1128−1130] CaF_2,[1131,1132] MgO, $MgPO_4$, and $BaSO_4$.[1133−1135]

Bulk calcium phosphates and carbonates have long been used in orthopedic joint implants and dental fillings, and it has been shown that engineering on the nanoscale could significantly improve utility and biocompatibility. The pertinent concepts are reviewed extensively elsewhere.[1136−1139] For example, replacing microsized $CaCO_3$ with NP counterparts forms bone cement that more closely mimics physiological bone structure and improves the mechanical properties of joint replacements, including their maximum load, bending strength, and elastic or bending modulus.[1128] Current joint replacements have a typical lifespan of 10−20 years, and such improvements could lengthen this period, thus avoiding repetitive surgeries for patients. Adding NPs containing MgO, $BaSO_4$, and hydroxyapatite in bone-filling resins has further been shown to increase adhesion to osteoblasts.[1126,1135] Nanoscale MgO has also been shown to decrease the harmful exothermic conditions from bone cement solidification.[1135] In dentistry, CaP NPs are being incorporated into resins for dental implants and fillings,[1140] while CaF_2 NPs are being examined for use in fillings and for the delivery of beneficial fluorides.[1131,1132] Some studies have recently reported on using $BaSO_4$ NP additives in bone cement to allow for subsequent X-ray visualization of the implants.[1134,1135]

The development of alkaline earth NMs has been advancing rapidly, and further bioconjugation should improve their biocompatibility and aid in targeting pharmaceutical agents to specific bone regions, for example, weak bone regions in osteoporosis patients or localized bone tumor delivery.[1136−1138] The unique delivery capabilities of CaP NPs have also made them of specific interest for cancer therapy. This is because they are insoluble at physiological pH but have increasing solubility in acidic environments, such as those found in and around solid tumors, thus facilitating localized drug release in these harmful tissues.[1141] Due to their altered metabolism and protein expression profiles, solid tumors generally display higher acidity in their extracellular and interstitial environments and, in conjunction with their relatively invariant internal pH, this results in a pH gradient between the two tissues that has long been of interest for exploitation in targeted chemotherapeutic delivery.[1142] The pH stability of alkaline earth NPs is also of interest for oral insulin delivery in diabetes treatment, because the insulin can be designed to be released specifically in the latter part of the digestive tract after exiting the highly proteolytic, acidic stomach area.[1122,1143] Insulin, a 51 residue polypeptide, undergoes considerable digestion in the stomach precluding oral consumption as an administration route without the protection of a NP or other matrix. The same pH-dependent solubility properties also make alkaline earth, and especially CaP NPs, attractive for targeted gene therapy. In these applications, a nucleic acid (e.g., siRNA or plasmid) would be substituted for a drug. Furthermore, Ca(II) is involved in many intracellular processes important to DNA transfection, including endosomal escape, cytosolic stability, and formation of nuclear pore complexes for DNA translocation into the nucleus, suggesting the possibility of cooperative effects with CaP NPs. The use of CaP NPs specifically for gene delivery is reviewed elsewhere.[1144] $MgPO_4$ has also been investigated for potential use as a gene delivery agent.[1133,1145]

While the focus of this section is primarily on CaP NPs due to their known biocompatibility, NPs derived from $MgPO_4$ show similar characteristics and may be able to undergo many

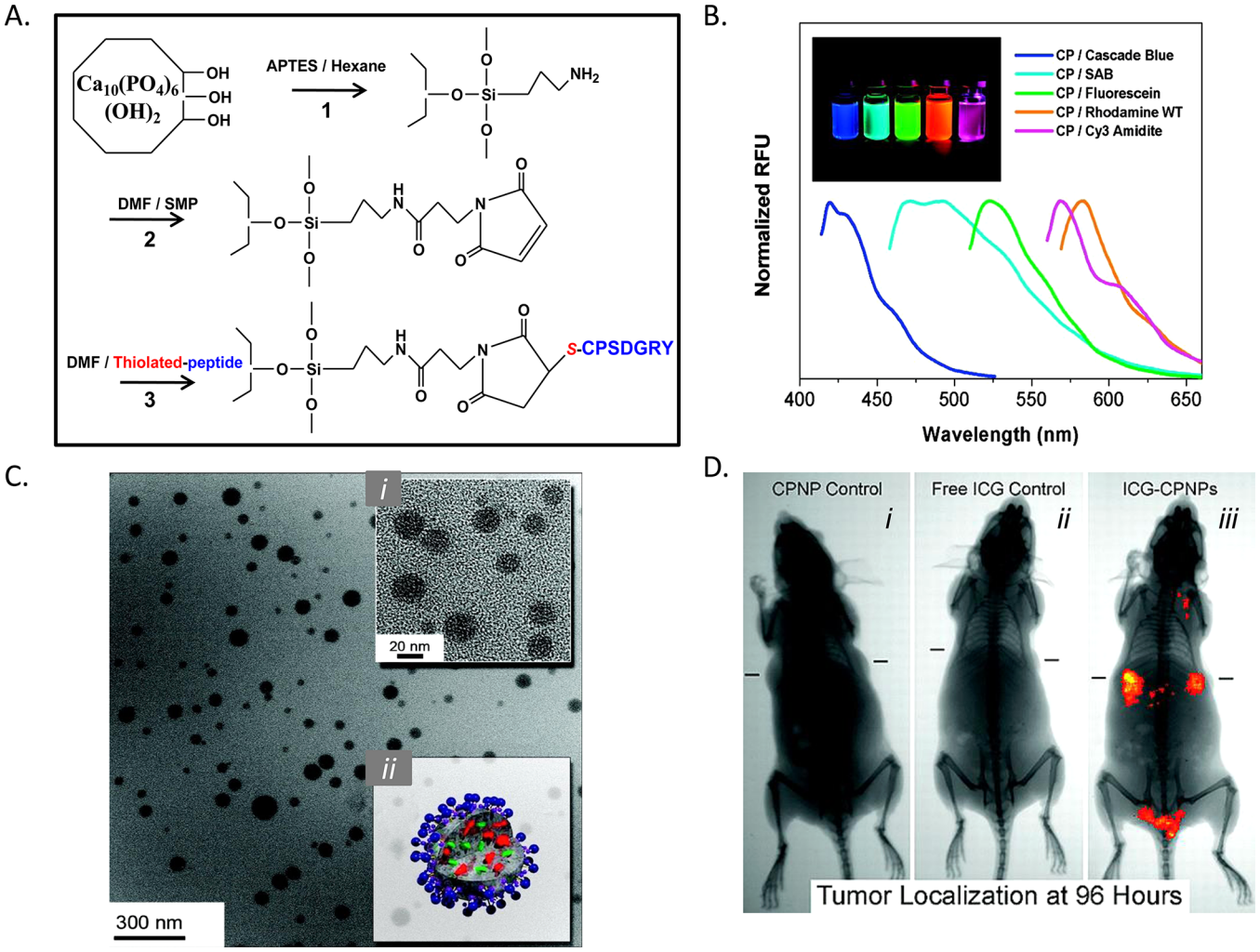

Figure 49. Modification of utility of $CaPO_4$ NPs. (A) Reaction scheme for functionalizing peptides onto $CaPO_4$-based NPs: (1) surface hydroxyl groups are activated with APTES to yield attached amino-silanes, (2) amines are activated to maleimides with SMP, which are (3) coupled to the free thiols on the peptide. Adapted from ref 1123. (B) Spectra and UV-excited photograph of dye-encapsulated CAP NPs (CP in figure) showing Cascade Blue, 10-(3-sulfopropyl) acridinium betaine (SAB), fluorescein, rhodamine WT, and Cy3 amidite. Reproduced from ref 1141. Copyright 2008 American Chemical Society. (C) TEM of dye-encapsulated indocyanine green (ICG-CPNP) dispersion with (i) inset showing the magnified particles and (ii) schematic CPNP architecture showing encapsulated imaging agent (green) and alternate payload (red) with full surface functionalization (blue). (D) NIR transillumination images (ex. 755 nm, em. 830 nm) for the ICG-CPNPs and controls delivered systemically via tail vein injections in nude mice implanted with subcutaneous human breast adenocarcinoma tumors. Hash marks next to each mouse indicate the position of the 5 mm tumors. Two control samples, (i) carboxylate-terminated CPNPs without ICG and (ii) free ICG, match the particle concentration and fluorophore content of a (iii) PEGylated ICG-CPNP sample. ICG-CPNP sample retains significant signal localized in tumors even after 96 h. Panels C and D reproduced from ref 1154. Copyright 2008 American Chemical Society.

of the same bioconjugation processes listed below. Most of the Ca NPs (Ca-based NPs) have been synthesized via microemulsion[1146−1148] or precipitation protocols,[1124,1149,1150] although alternative methods involving mineralization from aqueous solution[1151] and micelle-templated synthesis[1127] have also been described. Once synthesized, alkaline earth NPs are primarily biofunctionalized in three ways: (1) covalent surface chemistry, (2) coencapsulation of biomolecules, or (3) electrostatic interactions, especially with DNA for gene delivery applications. Examples of covalent functionalization are far more rare, and it appears that encapsulation and electrostatic interactions are currently the preferred bioconjugation methods. In the case of NPs that will undergo electrostatic interactions, postsynthetic surface modifications are usually not required.

3.7.1. Covalent Bioconjugation. In one of the few examples of covalent modification of alkaline earth NPs with biologicals, the Webster group biofunctionalized hydroxyapatite NPs and amorphous CaP NPs with peptides to investigate cellular interactions, see Figure 49A.[1123,1125] Hydroxyl groups present on the hydroxyapatite NPs were activated by surface silanization with APTES followed by reaction with the heterobifunctional cross-linker *N*-succinimidyl-3-maleimidopropionate. This provided maleimide groups on the NPs for subsequent attachment to the cysteine residues on peptides expressing either RGD or KRSR motifs, which are both known to influence adhesion to osteoblasts. While RGD showed a statistically significant increase in osteoblast adhesion of hydroxyapatite NPs compared with a nonadhesive RGE peptide control, the KRSR peptide only showed increased adhesion of the amorphous CaP NPs, but not crystalline hydroxyapatite NPs. Although only preliminary, these results again highlight the subtleties encountered in these systems and suggest that the

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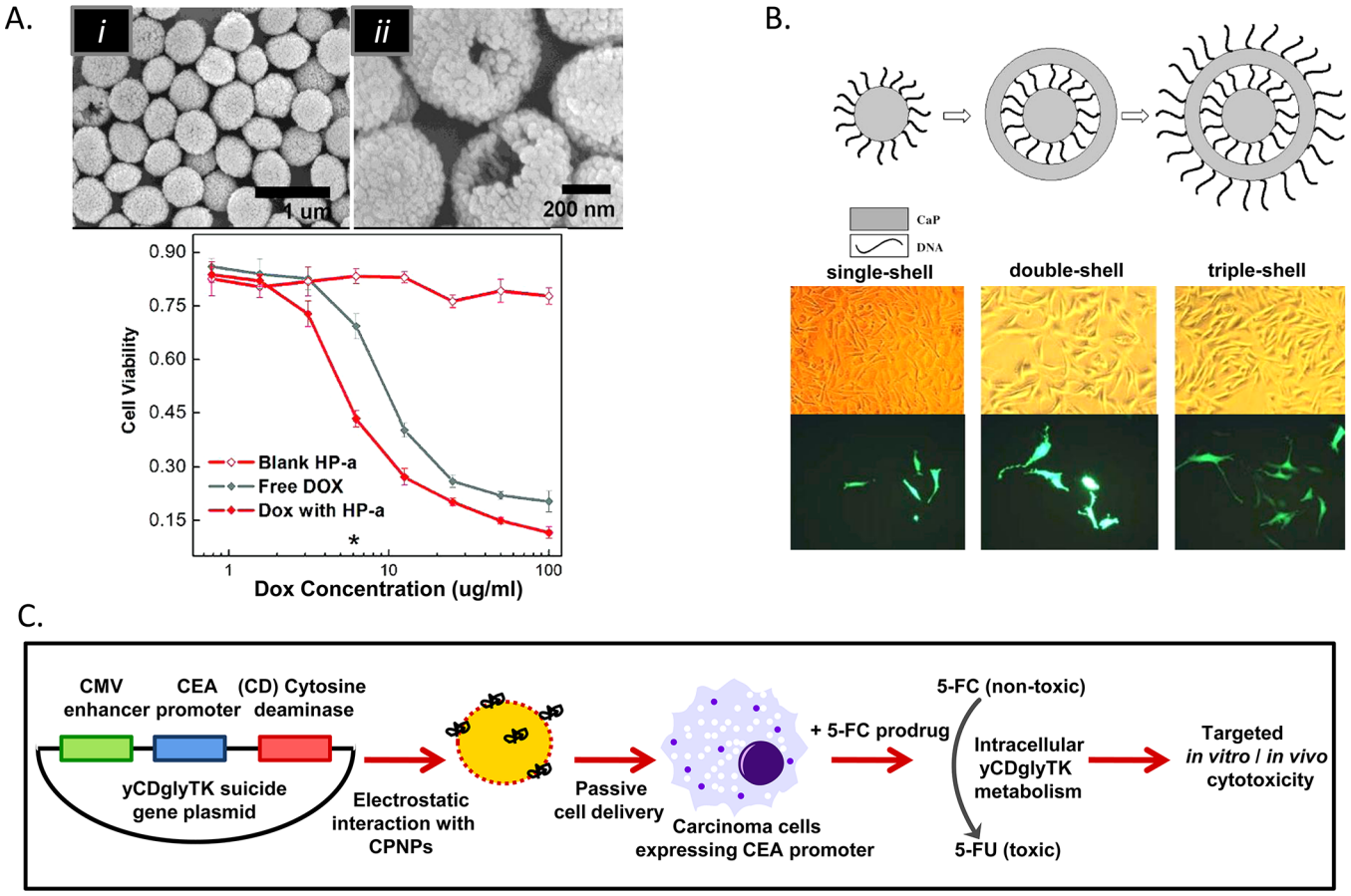

Figure 50. Calcium-based NPs. (A) SEM images showing (i) the uniform size of the $CaCO_3$ hierarchical particles (HPs) synthesized at 30 °C and (ii) some defects on the surface indicating the hollow structure of the spheres. Corresponding cytotoxicity of HepG2 cells exposed to free DOX, blank HP, and DOX-loaded HP for 48 h (mean ± SD, n = 4). Reproduced from ref 1130. Copyright 2008 American Chemical Society. (B) Schematic of the three types of $CaPO_4$–DNA NPs created by passively layering DNA over the NP and corresponding T-HUVEC cells transfected with each of the $CaPO_4$–DNA NPs encoding the gene for eGFP. An increase in transfection efficiency is noted when switching from single- to triple-shell NPs. Figure reproduced from ref 1155 with permission from Elsevier. (C) Schematic of the process for delivering the yCDglyTK suicide gene to cancer cells and selective activation of a 5-FC prodrug to a toxic 5-FU chemotherapeutic. Adapted from ref 1146.

continuing development of this bioconjugation strategy could impact some applications discussed above. In particular, further benefits may be derived from the introduction of bioorthogonal chemical handles, enabling greater control over the covalent conjugation of many biologicals.

3.7.2. Encapsulation. CaP NPs have also been used to encapsulate organics and especially fluorescent dyes for cellular imaging applications. These NPs are created primarily by coprecipitation followed by laundering to remove the residual precursors; the example given here utilizes van der Waals chromatography (vdW-HPLC laundering).[1152] Morgan et al. encapsulated numerous dyes (including Cascade Blue, 10-(3-sulfopropyl)acridinium betain, fluorescein, rhodamine, and Cy3 amidite) into citrate-capped CaP NPs by precipitation, as shown in Figure 49B, and also showed efficient delivery of Cy3 cargo to bovine aortic endothelial cells. The same group concurrently derivatized CaP NPs with PEG and amine groups via APTES as an example of secondary modification, though further biofunctionalization was not shown.[1141] Muddana et al. also described the photophysical properties of Cy3 encapsulated in analogous 7–10 nm CaP NPs.[1153] Altinoglu et al. used a similar method to encapsulate indocyanine green, a near-IR fluorophore useful for *in vivo* imaging, in 16 nm CaP NPs expressing surface carboxyl groups that were further functionalized with PEG using EDC chemistry.[1154] They were able to target the NPs to xenograft breast adenocarcinoma tumors implanted in nude mice by utilizing the intrinsic enhanced retention and permeability properties, see Figure 49C,D. The delivered NPs retained fluorescence in the tumor for up to 96 h suggesting strong potential for both cancer imaging and treatment.

Similar applications have been studied with $CaCO_3$ NPs, which have been shown to form hollow spherical shells or hierarchical particles, when starch is mixed in with the $CaCl_2$ and Na_2CO_3 precursors.[1130] Like CaP NPs, these NPs degrade under acidic conditions but not at physiological pH levels. Seeking to exploit the inherently lower pH of tumor environments, Wei et al. encapsulated DOX in $CaCO_3$ NPs for targeted drug delivery. The results demonstrated that incubating the DOX-loaded particles in cell culture media for 48 h can allow them to passively enter HepG2 human hepatocellular carcinoma cells with resulting cytotoxicity, see Figure 50A. Interestingly, some NPs were localized in and around the nucleus confirming some potential release from the endosomes after acidification.

In a modification of standard encapsulation chemistry, Sokolova et al. have described a popular method for creating multishell CaP–DNA NPs that involves creating an outer protective layer of CaP over the DNA to protect it from lysosomal

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nucleases during cellular delivery.[1155] A CaP NP serving as a core is first passively coated with DNA, which provides colloidal stability. The DNA-coated CaP NPs were then mixed with $Ca(NO_3)_2$ and $(NH_4)_2HPO_4$, to create the outer CaP shell. Triple-layer NPs were formed by mixing the double shell with more DNA to prevent aggregation and retain colloidal stability. The authors noted that this process can be repeated to add more layers as needed. Multiple cell lines were transfected with the gene encoding enhanced green fluorescent protein (eGFP) using the commercial agent Polyfect. Transfection efficiency results in T-HUVEC cells showing increased efficiency from the multishell NP formulations are presented in Figure 50B. Interestingly, Ca-containing drugs have undergone encapsulation into polymeric NPs[1156] (see section 4.3.1) and have been directly reformulated as NPs for testing of delivery efficacy. One prominent example is the lipid lowering drug atorvastatin-Ca, commercially known as Lipitor. Kim and co-workers have shown that atorvastatin-Ca can be formulated from its crystals into anhydrous, amorphous NPs using a supercritical antisolvent process;[1157] the process itself is thoroughly reviewed elsewhere.[1158] The amorphous atorvastatin-Ca NPs were shown to have better *in vivo* solubility, dissolution rates, and adsorption in rats compared with crystalline unprocessed drug, and this was primarily ascribed to the increased NP surface area.[1157]

3.7.3. Electrostatic Interactions. Due to their high intrinsic ζ potential, cationic Ca NPs are especially amenable to functionalization with anionic DNA by electrostatic interactions. This process has been described extensively in the literature for assembling Ca NP bioconjugates with DNA[1133,1146,1148,1150,1159−1161] and siRNA,[1162] as well as $MgPO_4$ DNA bioconjugates.[1133] DNA attachment is often done after NP synthesis by simple electrostatic interaction or by microemulsion during the synthetic process.[1148] Such DNA containing Ca NPs appear to function as relatively efficient transfection agents.[1163] A basic understanding of Ca NP morphology effects on cellular uptake[1150] and the intercellular fate of the NPs has been elucidated using CaP NPs functionalized with TRITC dye and a DNA plasmid encoding GFP.[1161] The TRITC dye provided a label to monitor intracellular NP fate, while GFP expression was used to monitor transfection efficiency.

Clinical pursuits based upon DNA transfection with alkaline earth NPs are typified by selective delivery of suicide genes to solid tumors. Suicide genes can activate nontoxic prodrugs into their toxic chemotherapeutic metabolites providing a mechanism of delivering a specific, localized therapy. One prominent example utilized the yCDglyTK suicide gene, a fusion of yeast (y) cytosine deaminase (CD) and herpes simplex type 1 thymidine kinase genes, which activates the nontoxic 5-fluorocytosine (5-FC) prodrug to toxic 5-fluorouracil and also increases cellular sensitivity to radiation therapy (Figure 50C). In this example, the suicide gene was genetically fused to a carcinoembryonic antigen (CEA)-specific promoter sequence. This binding protein is commonly overexpressed in colon and gastric cancer cells; thus, cells specifically expressing the suicide gene from the transfected construct will selectively activate the drug.[1146] A cytomegalovirus enhancer element was also attached to stimulate gene expression. CaP NPs ranging from ∼23.5 to 31.4 nm with a ζ potential of +25.1 mV were made by microemulsion, and the strong charge provided facile association with the yCDglyTK gene construct for subsequent cellular delivery. *In vitro* cell survival studies showed that SGC7901 gastric carcinoma cells had significantly decreased survival after delivery of 5-FC prodrug when transfected with CEA-yCDglyTK, while HeLa cells were not affected. The same DNA−CaP NPs induced a decrease in CEA expressing xenograft tumor volume after intratumoral injection in nude mice, indicating that the suicide gene is also expressed *in vivo* in live subjects.[1146] A follow-up study confirmed both the *in vitro* and *in vivo* results in colon cancer cells and nude mice using a LoVo human colorectal cancer xenograft,[1164] see process schematic in Figure 50C. Overall, the biocompatibility of the CaP NPs, the facile noncovalent bioconjugation chemistry, and the efficiency and specificity of this treatment highlight the potential for combining NMs with gene therapy to create synergistic treatments.

The use of alkaline earth NPs alone have increased osteoblast adhesion to artificial bone resins;[1126,1135] however, methods for increasing the proliferation of the osteoblast cells themselves (i.e., targeted osteogenesis) is also an area of considerable interest. In pursuit of this, CaP NPs have been used as a delivery system for the gene encoding bone-morphogenic protein 2, which stimulates bone and dental growth. DNA was incorporated into the NPs using electrostatic coating and mixed with osteoblast cells in an alginate hydrogel.[1160] The hydrogel allows for a minimally invasive injection of both the bioconjugated NPs and the osteoblasts directly into the injury site. Results showed that when injected into rat spines, hydrogel-containing osteoblasts and bone-morphogenic protein 2 CaP NPs stimulated bony tissue formation, while rats injected with hydrogel and osteoblasts alone did not have any bone formed. The DNA was found to release slowly over the course of two months from the hydrogel, which would be beneficial for long-term bone growth by providing continuous stimulation of the surrounding osteoblasts.[1160]

The electrostatic potential of CaP NPs has also proven useful for investigating drug delivery applications. Again, this takes advantage of the pH-dependent properties of CaP NPs but also combines them with noncovalent drug association. For example, the chemotherapeutic agent cisplatin has been shown to bind to CaP NPs by electrostatic interactions,[1124,1149] and the resulting conjugates showed significant toxicity against murine osteosarcoma cells and human ovarian cancer cells. Similar CaP NP-delivery characteristics are also of interest in the treatment of diabetes. While CaP NPs usually degrade at acidic pH, Ramachandran et al. showed, by monitoring *in vitro* release kinetics, that PEGylated CaP NPs could theoretically pass through the gastric region intact and release insulin specifically at intestinal pH levels.[1143] Besides providing stability, the PEG coating also functions to make the NPs hydrophilic in character thus allowing for increased interaction with the insulin. The NPs utilized were synthesized from $CaCl_2$, $Na_3C_6H_5O_7$, $Na_4P_2O_7$, and Na_2HPO_4; the phosphates were activated using EDC and conjugated to diamino-PEG. Insulin was loaded by a diffusion filling method, and then coated with Eudragit S 100 polymer.[1143] Using a similar insulin delivery approach with tricalcium phosphate microspheres that lacked the PEG coating but still used Eudragit S 100, Paul et al. showed decreased glucose levels in diabetic rats and mice upon oral administration. The authors suggested that engineering the treatment into a NP formulation could significantly improve overall oral insulin delivery efficacy.[1122]

3.8. Silicon and Germanium Nanoparticles

Group IV NPs are an emerging class of semiconductor nanocrystals with size-dependent PL properties stemming from quantum confinement. Although the PL QY at room temperature

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is lower than that of the more common group III−V (e.g., GaAs) or group II−VI (e.g., CdSe) QDs, their biocompatibility potentially makes them superior candidates for biomedical applications, and these materials are also eliciting a great deal of interest for diagnostics and electronic applications.[1165−1171] As an added benefit, some group IV NPs can be synthesized as mesoporous NP materials for the loading of drugs and other small molecules within their pores.[1167,1172] Several excellent reviews of group IV semiconductor NPs are available,[1165,1168,1171] and the reader can refer to these publications for additional insight on their synthesis,[877,935,1173−1177] properties, and applications. We remind the reader of the distinction between silicon (Si) and silica (SiO_2); the latter is described in more detail along with the other oxide NP materials in section 3.3.

Several methods for the functionalization of silicon NPs (Si-NPs) with organic molecules through the formation of Si−C bonds have been developed. Early approaches relied on surface functionalization through covalently bound organic layers introduced concomitantly to the synthesis of the Si-NPs, usually under harsh conditions and in nonaqueous environments.[1178] For instance, the reduction of chloro- and alkylsilanes with Na at high pressure and temperature[1179] yielded covalent alkyl-derivatized Si-NPs. Alternatively, chloride-capped Si-NPs obtained via the reaction of NaSi (Zintl salt) with $SiCl_4$ have been directly alkylated by addition of Grignard (RMgX) or organolithium (RLi) reagents (where R = alkyl, X = halogen).[1180] The latter method is relatively simple and can be applied to the addition of various alkyl groups provided that the organolithium or Grignard reagent is commercially available or synthetically accessible. However, the preparation of the Zintl salt is not trivial and might limit the implementation of this synthetic procedure. Depending on the nature of the organic molecule to be conjugated, these earlier functionalization approaches might not be widely applicable, and this is especially true if the conjugation partner is a biomolecule. Milder and more versatile methods are therefore desirable. Probably the simplest and most direct methods to conjugate organic molecules to Si-NPs are the thermal,[1181,1182] catalytic,[1183−1186] or UV-photoinitiated[1187−1190] hydrosilylation of terminal alkenes or alkynes with hydride-terminated (Si−H) Si-NPs. For preparing the latter, the Si-NPs can be synthesized directly with a Si−H passivated surface.[1184,1191] Alternatively, Si−H surface passivation can be easily achieved by treatment with HF[1185,1187] or $LiAlH_4$[1183] prior to alkylation. Si-NPs prepared in organic solvents can be converted into water-soluble suspensions by hydrosilylation with allylic amines[1186] or acrylic acids.[1192] The length of the aliphatic chain has an effect on the chemical stability and solubility of the capped Si-NPs, with longer chains yielding more stable and suspendible formulations.[1192] Although the main reason for introducing terminal amines or carboxylic acids was to increase water dispersibility, these moieties could be, in principle, exploited as anchoring points for functional group interconversion using any of several amine-reactive homo- or heterobifunctional linkers. Several preliminary Si-NP bioconjugates have been prepared via the hydrosilylation route including those displaying DNA,[1193] siRNA,[1194] and antibodies.[1195] Very recently, Si-NPs conjugated with FA and apo-ferritin have been obtained through the incorporation of an intermediate heterobifunctional cross-linker to a covalently attached layer of undecylenic acid.[1181] Linear aliphatic chains introduced by UV-initiated hydrosilylation have also been utilized as anchoring points for the UV-triggered addition of a heterobifunctional linker, leading eventually to SA-functionalized Si-NPs.[1196] Alternatively, alkyl-terminated Si-NPs can be oxidized with *m*-chloroperbenzoic acid to yield terminal epoxides or vicinal diols.[1197] Both groups can be further reacted with appropriate conjugation schemes, such as nucleophilic addition for the epoxides or the periodate oxidation/reductive amination sequence for the diols.

An alternative procedure to coating Si-NPs with organic molecules is the controlled oxidation of the Si-NP surface to obtain a passivated SiO_2 or SiOR layer (R = alkyl). Following passivation, a silanization step with organosilane agents ($RSiOR'_3$) permits access to a variety of functionalized Si-NPs.[1198,1199] A drawback of this method is the relative complexity in controlling the oxidation and silanization step, which might result in a significant increase of the NP size and aggregation. Although the commercial and synthetic accessibility of organosilanes is quite large, the available molecules and functional groups that can be introduced by this route are far smaller and impose a limit on this approach. One important additional issue to consider when functionalizing Si-NPs is the change in the photophysical properties of the resulting construct.[1200]

Because both Ge and Si are group IV elements, the conjugation chemistries available for the functionalization of GeNPs are very similar to those available for Si-NPs. However, the conjugation examples reported for GeNPs are much more limited because this NM has only recently started to attract interest. At present, GeNPs have been synthesized and passivated as Ge−H surfaces and then catalytically hydrogermylated with allylamine to obtain amine-terminated and water-soluble Ge-NPs[1201] or those with alkylic chains.[1202] PEGylated Ge nanowires have also been reported and obtained through preliminary attachment of a thiocarboxylic acid by thermal thiolation, followed by EDC/NHS-mediated attachment of amino-terminated PEG.[1203]

4. BIOCONJUGATION OF OTHER NONTRADITIONAL AND ORGANIC NANOPARTICLE MATERIALS

4.1. Carbon Allotropes

Carbon NMs come in a wide range of forms including carbon nanotubes (CNTs), spherical fullerenes, nanodiamonds (NDs), graphene NMs, carbon nano-onions, -peapods, -horns, -cups, -rings, and -containers.[1204−1206] Each NM is characterized by widely differing physical and chemical properties. This diversity represents a variety of allotropic forms including ND, the fullerene spheres and nanotubes, graphite and graphene, and amorphous carbon. Carbon NMs have shown great potential in a number of bioapplications including vectors for drug and gene delivery, contrast agents, therapeutic agents, and components of biosensing configurations; however, progress has been hampered by their inherent insolubility.[1207−1211] Although many of these materials are often synthesized in the research laboratory, a significant advantage in their development is that many major allotropes (e.g., CNTs, fullerenes, and NDs) are now available commercially, often premodified with desirable functional groups that aid in solubilization and bioconjugation. For example, Sigma-Aldrich, Carbon Solutions Inc., and Nano-C Inc. offer a wide range of unmodified and functionalized fullerenes and CNTs. Element Six, Microdiamant, and NanoAmor: Nanostructured and Amorphous Materials Inc. are commonly used sources for ND materials. See also Supporting Table 1, Supporting Information.

4.1.1. Carbon Nanotubes. Of all carbon-based NMs, CNTs are probably the most studied to date in bionanotechnology and

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have been applied to a range of applications, which include imaging, therapeutics, sensing, and bioelectronics.[9,38,39,1208,1209,1212−1216] CNTs are members of the fullerene structural family and comprise graphene tubes that come in two main forms: SWCNTs with an outer diameter of 0.6−2.4 nm and multiwalled nanotubes (MWNTs), which consist of multiple concentric layers of SWCNTs with an outer diameter ranging from 2.5 to 100 nm.[1210,1211] CNT lengths typically range from nanometers up to millimeters, leading to extraordinary aspect ratios. SWCNTs, in particular, possess a number of unique properties, including electrical, PL, and Raman scattering, all of which directly aid in characterization and particle tracking. Along with straight tubular structures, CNTs containing surface defects yield alternative tubular morphologies such as branched, waved, beaded, or coiled structures, where each may also possess unique mechanical and electrical properties, such as tensile strength or hardness, metallic or semiconducting behavior, or anisotropic thermal and electrical conductivity.[1217] CNTs are typically synthesized using one of three techniques: chemical vapor deposition, electric arc discharge, or laser ablation, with the properties of the resulting CNTs being somewhat dependent on the synthetic route used.[1210,1211,1218−1220] Purification of synthesized CNTs typically involves strong acid oxidation treatment to remove metal catalysts and carbonaceous impurities, although less harsh alternatives, such as chromatography, are under investigation.[1218,1221,1222] In an attempt to allow greater control over the aspect ratios and resulting structures, Chun and co-workers developed a technique using anodized aluminum oxide templates that allowed the synthesis of unique morphological graphitic carbon materials, including nanocups, nanorings, nanocontainers, and short nanotubes.[1204] The chemical and biological modification of CNTs has been previously reviewed,[9,39,1214,1223] with biofunctionalization

typically involving either covalent modifications, noncovalent interactions, or, rarely, a combination of both. It is important to note that the introduction of chemical handles or functional groups onto the CNT for subsequent modification purposes can potentially disrupt the native structure and introduce defects.[9,39,1214,1223] Despite their potential in a variety of biomedical applications, as with most NMs intended for *in vivo* use, the debate surrounding CNT toxicity is ongoing and would certainly benefit from better characterization and consistency, as discussed in section 7.[1224]

4.1.1.1. Noncovalent Modification. Noncovalent functionalization of CNTs is a popular modification method and generally relies on van der Waals, $\pi-\pi$ stacking, and less well-defined hydrophobic interactions. Pyrene-derived materials have been shown to easily interact with CNTs via $\pi-\pi$ stacking.[1225−1227] Likewise, the aromatic nucleobases on single-stranded DNA (ssDNA) are found to interact directly via $\pi-\pi$ stacking, with DNA actually wrapping itself around the CNT.[1228−1231] Researchers using phage display technology have found that peptide sequences comprising repeat units of the aromatic amino acid tryptophan have a high affinity for CNT side walls, likely due to $\pi-\pi$ stacking interactions, leading to the possibility of unique peptide tags that could aid in additional bioconjugation.[1232,1233] DNA can also undergo electrostatic interactions with CNTs modified to contain amine functional groups. Karmakar and co-workers used ethylenediamine-functionalized SWCNTs to deliver the oncogene suppressor p53 gene to MCF-7 breast cancer cells and subsequently induced apoptosis.[1234] The potential impediment to this approach would be the lack of control over attachment orientation, DNA ratio, and structure within the conjugate. Lipid-based surfactant structures are also known to interact with CNTs via hydrophobic interactions. A number of groups have used this to their advantage to functionalize CNTs with surfactant−PEG−polymer species, which improved *in vivo* biocompatibility,[1235,1236] or surfactant−PEG/poly(ethylene oxide) derivatives that have terminal amines, hydroxyls, or biotin, which can allow further protein or DNA attachment.[1237−1239] For example, McCarroll and co-workers synthesized a lipid-based lysine dendrimer to functionalize the surface of SWCNTs with siRNA, which was then used for systemic delivery into mice models.[1239] Here, the lipid portion of the molecule bound to the hydrophobic walls of the oxidized SWCNTs, leaving the lysine dendrimer portion to electrostatically interact with the siRNA. Loading of the lipid-based dendrimer molecule to the oxidized SWCNTs could be increased through the addition of EDC, which covalently coupled the carboxylic acids present on the SWCNT ends with the amines on the lysine dendrimer portion. Interestingly, this example represents one of the few where covalent and noncovalent chemistry are both used, with the former acting to potentiate the functionalization.

Proteins are also capable of interacting with CNTs via hydrophobic or electrostatic interactions; however, depending on the specific protein in question, this is often accompanied by structural changes that can lead to undesired inactivation of function.[1240−1243] Tsai and co-workers modified SWCNTs with GOx and determined that the enzyme retained 75% activity upon adsorption.[1242] The resulting GOx−SWCNTs were used in layer-by-layer electrochemical biosensors for glucose, demonstrating enhanced sensor response that was attributed to increased electron diffusion through the films enhancing the electrochemical surface area of the GOx−SWCNT-modified sensors. Electrostatic interactions can take advantage of the anionic carboxylate groups that are readily introduced to CNTs. For example, Yang and co-workers functionalized carboxylated CNTs with a positively charged poly(diallyldimethylammonium) chloride polymer, followed by exposure to a negatively charged antibody for conjugate formation.[1244] Alternatively, Kang and co-workers used electrostatic interactions to noncovalently bind the natural polymer CHI to CNTs, and the excess amines displayed on the polymer were further used to covalently functionalize the CNTs with FA via EDC/NHS chemistry, thereby allowing for targeting of HepG2 cancer cells.[1245] Zhang and co-workers modified SWCNTs with various combinations of the polysaccharides sodium alginate (ALG), CHI, or both, followed by adsorption of DOX in pursuit of assembling a drug delivery vector.[1246] The CHI/ALG-modified SWCNTs were covalently functionalized with FA using EDC chemistry prior to DOX adsorption. Drug loading and release from the various polysaccharide-modified SWCNTs was then characterized before incubating the DOX-FA-CHI/ALG-SWCNTs with HeLa cells for cytotoxicity testing. The use of mixed CHI and ALG polysaccharide surfaces on the SWCNTs also allowed for control over the release times of the positively charged DOX in acidic environments.

4.1.1.2. Chemical Modification. Covalent modification of CNTs often begins with an oxidation treatment that selectively introduces carboxylic acid functionalities at the CNT tips or exterior wall defects.[9,1247] The resulting carboxylic acids are then easily coupled, using EDC/NHS chemistry, to polymers, proteins, or other biomolecules with reactive amine groups. Examples of materials with which CNTs have been covalently

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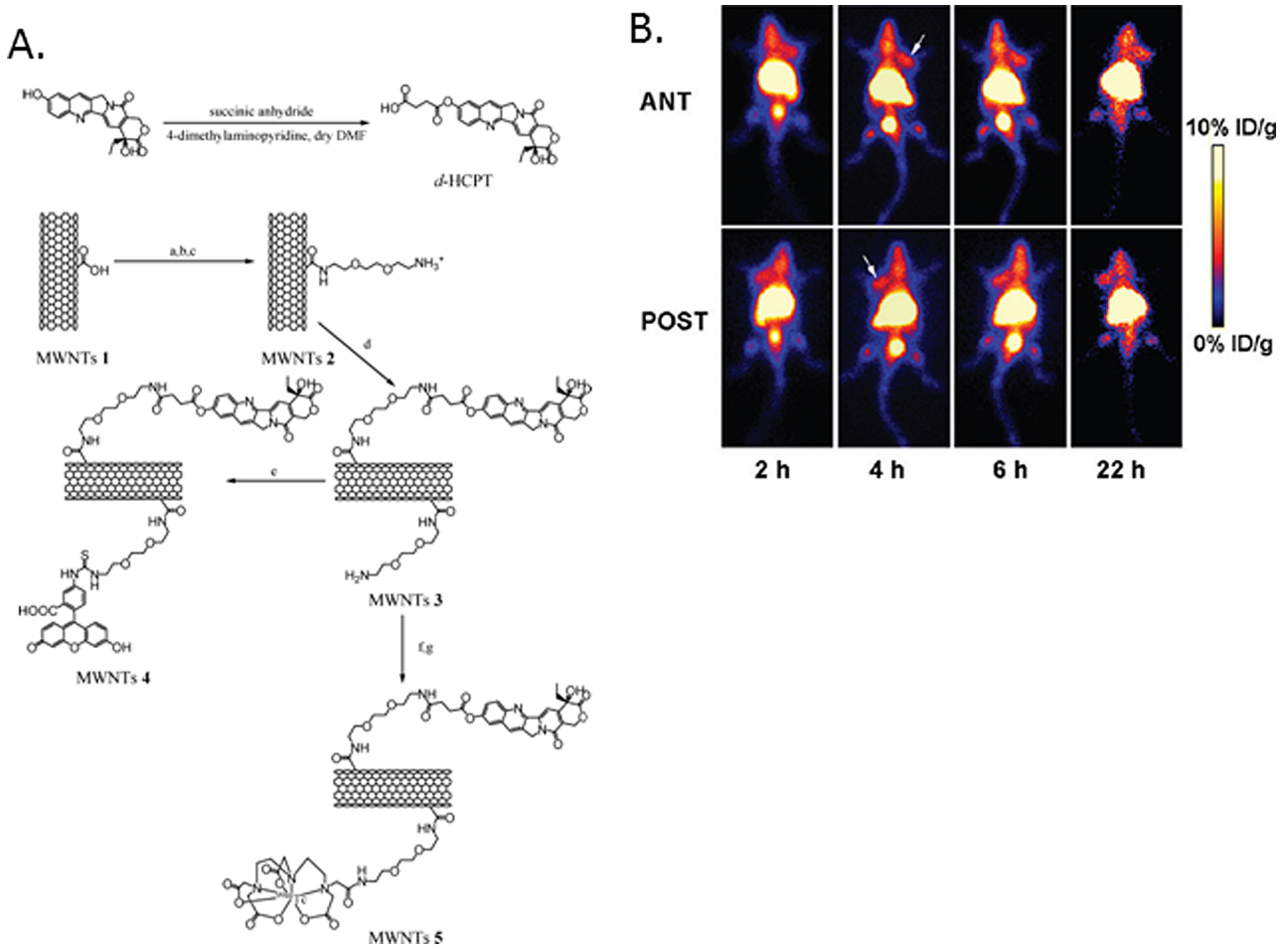

Figure 51. MWNT delivery of antitumor agent HCPT. (A) Synthesis route of the MWNT−HCPT conjugates: (a) thionyl chloride, reflux; (b) Boc-$NH(CH_2CH_2O)_2$−$CH_2CH_2NH_2$, triethylamine, anhydrous THF, reflux; (c) 4 M HCl in dioxane; (d) *d*-HCPT, EDC, NHS, triethylamine, anhydrous DMF; (e) FITC, anhydrous DMF; (f) DTPA dianhydride, triethylamine, anhydrous dimethyl sulfoxide (DMSO); (g) stannous chloride, $^{99m}TcO_4$. (B) SPECT images of a hepatic H22 tumor-bearing mouse at various time points after injection of MWNTs **5** [see panel A] via the tail vein obtained from anterior (top) and posterior (bottom) acquisition under anesthetized conditions. The arrows denote the regions of the tumor. Reprinted with permission from ref 1255. Copyright 2009 American Chemical Society.

bioconjugated, via EDC/NHS chemistry, include BSA,[1248] various enzymes,[40] ferritin,[1248] epidermal growth factor (EGF),[1249] antibodies,[1250] biotin,[1251] various protein biomarkers for prostate cancer,[1252] and cationic glycopolymers.[1253] However, in a critical assessment of this conjugation technique for CNTs, Gao and Kyratzis noted the importance of appropriate controls to ensure the attachment was truly covalent and not a result of intrinsic hydrophobic and electrostatic interactions.[1254] Carboxylic acid groups have also been used to covalently couple the drug 10-hydroxycamptothecin (HCPT) to the surface of MWNTs, see Figure 51, followed by further modification with either fluorescein or radioactive ^{99m}Tc in order to facilitate fluorescence or SPECT imaging and gamma scintillation counting, respectively.[1255] During *in vivo* injection and biodistribution studies in hepatic H22 tumor-bearing mice, the highest organ concentrations of these MWNTs were found in the liver and spleen, although significant levels were also found in the lung, kidney, stomach, femur, and tumor. Subsequent antitumor studies in the same animal model revealed a reduction in overall tumor weight in mice treated with HCPT−MWNTs relative to mice injected with only HCPT and saline, demonstrating the enhanced antitumor effect of the MWNT−drug complex. This is a particularly interesting finding given that the drug was covalently coupled to the MWNT.

Studies have also shown that nitric acid treatment of CNTs actually leads to higher levels of aldehyde/ketone functionalities than carboxylic acids, suggesting that aldehyde/ketone-reactive chemistries such as hydrazine or Schiff base formation may be better suited to CNT modification.[80,1256] Various covalent cycloaddition-based techniques for CNT side wall modification have also been demonstrated.[9,1213,1214,1238,1257,1258] For example, Mackeyev and co-workers used cyclopropanation to attach a short peptide sequence to the surface of Gd-modified SWCNTs.[1258] Others have introduced pyrrolidine moieties with terminal amines to MWNTs using 1,3-dipolar cycloaddition of azomethine ylides.[1238,1257] Herrero and co-workers used these groups to generate dendron-functionalized MWNTs containing peripheral tetraalkyl ammonium salts that facilitated electrostatic interaction with siRNA for application in gene silencing and knockdown therapies.[1257] Chen and co-workers used amine-terminated pyrrolidine-functionalized SWCNTs to covalently attach a taxoid drug incorporating a disulfide linker, which allowed for hydrolytic/reductive drug release upon receptor-mediated endocytosis into a leukemic cell line, see Figure 52.[1238] Zhang and co-workers used zwitterion-mediated

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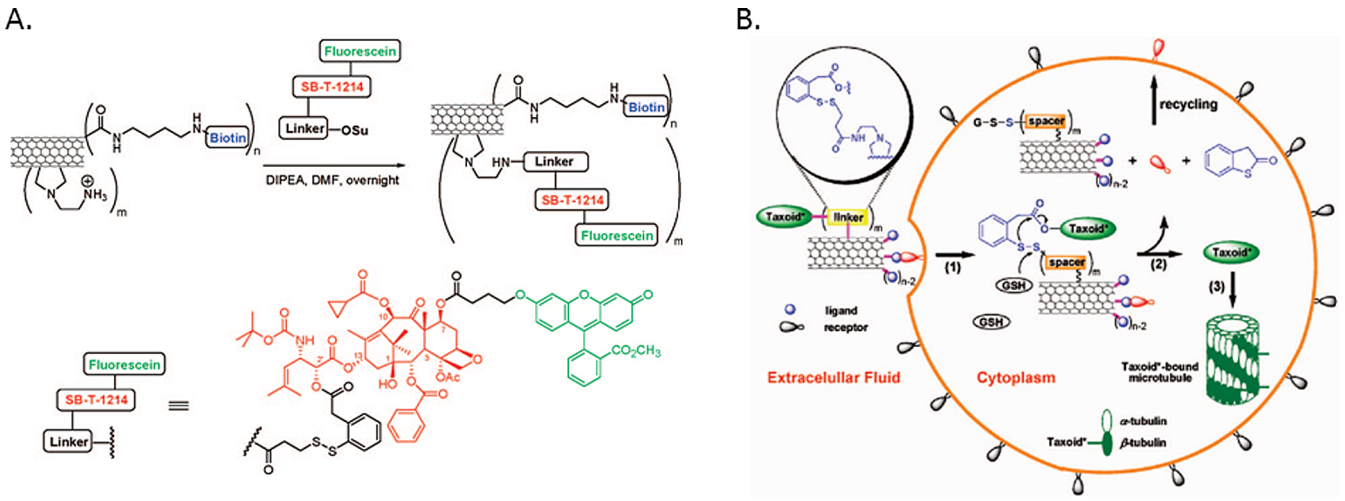

Figure 52. Targeted delivery of the prodrug taxoid using SWCNTs. (A) Synthesis of biotin−SWCNT−linker−(taxoid−fluorescein) conjugate. (B) Schematic illustration of three key steps involved in the tumor-targeting drug delivery of biotin-linker−taxoid conjugates. Reprinted with permission from ref 1238. Copyright 2008 American Chemical Society.

reactions, under relatively mild conditions, to introduce alkyne groups that could be further functionalized using CuAAC.[1259] Click chemistry was also demonstrated for the layer-by-layer functionalization of MWNTs and could be readily applied for additional bioconjugation of the CNTs.[1260]

Covalent modification of SWCNTs, in particular, while robust in nature, can disrupt their unique optical and electronic characteristics, which can be problematic depending on the desired application. Double-walled carbon nanotubes may address some of these concerns because they have similar optical and electrical properties to their SWCNT counterparts, but the additional outer tube wall can sometimes be functionalized without disrupting the underlying function.[1261,1262] It should be noted, however, that a study by Tsyboulski and co-workers evaluating the fluorescence properties of these materials attributed the fluorescence to SWCNT impurities in the sample.[1263]

4.1.1.3. Biotin-Based Modification and Encapsulation. Liu and co-workers used the carboxylic acids on oxidized SWCNTs to covalently attach biotin.[1251] Following biotinylation, the SWCNTs were exposed to SA and subsequently modified with biotin-labeled DNA, fluorophores, or AuNPs in a layered assembly approach. Extensive characterization of the resulting bioconjugates demonstrated this method as a promising generic tool for modifying SWCNTs with a wide range of materials. In a fascinating demonstration, Chen and co-workers developed and applied a cellular nanoinjector based on CNTs, allowing the introduction of materials into the cell with minimal disruption of the cell membrane.[1227] They took advantage of the known $\pi-\pi$ stacking interaction between a pyrene moiety and the surface of CNTs to functionalize MWNTs with a terminal biotin linked to the pyrene by a disulfide linker. The MWNTs, which were attached to an AFM tip, were then functionalized with SA−QDs and injected into the cytosol of HeLa cells, where cleavage of the disulfide bound occurred and resulted in the release of the SA−QDs from the MWNT nanoinjector, see Figure 53. Although less studied to date, CNTs have also demonstrated the potential to encapsulate a range of biomolecules of interest including drugs,[1264] proteins,[1265,1266] and DNA.[1267]

4.1.1.4. Modification with Other Nanoparticle Materials. Researchers have used biotin−SA affinity to modify biotin−MWNTs with SA−QDs[1227] and biotin−SWCNTs with biotin−AuNPs via a SA bridge.[1251] Lo and co-workers used an electrochemical method to decorate SWCNTs with Ni-NPs.[1268] The Ni-NPs were subsequently used to orientate and immobilize antibody single chain variable fragments (scFVs) containing a His-tag onto the SWCNT−Ni-NPs surface creating a field effect transistor sensor capable of detecting the tumor marker CEA. SWCNTs functionalized with pendant amine groups, achieved through pre-exposure to amine containing polymers, were found to act as nucleation sites for gold, resulting in facile deposition of AuNPs onto the surface of the CNTs.[1269] GA-modified magnetic NPs were introduced to the surface of poly(diallyldimethylammonium) chloride-functionalized MWNTs through simple mixing.[1270] The resulting GA-modified magnetic NP−MWNTs were then deposited onto glassy carbon electrodes before functionalization with cholesterol oxidase and HRP to generate an electrochemical sensor for cholesterol demonstrating a dynamic range between 0.01 and 0.95 mM.

4.1.2. Spherical Fullerenes. Since the first description of C_{60} in 1985,[1271] extensive research has led to a wide range of potential fullerene applications in the fields of biology and medicine.[1272−1274] Spherical fullerenes consist of a carbon cage approximately 1 nm in diameter and share much of the potential and challenges (e.g., aqueous solubility) associated with their CNT counterparts.[1275−1277] Fullerenes are typically synthesized in the laboratory, using approaches such as arc discharge between graphite rods or the combustion of benzene, although they have also been found occurring in nature.[1278,1279] Most initial modifications of fullerenes were designed to aid in solubility and bioconjugation and relied primarily on covalent chemistry.[1259,1280] Fullerene-bioconjugate applications to date are primarily as gene delivery vectors,[1274,1280−1282] drug delivery vectors,[1274,1283−1286] antibody probes for cancer targeting,[1287] and dual antibody/drug constructs for targeted therapy.[1288]

DNA has also been conjugated to fullerenes either covalently[1275] or through electrostatic interactions with cationically modified fullerenes.[1275,1280−1282] Maeda-Mamiya and co-workers developed a cationic tetraamino-modified fullerene that facilitated DNA adsorption through electrostatic interactions for *in vivo* gene delivery and demonstrated its effectiveness by transfecting the eGFP and insulin 2 genes in mouse

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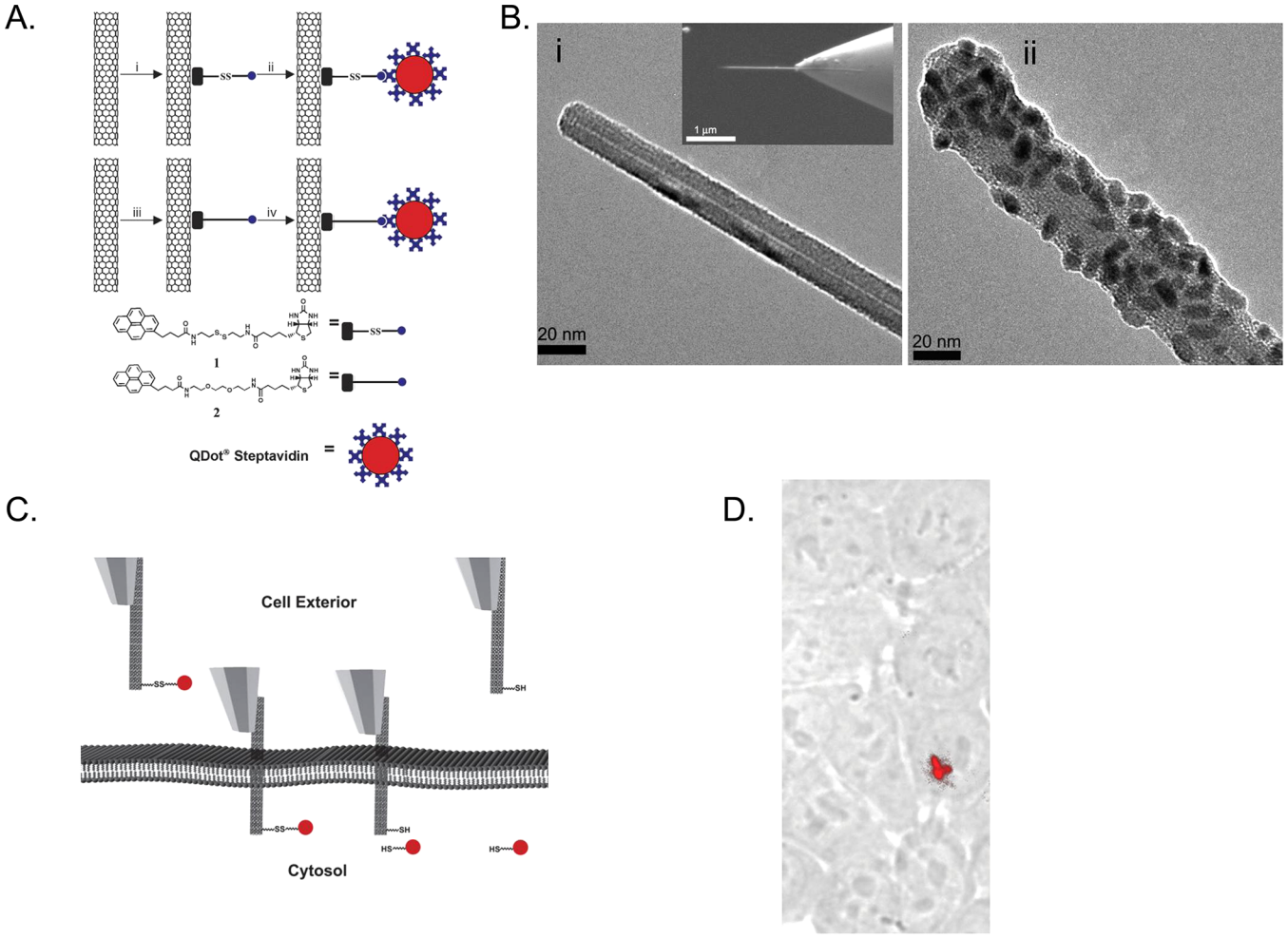

Figure 53. MWNTs for nanoinjection into cells. (A) Functionalization of MWNT−AFM tips. QD−SA was attached to the MWNT surface through linker **1** containing a disulfide bond: (i) **1**, MeOH; (ii) QD−SA, borate buffer. QD−SA was attached to the MWNT surface with linker **2** containing no disulfide bond: (iii) **2**, MeOH; (iv) QD−SA, borate buffer. (B) Characterization of nanoneedles before and after loading the cargo. (i) TEM image of the MWNT tip region; inset shows SEM image of a whole MWNT−AFM tip. (ii) TEM image of a MWNT−AFM tip coated with linker **1** and conjugated with QD−SA. (C) Schematic of the nanoinjection procedure. A MWNT−AFM tip with cargo attached to the MWNT surface via a disulfide linker penetrates a cell membrane. After disulfide reduction within the cell's cytosol, the cargo is released, and the nanoneedle is retracted. (D) Combined bright-field and fluorescence image of the cells after the nanoinjection of QD−SA conjugates into a target HeLa cell. Images reproduced with permission from ref 1227. Copyright 2007 National Academy of Sciences, USA.

models.[1282] Drugs are generally covalently attached to fullerene adducts using more "classical" synthetic organic chemistry methods,[1283,1285,1288] while antibodies are usually attached using either a NHS ester functionality introduced onto the fullerene surface[1288] or a disulfide exchange mechanism with SPDP-modified fullerenes and thiol-functionalized antibodies.[1287]

4.1.3. Nanodiamonds. ND particles have recently garnered strong interest due to their superior biocompatibility and hydrophilicity compared with other carbon NMs, along with bright fluorescence induced by radiation damage.[1289−1291] Bioconjugated NDs have a number of potential applications including imaging, drug delivery, protein separation, and biosensing.[1292−1294] For an excellent review covering the synthesis, properties, biocompatibility, and potential bioapplications of ND particles, see ref 1290. NDs have been produced by a number of methods, such as milling of larger grain diamonds, high pressure−high temperature, and detonation of carbon-containing explosives, where the resulting particle size (∼5−100 nm) depends on the technique.[1290,1291] NDs may contain nitrogen (N) impurities, which lead to their classification as type Ia, Ib (0.001−0.300% N), or II (0% N) depending on the nitrogen content.[1292] Radiation or ion beam damage followed by thermal annealing of ND particles produces nitrogen-vacancy (NV) point defects in the nanocrystalline structure and leads to intense fluorescent properties, typically referred to as NV color centers. Unlike QDs, where the emission wavelength depends on size and material, ND emission is typically determined by the structure and composition of the color center. Bright red fluorescence (∼700 nm) originates from type Ib NDs containing (NV) defects, and green fluorescence (∼530 nm) originates from type Ia NDs containing N−V−N defects.[1289,1295−1298] Blue fluorescent NDs have been produced by covalently linking octadecylamine to the surface of 5 nm NDs synthesized by detonation, and similar surface passivation of ND materials with PEG-based polymers has also been found to generate green/blue fluorescence, although in both cases the mechanism responsible for emission remains unclear.[1299,1300]

Generally, the fluorescence of larger scale NDs (>7 nm) is bright and does not photobleach or blink (cf. QDs), although Bradac and co-workers observed blinking of NV color centers

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in discrete 5 nm NDs that could be prevented by surface modification.[1301] The ND emission bands are spectrally broad (~200 nm), and this makes multiplexing with ND particles challenging to implement.[1296] Ultimately the surface functionality of the final ND depends, to some extent, on the method of synthesis, treatment, and purification. Strong acid oxidation treatment, reduction, high temperature or a bead-assisted sonic disintegration process, for example, can lead to a range of surface groups including carboxylic acid, hydroxyl, carboxylate, ester, alkene, or ether groups.[1290,1291,1302,1303] Chang and co-workers introduced a range of surface functionalities to surface-graphitized NDs using ultrasonication and microwave-initiated free radical copolymerization of oligomers.[139] In addition, some organic modification schemes have been developed to functionalize the ND surface with amines.[1304,1305] However, most methods rely on carboxylic acid or hydroxyl functions, as highlighted below.

4.1.3.1. Noncovalent Adsorption. Carboxylic acid functionalized NDs have been used to improve the delivery of various water-insoluble and other therapeutics including, DOX, purvalanol A, 4-hydroxytamoxifen, and dexamethasone by complexing with the drug molecules and improving their overall dispersion in water.[1293,1306] Carboxylic acid-functionalized NDs have also been demonstrated as an effective platform for gene delivery[139,1307] and protein adsorption.[1308] For gene delivery, the ND surface was first exposed to a positive polymer, such as PEI, which subsequently facilitated the electrostatic interaction of siRNA[139] or plasmid DNA,[1307] see Figure 54.

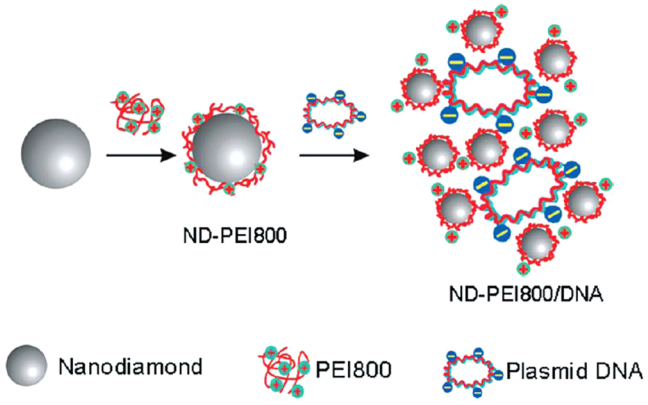

Figure 54. Schematic of polymer PEI800-functionalized ND platforms used for plasma DNA delivery. Reprinted with permission from ref 1307. Copyright 2009 American Chemical Society.

PEI–NDs functionalized with luciferase plasmid DNA (pLuc) were found to be 400 and 800 times more efficient at pLuc transfection than amine-modified and unmodified NDs, respectively.[1307]

4.1.3.2. Covalent Modification. The carbonyl functions typically present on the surface of detonated NDs can be converted to carboxylic acids via oxidation in the presence of strong acids. Similar to CNTs, carboxyl groups are frequently employed for covalent bioconjugation to the ND surface using EDC/NHS chemistry. Example conjugates include Tf,[1309,1310] BSA,[1311] poly-L-lysine (PLL) and dextran,[1311] PEG and FA,[1312] PLL followed by electrostatic interaction with DNA,[1296] and a Gd(III) MRI contrast agent.[1313] Fluorescent NDs functionalized with Tf[1309] and FA[1312] demonstrated receptor-mediated uptake in HeLa cells and were imaged using fluorescent microscopy, see Figure 55. In both cases, the addition of excess

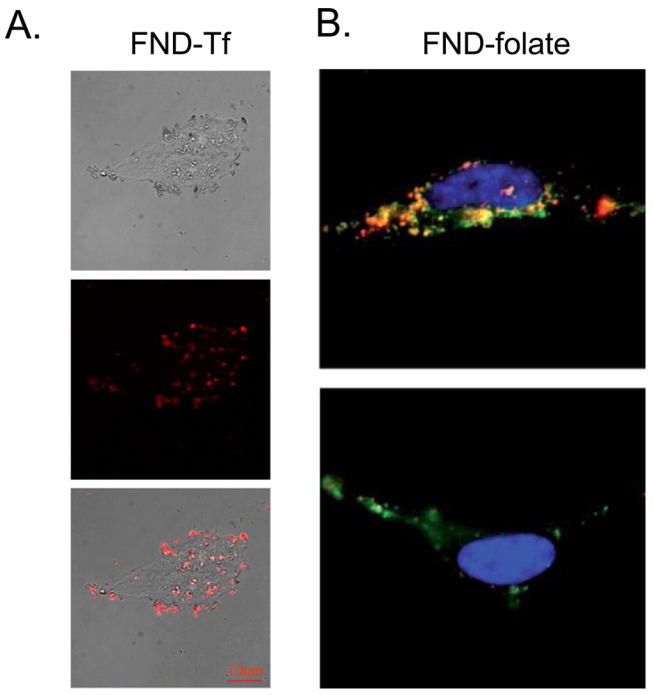

Figure 55. Fluorescent nanodiamond (FND) bioconjugates for targeted imaging. (A) Comparison of confocal fluorescence images of HeLa cells treated with FND–Tf bioconjugates; bright-field, fluorescence, and merged images (top to bottom). Reprinted from ref 1309, Copyright 2009, with permission from Elsevier. (B) Images of FA-conjugated FNDs internalized by HeLa cells without or with free FA in the media (top and bottom, respectively). Confocal fluorescence of cells with their membrane and endoplasmic reticulum stained in green with wheat germ agglutinin Alexa Fluor 488 conjugates, and nuclei stained in blue with Hoechst33258, FNDs in red. All the cells were incubated with FNDs for 3 h at a particle concentration of 10 mg/mL. Reprinted with permission from ref 1312. Copyright 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

FA or Tf for competitive purposes in the cell media reduced uptake of the ND bioconjugate and demonstrated the specificity of the receptor-mediated uptake process.

The carbonyl functions can also be converted to hydroxyl groups either by reduction in the presence of borane[1302,1303,1312] or a process referred to as the Fenton treatment, which consists of reacting the NDs with H_2O_2 and $FeSO_4$ under strongly acidic conditions; this also removes undesirable soot matter while increasing the density of surface hydroxyl groups for further functionalization chemistry.[1314,1315] Silanization of the subsequent hydroxyl groups is also a popular method to introduce a wide range of functionalities to the ND surface including amines,[1312] amines followed by biotin (see Figure 56),[1302] isocyanate,[1314] and acrylate.[1303] Martin and co-workers used isocyanate-modified NDs to attach the fluorescent dye thionine, via its amines, and 3-iodopropyl triethoxysilane-modified NDs to attach triethylamine.[1314] The triethylamine–NDs were then functionalized with eGFP plasmid DNA through electrostatic interactions and incubated with HeLa cells, which subsequently exhibited green fluorescence from the expressed protein. Hydroxylated NDs have also been used to support Au- and PtNPs, demonstrating high antioxidant activity against ROS-induced damage in a hepatoma cell line.[1314] Barras and co-workers functionalized the surface of NDs using dopamine derivatives containing terminal azide groups. The dopamine derivatives chemically reacted with hydroxyl-terminated NDs, allowing for subsequent click chemistry

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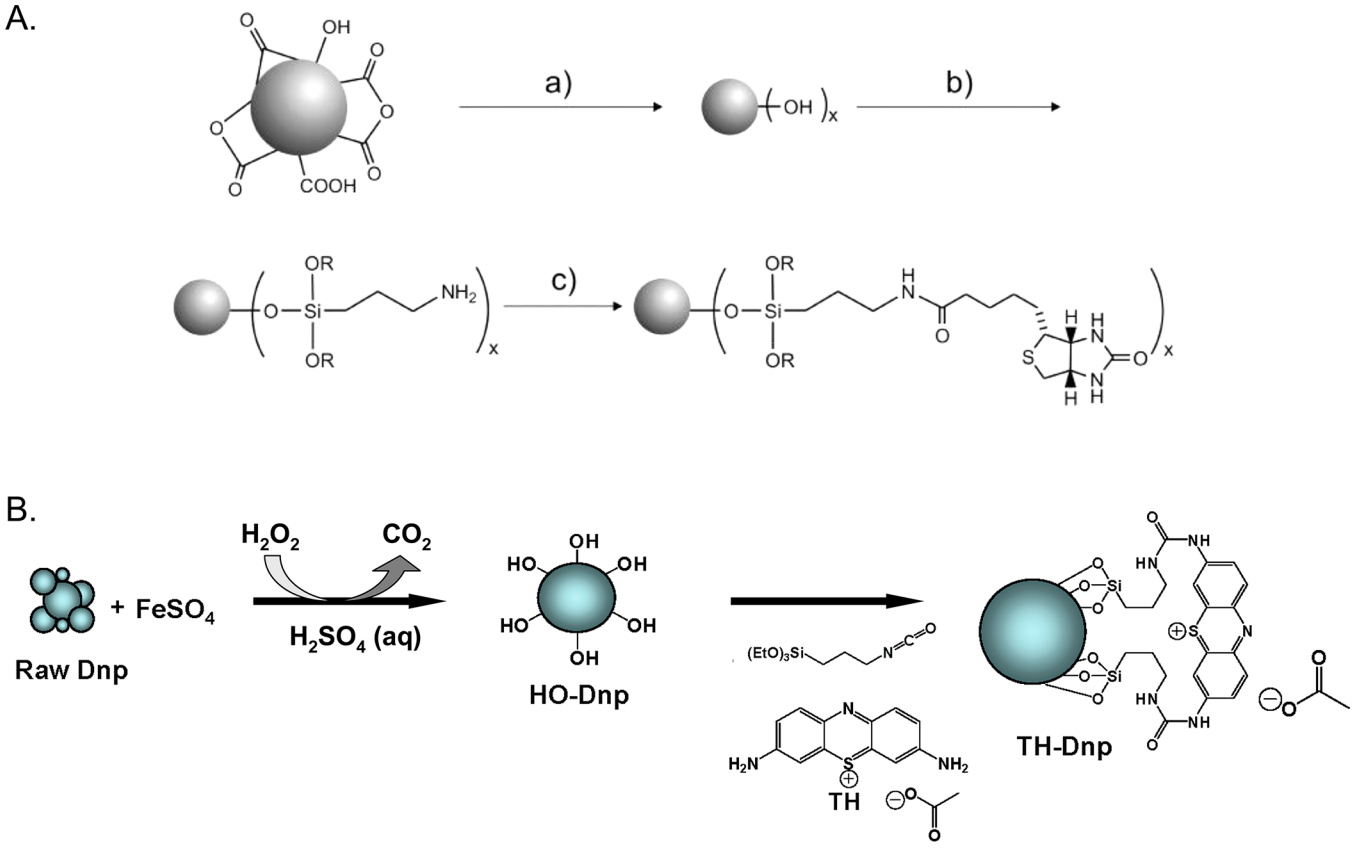

Figure 56. Modification of ND surfaces. (A) Biotinylation via silanization (a) BH_3·THF, THF, reflux, 72 h; (b) APTES, acetone, room temperature, 16 h; (c) biotin, EDC, DMAP, CH_2Cl_2, 0 °C to room temperature, 65 h. Reprinted with permission from ref 1302. Copyright 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (B) Fenton treatment of NDs (abbreviated Dnp in cited publication) followed by the synthetic route used to obtain NDs labeled with the fluorophore thionine (TH). Reprinted with permission from ref 1314. Copyright 2010 American Chemical Society.

modification with an alkynyl-pyrene fluorescent probe or a poly-*N*-isopropylacrylamide polymer.[1316]

As mentioned, modification schemes have also been developed to functionalize ND surface amines.[1304,1305] Hens and co-workers used NHS-derivatives of TAMRA dye and biotin to functionalize the surface of amine-modified NDs.[1305] Amine-modified NDs were also converted to azide-terminated particles using 4-azidobenzoic acid and DCC.[1316] Various alkyne-containing species, including decyne, ethynylferrocene and *N*-propargyl-1-pyrenecarboxamide, were subsequently coupled using CuAAC and extensively characterized using various techniques such as FT-IR, UV−visible spectroscopy, fluorescence spectroscopy, cyclic voltammetry, TGA, and dynamic light scattering. Aromatic diazonium salts have also been shown to react with the ND surface and introduce new functionalities such as carboxylic acids and NHS-esters.[1303,1317] Dahoumane and co-workers used such an approach to demonstrate modification with the protein BSA, which improved the overall stability of the ND materials.[1317]

4.1.4. Other Carbonaceous Nanomaterials. There appears to be an ever-expanding number of newly described carbon-based NMs that have interesting shapes or properties, including graphene-based materials[1318] and carbon nanohorns,[1206,1319] -rings, -cups, -containers, -ribbons,[1204,1205] and -clusters,[1320,1321] along with fluorescent carbon nanodots (C-dots).[1322,1323] The recently described C-dots, which represent sub-10 nm particles that become intensely fluorescent upon surface passivation, have a range of possible applications in bioimaging and biosensing.[1323−1325] The C-dot PL is broad, and interestingly, the emission maximum is dependent on the excitation wavelength. Sun and co-workers have shown that doping their carbon NPs with ZnO or ZnS produced much brighter PL upon surface passivation.[1326] The surface passivation agents are typically polymers, such as diamino-terminated oligomeric PEG or poly(propionylethyleneimine-*co*-ethyleneimine),[1226,1324,1325] which should make biostability and further bioconjugation a fairly simple progression. Li and co-workers used the terminal amines of C-dots passivated with PEG to conjugate Tf using EDC chemistry.[1327] Cellular uptake and targeting was studied in HeLa cells by exploiting C-dot fluorescence for imaging.

Zhao and co-workers recently demonstrated the electrooxidation of graphite to produce graphitic blue and yellow fluorescent carbon NPs with 1.9 and 3.2 nm diameters, respectively.[1328] Here, unlike the C-dots and more akin to semiconductor QDs, the fluorescence appeared to be size-dependent, and the emission maximum was independent of the excitation wavelength, although no bioconjugation of these materials has been reported to date. Zhang and co-workers employed carboxylic chemistry to functionalize carbon nanohorns, immobilized on glassy carbon electrodes, with antibodies against the cyanotoxin microcystin-LR (MC-LR) produced by cyanobacterial blooms.[1319] The modified electrodes were then used as electrochemical immunosensors for MC-LR detection in polluted waters, and the determined MC-LR concentrations matched those from HPLC analysis, but in a quicker time

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frame. Nanohorns have also been used as multimodal imaging agents by encapsulating trimetallic nitride template endohedral metallofullerenes, containing either Gd (MRI contrast) or Lu (X-ray contrast), and further conjugating QDs to the exterior of the nanohorns.[1206] Amine-modified CdSe/ZnS QDs were also coupled to the carboxyl groups on the surface of the nanohorns using EDC/NHS chemistry. Such materials allowed for both fluorescent and phase contrast imaging of *in vitro* and *in vivo* samples. Berlin and co-workers developed carbon nanoclusters as drug delivery vectors for the drug paclitaxel (PAX).[1320,1321] They found that by further modifying the PAX-loaded and PEG-functionalized carbon nanoclusters with cetuximab (a monoclonal antibody against EGFR) through noncovalent interactions, *in vitro* targeting to tumor cells overexpressing EGFR was greatly enhanced.[1321]

4.2. Biopolymeric Nanomaterials

Biopolymeric or natural polymeric NMs, derived from polysaccharides, nucleic acids (polynucleotides), proteins, polypeptides, viruses, and the like are being developed as alternatives to synthetic and lipid-based materials.[1329−1333] Biopolymeric NMs are prepared using a variety of methodologies that mirror those of synthetic polymer NM formation, such as emulsion/solvent evaporation, solvent displacement, complex coacervation, and salt-induced desolvation (salting out).[1331,1334,1335] In addition, due to either the highly charged nature of some of these materials or their amphiphilic properties, ionic gelation (induced by small ions), electrostatic interactions (between polyelectrolytes of opposite charge), or simple self-assembly can drive NM formation.[1331,1334] While there are issues associated with biopolymer purification, processing, batch reproducibility, immunogenicity, and potential disease transmission from source materials,[1330] a number of biopolymer-derived materials do possess inherent biocompatibility, come from renewable sources, are naturally biodegradable, and oftentimes are considered less toxic than their synthetic counterparts. Biopolymeric NMs, and in particular those derived from polysaccharides, proteins, or polypeptides, have been proposed and applied to a variety of medical applications, such as therapeutic delivery, imaging, and tissue engineering.[1329,1332,1333] Being biopolymeric in nature, they can access a wide variety of possible modification chemistries for biofunctionalization, including direct chemical labeling of internal and external surfaces, cross-linking, noncovalent adsorption, encapsulation, chemoselective ligation, mutagenesis, and genetic fusion. In the majority of projected bioapplications, multifunctional NMs are desired to increase the overall utility of the final complex. Biopolymers lend themselves well to the multiple modifications needed to prepare these materials because they typically contain more than one functional group or characteristic "handle" that can be targeted, as will be highlighted throughout the following sections. Given the vastness of this materials field, only representative examples are utilized here for illustrative purposes.

4.2.1. Polysaccharides. Polysaccharides are comprised of repeat mono- or disaccharide units that can be linear, branched, or in some cases even cyclic in nature, see Table 11. A number of these have previously been used in bead or micrometer-particle size formats but are now increasingly being formulated as NMs (primarily NPs). Of the materials highlighted in Table 11, chitosan (CHI) represents the most common polysaccharide NM combined with biomolecules (including drugs) for biomedical applications such as drug or gene delivery and

Table 11. Common Polysaccharides Formulated as Nanoparticles

material	source and details	nanoparticle applications	references
alginate (ALG)	linear anionic polysaccharide; polymeric blocks of β-(1−4)-linked D-mannuronate and α-L-glucuronate; extracted from seaweed (e.g., kelp) and produced by *Pseudomonas* and *Azotobacter* bacteria	drug delivery	1341, 1348−1352
cellulose	linear polysaccharide; β-(1−4)-linked D-glucose; structural component of the cell walls of higher plants, several marine animals, and some algae, fungi, bacteria, and invertebrates	drug delivery, imaging, sensing, and NP synthesis	1343, 1344, 1353−1355
chitosan (CHI)	linear cationic polysaccharide; random distribution of β-(1−4)-linked D-glucosamine and *N*-acetyl-D-glucosamine; produced by deacetylation of chitin, a structural component of the exoskeletons of crustaceans and fungi cell walls	drug, protein, and gene delivery, imaging, and contrast agents	45, 242, 1336−1340, 1348−1351, 1358−1380
dextran	branched polysaccharide; α-(1−6)-linked D-glucose linear chain with α-(1−3)-branching; synthesized by lactic acid bacteria, *Streptococcus mutans*, *Leuconostoc mesenteroides*	drug delivery	1381−1383
dextrin	low molecular weight polysaccharide; cyclodextrins also possible; α-(1−4)-linked D-glucose linear chain with α-(1−6)-branching; synthesized from the hydrolysis of starch.	drug delivery	1384
heparin	anionic polysaccharide, linear chain that is highly sulfated; most common disaccharide unit comprised of 2-*O*-sulfated iduronic acid and 6-*O*-sulfated, *N*-sulfated glucosamine; extracted from mucosal tissues of porcine intestine and bovine lung; highest average negative charge density of any known biomolecule	drug delivery anticoagulant	1385−1387
hyaluronan (HAA)	anionic polysaccharide, linear chain; alternating β-(1−4) and β-(1−3)-linked D-glucuronic acid and D-*N*-acetylglucosamine; main component of extracellular matrix in humans.	drug delivery	1345−1347, 1374
starch	polysaccharide composed of amylose (linear) and amylopectin (branched) chains; α-(1−4)-linked D-glucose linear chain with α-(1−6)-branching; produced by many plants to store energy; extracted from maize, wheat, potatoes, and cassava	drug delivery	1342, 1388, 1389

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especially for oral and ocular therapeutics.[1336−1340] The popularity of CHI likely stems from its highly cationic nature, which originates from the numerous primary amines comprising the polymer backbone, making it a natural complement to DNA for electrostatic self-assembly. Other polysaccharides have been formulated as NPs, see Table 11, although to date and compared with CHI, their subsequent conjugation has been more limited. Alginate (ALG), for example, is routinely used as capsules, beads, and microspheres for drug delivery applications;[1341] however, apart from its use in conjunction with CHI, ALG is less commonly formulated as a nanoscale material.

Cellulose and starch represent the two most abundant renewable polymer resources available. Starch NPs[1342] and cellulose nanocrystals[1343,1344] have mostly been used in biocomposites to improve mechanical and barrier properties of materials for industrial applications, although there are some examples of their application to drug delivery, imaging, and sensing. Another promising polysaccharide material is hyaluronan (HAA; also known as hyaluronic acid), which, similar to ALG, has previously been used as microspheres for drug delivery but has recently been formulated as NPs.[1345−1347] HAA is of particular interest because cancer cells are found to overexpress certain HAA receptors, making HAA-derived materials natural targeting agents.[1345] Polysaccharides often contain one or more additional functional groups that can be targeted for modification (e.g., amines, hydroxides, carboxylic acids) although encapsulation still represents the most common method of incorporating biomolecules to date.

4.2.1.1. Chemical Modification of Naturally Occurring Saccharide Groups. The principal naturally occurring functional groups present on polysaccharide-based NMs that have been targeted for biofunctionalization include hydroxides and, where available, amines. CHI, a linear polysaccharide comprised of randomly distributed β-(1−4)-linked D-glucosamine and *N*-acetyl-D-glucosamine, is considered cationic due to the presence of the primary amine in the glucosamine residues.[1338] These amines impart charge and functionality to the CHI biopolymer that can be readily used for conjugation. Given the many amines present on the CHI polymer, it is becoming increasingly common to modify the polymer chain with drugs, dyes, or other targeting agents prior to the production of the NPs. For example, Yang and co-workers conjugated FA to CHI using EDC chemistry prior to NP formation via ionic gelation with either tripolyphosphate (TPP) or 5-aminolevulinic acid.[1358] FA was used to target the NPs to HT29 and Caco-2 colorectal cancer cell lines that overexpress FA receptors, and once taken up, the 5-aminolevulinic acid was converted to fluorescent protoporphyrin IX that was monitored with confocal microscopy. Nam and co-workers modified glycol CHI amines with 5β-cholanic acid via EDC/NHS with an NHS-modified Cy5.5 and a NHS-modified Gd(III)−DOTA chelate to generate a multifunctional CHI polymer, see Figure 57.[1359] The 5β-cholanic acid portion is hydrophobic and results in an amphiphilic CHI chain that spontaneously self-assembled when dissolved in aqueous solution to produce ∼350 nm diameter particles. These NPs were injected intravenously into mice bearing pectoral subcutaneous murine squamous carcinoma cell (SCC7) tumors, where they were taken up via the enhanced permeation and retention (EPR) effect. Both near-IR fluorescence optical (via Cy5.5) and MR (via Gd(III)-DOTA) imaging could detect tumors in mice one day post injection. Yousefpour and co-workers conjugated a carboxylic acid form of DOX to CHI using EDC/NHS chemistry. Following self-assembly of the DOX−CHI NPs, the thiol-modified monoclonal antibody trastuzumab, which targets HER2, was conjugated using sulfo-SMCC.[1379] The resulting DOX−CHI−mAb NPs showed specificity for HER2-overexpressing SKOV-3 cells over HER2 negative MCF-7 cells, highlighting the potential for targeted drug delivery in certain breast and ovarian cancers.

Lallana and co-workers exploited a two-step strategy to demonstrate that CHI polymer chains could be initially functionalized with carboxyl-PEG−azides using EDC chemistry and that dyes or antibodies carrying terminal alkyne groups could then be readily conjugated using CuAAC.[1361] Here CHI NPs were cross-linked using citric acid that was activated with EDC for increased stability. Interestingly, the authors noted that the CuAAC was detrimental to the CHI polymer backbone, causing depolymerization, and so formulated an alternative route by using cyclooctynes in a strain-promoted azide−alkyne cycloaddition reaction (SPAAC).[242,1361] Various dyes or antibodies containing a PEG linker with strained cyclooctynes were prepared and successfully clicked to either the azide-functionalized CHI polymer backbone prior to NP formation or to the preformed NP.

In contrast, cellulose nanocrystals are commonly prepared by acid hydrolysis followed by exposure to strong hydroxide solutions, which act to functionalize the rod-shaped nanocrystals with hydroxyl groups. These hydroxyl groups have been used as initial starting points to modify the cellulose nanocrystals with dyes for imaging,[1353,1354] see Figure 58, and pH sensing.[1355] This same approach allowed the chemotherapeutic PAX to be chemically incorporated into heparin NPs by targeting hydroxyl groups that were first chemically succinylated using 4-(dimethylamino)pyridine and succinic anhydride and then modified with either PAX or amino-FA, via DCC coupling, for targeting.[1385,1386]

4.2.1.2. Affinity-Based Modification. Cellular uptake of siRNA has been achieved using cyclodextrin-based polymeric NPs labeled with human Tf for targeted delivery.[1384] First, siRNA was encapsulated within cyclodextrin NPs resulting in the cyclodextrin portion of the polymer chain ultimately residing on the exterior surface of the NP. Using the known affinity of β-cyclodextrin for adamantine, conjugates with either PEG or human Tf−PEG were prepared and used to functionalize the surface of the cyclodextrin NPs to provide stability while still targeting Tf receptor expressing cancer cells. Alternatively, biotin−avidin interactions were used by Aktas and co-workers to develop targeted CHI-NPs that facilitated delivery of therapeutic peptides across the blood−brain barrier.[1360] The CHI amine groups were first modified with either carboxyl-PEG or carboxyl-PEG−biotin via EDC chemistry, and a caspase inhibitor peptide was then mixed in solution with TPP to generate the CHI-NPs via ionic gelation. The amount of PEG−biotin displayed on the surface of the resulting NPs could be varied by changing the ratio of CHI−PEG to CHI−PEG−biotin during the reaction mixture. The peptide-loaded NPs were then functionalized with a SA-labeled monoclonal QX26 antibody, specific for the Tf-receptor, using biotin−SA affinity. Brain specimens from exposed mice demonstrated that the QX26 antibody was critical for facilitating translocation into the brain tissue following iv injection.

4.2.1.3. Noncovalent Adsorption and Encapsulation. These techniques represent perhaps the most common method of introducing gene, drug, and dye species into polysaccharide-based NMs. Due to the cationic nature of CHI and the anionic nature of DNA and RNA, the electrostatic interaction of these

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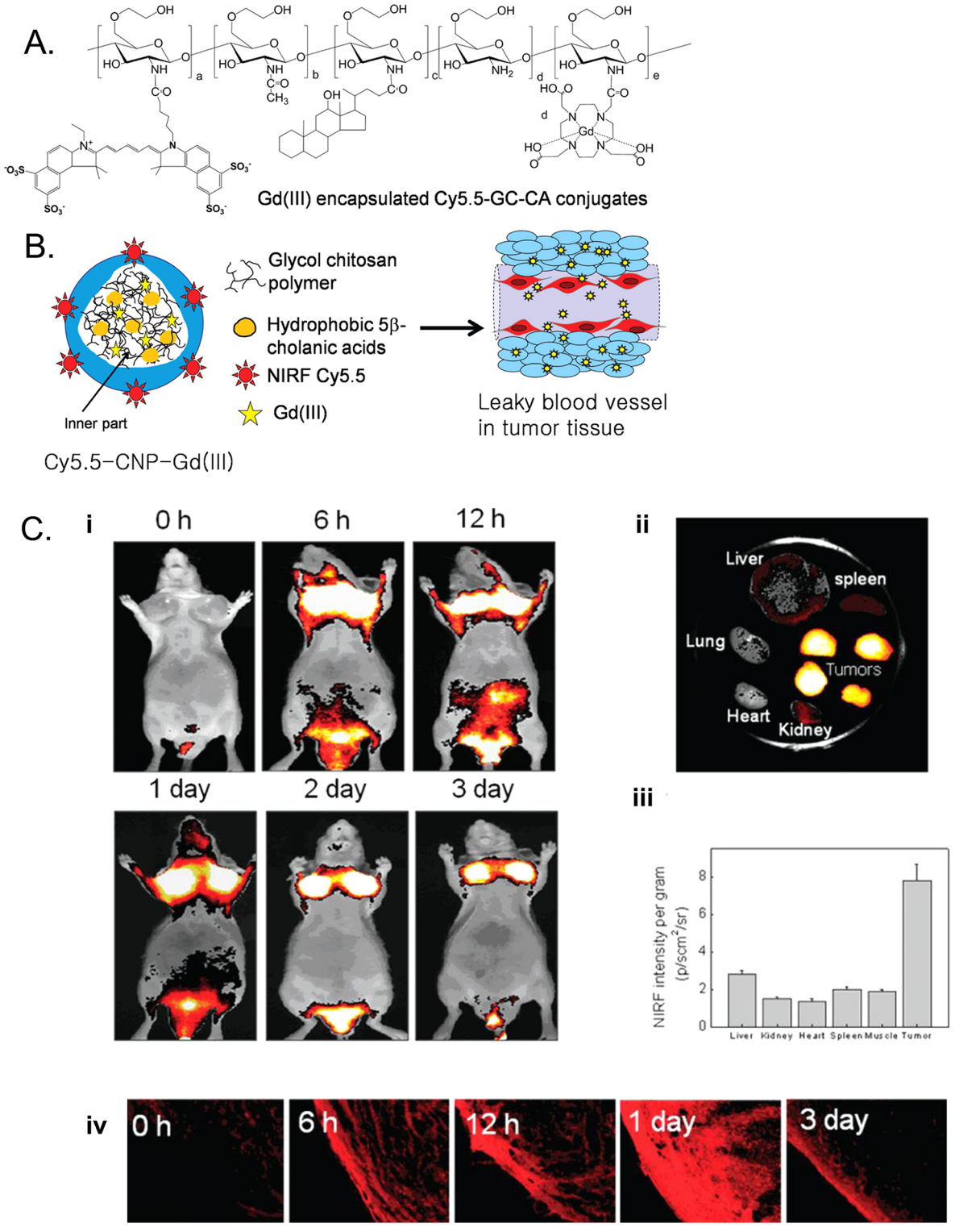

Figure 57. Chitosan NPs (CNPs) for tumor targeting and imaging using optical/MR imaging probes. (A) Chemical structure of Cy5.5–CNP–Gd(III). (B) Schematic illustration of self-assembled CNPs (blue, glycol chitosan shell; white, inner part of CNP) and subsequent tumor targeting. (C) *In vivo* time-dependent biodistribution of Cy5.5–CNP–Gd(III) in SCC7 tumor-bearing mice (diameter 8–10 mm): (i) *In vivo* time-dependent whole body imaging after iv injection of Cy5.5–CNP–Gd(III) (5 mg/kg). (ii) *Ex vivo* images of normal organs (liver, lung, spleen, heart, and kidney) and tumors excised 1 day postinjection. (iii) A quantification of *in vivo* biodistribution of Cy5.5–CNP–Gd(III) was recorded as total photon counts per centimeter squared per steradian (p/scm^2/sr) per each excised organ at 1 day postinjection. All data represent mean (se). (iv) Near-IR fluorescence micrographs of tumors excised from Cy5.5 (5 mg/kg)-labeled CNP-treated SCC7 tumor-bearing mice. Magnification is ×100. Reprinted with permission from ref 1359. Copyright 2010 American Chemical Society.

two polyelectrolytes is the most common method of incorporating DNA or RNA into CHI NPs. In many cases, the two components are simply mixed in deionized water or buffer and allowed to incubate before the resulting NPs are analyzed, and

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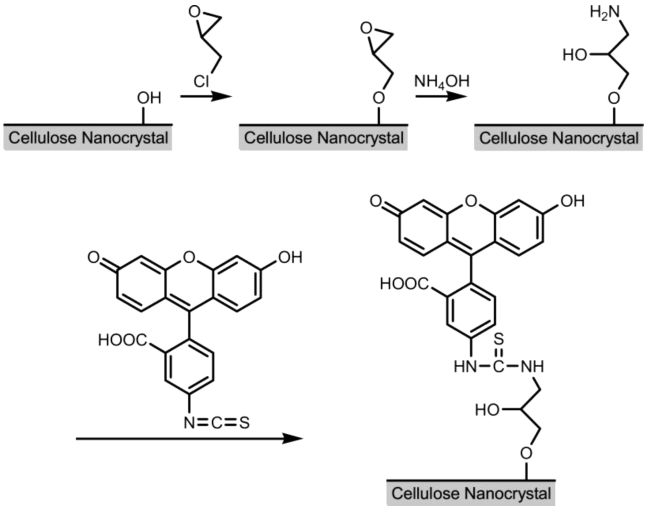

Figure 58. Schematic of labeling protocol used to label cellulosic NMs with fluorescein. Reprinted with permission from ref 1353. Copyright 2007 American Chemical Society.

this is referred to as the incorporation method.[1362,1363,1380] CHI-NP formation is also commonly initiated through ionic gelation via addition of polyanions such as TPP and has been used in the presence of DNA to generate antisense oligonucleotide-loaded NPs for topical therapeutic drug delivery.[1364] DNA or RNA has also been incubated with preformed CHI-NPs, which is called the incubation method, and the resulting complex was used to transfect various cells with luciferase or β-galactosidase expressing genes.[1365] Similar to DNA and RNA, protein loading either follows an incorporation or incubation type method, as demonstrated by Gan and Wang, when preparing BSA-loaded CHI-NPs.[1366] Here, the NPs were formed using TPP in a polyionic coacervation fabrication process. Hu and co-workers studied the nanocomplexes formed between caseinophosphopeptides, prepared via tryptic digestion of casein proteins, and CHI, finding electrostatic interactions to be the dominant assembly driving force.[1367] Often, chemotherapeutics, such as DOX and cisplatin, are incorporated into CHI-NPs using the incubation method,[1368,1369] although the incorporation method has also been used.[1370,1380] Cui and co-workers prepared fluorescent drug-loaded CHI-NPs by combining three components: cationic CHI, an anionic fluorescent anthracene derivative, and a model blood pressure regulating drug, nicardipine, see Figure 59.[1370] Interestingly, the drug quenched the anthracene via FRET with drug release resulting in recovery of fluorescence and providing a useful sensor for monitoring drug release.

As highlighted above, CHI readily complexes with DNA or RNA to yield NP formulations.[1362,1363] However, due to the strong electrostatic interaction between these two polyelectrolytes, the resulting DNA or RNA release kinetics upon reaching the therapeutic site are not always ideal.[1348] One approach researchers have used to improve the release kinetics of the incorporated DNA/RNA therapeutic load has been to add a second anionic polymer into the formulation, and examples of the latter include polyguluronate[1371] and ALG.[1348,1349] CHI coupled with anionic polymers has also been successfully demonstrated for delivery of protein- or peptide-based therapeutic payloads. CHI/ALG composites have already been used for the delivery of insulin[1350,1351] and the model protein BSA,[1372] while CHI/carrageenan[1373] and CHI/HAA[1374] have been used to transport ovalbumin and vascular endothelial growth factor (VEGF)/platelet derived growth factor (PDGF), respectively. In a mixed payload approach, Khadair and co-workers demonstrated encapsulation of DOX and methylene blue (PDT drug) within Aerosol-OT (surfactant)−ALG NPs.[1352] The resulting chemotherapy/photodynamic combination therapy demonstrated

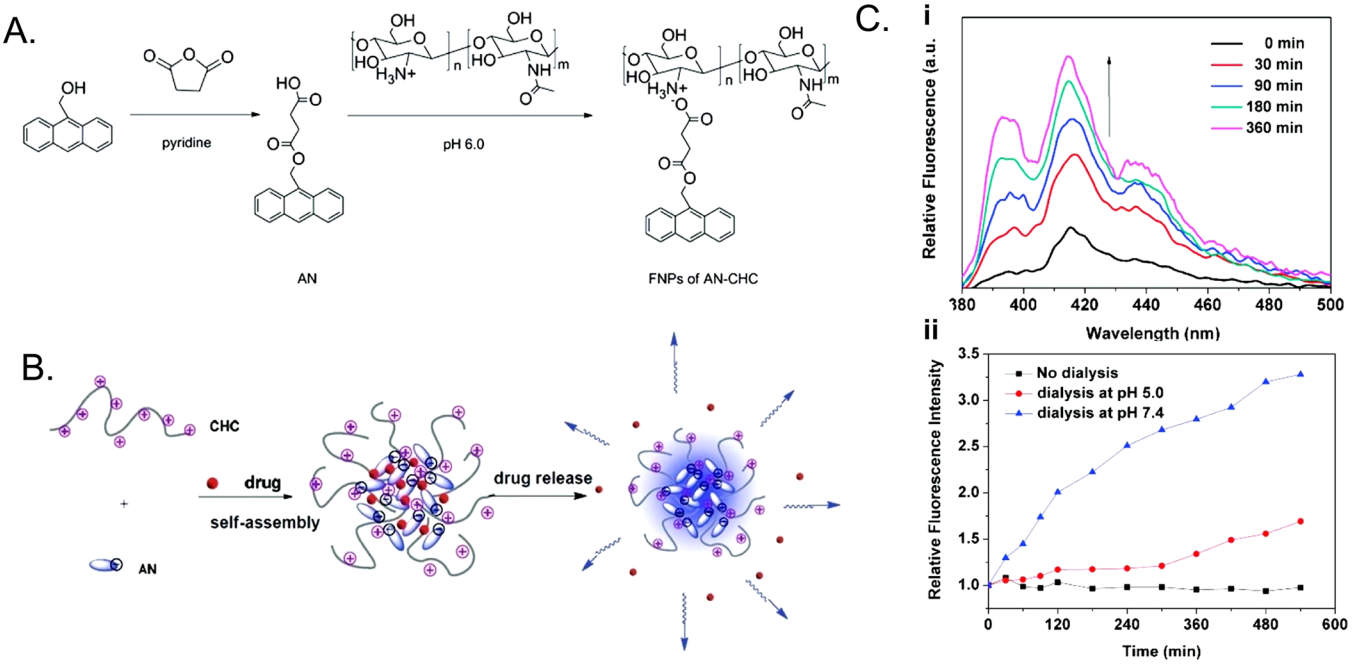

Figure 59. Preparation and characterization of drug loaded fluorescent chitosan NPs (FNPs). (A) Synthetic pathway of 4-(anthracen-9-ylmethoxy)-4-oxobutanoic acid (AN) and FNPs of AN-CHC and (B) formation of drug-loaded FNPs, where drug release from FNPs can be monitored through fluorescence recovery. (C) (i) Fluorescence recovery of the FNPs with increasing dialysis time at pH 7.4 and (ii) relative fluorescence of FNPs without dialysis (black line) and with dialysis at pH 5.0 (red line) and pH 7.4 (blue line) as a function of time. Reprinted with permission from ref 1370. Copyright 2011 American Chemical Society.

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therapeutic potential in mice bearing drug-resistant tumors, inhibiting tumor growth and improving animal survival. To potentially treat diabetes, Reis et al. prepared insulin-loaded ALG−dextran nanospheres coated with CHI−PEG for oral drug delivery.[1381] Various other starch derivatives have been proposed for drug delivery, including starch−oleic acid and starch−stearic acid graft polymers for delivery of indomethacin[1388] and propyl-starch for flufenamic acid, testosterone, and caffeine delivery.[1389]

Monitoring drug release kinetics and therapeutic effects are also a popular research focus when trying to understand the efficacy of these types of materials. In this vein, Al-Ghananeem and co-workers developed PAX-loaded HAA NPs using a desolvation method followed by GA cross-linking to stabilize the resulting NPs.[1345] Direct intratumoral injection of the PAX-loaded HAA NPs in rats demonstrated effective tumor growth inhibition. Wang and co-workers encapsulated PAX into targeted heparin NPs demonstrating marked reduction in tumor growth in a xenograft model of resistant human squamous cancer.[1386] Dextran sulfate has been shown to form 250−500 nm nanocomplexes with DOX via electrostatic and $\pi-\pi$ stacking interactions, with subsequent drug release profiles demonstrating an initial fast burst release followed by a slower and more sustained release leading to a total cargo delivery efficiency of 32% after 15 days.[1390] Jackson and co-workers investigated the adsorption and subsequent release of various hydrophilic and hydrophobic drugs in nanocrystalline cellulose fibers (10 nm × 500 nm) prepared as is or modified with the surfactant CTAB, respectively.[1356] Drug binding efficiency was found to be dependent on a number of factors including the dispersion medium and amount of CTAB coating. Cohen and co-workers modified acetylated-dextran polymers with spermine to generate acid-degradable cationic dextran particles, ∼180−230 nm in diameter when dry, which were then used to encapsulate siRNA.[1382] The acid-degradable nature of the NMs was key to siRNA release upon endosomal acidification during cellular uptake and provided efficient gene knockdown in HeLa-*luc* cells. In another example, pectin-based NPs were loaded with the antineoplastic drug 5-fluorouracil,[1391] while the two polyelectrolytes heparin and protamine were used to generate NPs that were further stabilized with dextran before fibroblast growth factor-2 was loaded.[1387]

4.2.1.4. Chemoselective Ligation. Malhotra and co-workers developed a chemoselective method of PEGylating CHI using sodium hydride-catalyzed etherification between chlorinated CHI and methyl-PEG.[1375] During PEGylation of the CHI polymer, the amines were protected using phthalic anhydride and later used along with TPP to encapsulate a transfection indicator siGLO gene via ionic gelation. Encapsulated DNA delivery and targeted delivery to HeLa cells was achieved with dextran NPs that were labeled with CPP via formation of stable oxime conjugates between acetal-modified dextran groups and alkoxyamine modified dye or peptide.[1383]

4.2.1.5. Modification with Other Nanoparticle Materials. The group of Zhang and co-workers demonstrated encapsulation of multiple AuNPs or QDs into larger CHI NPs using the electrostatic interaction of the cationic CHI and the negatively charged NPs.[1376,1377] Ultimately they were able to demonstrate gene silencing by immobilizing siRNA electrostatically onto the surface of preformed CHI NPs and delivering the conjugates via a targeting HER2 antibody to HER2 overexpressing SKBR3 breast cancer cells.[45] The HER2 antibody was covalently coupled to the CHI NPs with EDC/NHS chemistry and the encapsulated QDs were exploited to visualize successful cell targeting. Guo and co-workers demonstrated the development of a multifunctional nanocarrier based on CHI for cell imaging, drug delivery, and near-IR photothermal therapy.[1369] Here, Au nanorods with photothermal utility were encapsulated within CHI NPs using a non-solvent-aided, counterion complexation method. The NPs were cross-linked using GA, and remaining surface amine groups on the CHI/Au nanorods were then functionalized with FITC dye for imaging with cisplatin finally incorporated using the incubation method. Lee and co-workers prepared oleyl-functionalized CHI NPs with EDC chemistry between the carboxylic acid of the oleic acid and the amine groups on the CHI.[1378] Some of the remaining amine groups on the CHI backbone were further functionalized with NHS−Cy5.5 before the CHI NPs were formed. Oleic acid decorated 12 nm IONPs were then encapsulated within the CHI, and the resulting dual-labeled nanocomplex was used for both optical (NIR) and magnetic imaging of tumor-bearing mice. In a far different role, cellulose nanocrystals have been used to facilitate production of metallic NP chains of Cu, Ag, Au, or Pt via their surface hydroxyl groups.[1357]

4.2.2. Polynucleotide Nanoparticles. Polynucleotidyl NPs and nanostructures are a fairly recent but rapidly evolving area with much of the initial research to date focused on generating and characterizing a variety of DNA- or RNA-based nanostructures.[1392−1394] DNA origami, which relies on purely Watson−Crick base pairing to drive self-assembly, is emerging as a leader in this field for generating unique 2-D and 3-D DNA nanostructures with exquisite control afforded over the final architecture.[1394−1399] Other types of functionalized DNA structures are also being designed. Moran and co-workers, for example, demonstrated that DNA gel NPs could be prepared by nebulization of a mixture of DNA with an oppositely charged surfactant or protein.[1400] Recently, there have been increasing reports in the literature of the additional (bio)-conjugation of these DNA and, less frequently, RNA nanostructures for imaging, drug delivery, electronics, or plasmonics. Methods for conjugating proteins to DNA have recently been reviewed[1401] and often rely on standard bioconjugation techniques. In one example of RNA work, Khaled and co-workers developed a variety of 20−40 nm RNA NPs based on packaging RNA (pRNA) from bacteriophage phi29.[1402] The pRNA nanostructures were designed to incorporate various siRNA, FITC, and either aptamer or FA moieties that targeted cellular receptors. Binding of these NPs to cancer cells and leukemia model lymphocytes in cellular and *in vivo* mouse studies was demonstrated and resulted in cancer cell apoptosis. Using DNA origami, Walsh and co-workers assembled tetrahedral DNA cages using four 63-nucleotide sequences, one of which was labeled with Cy5 or biotin, and demonstrated substantial uptake of the DNA cage into HEK cells.[1403] FRET experiments using DNA cages comprising Cy5 and Cy3 component oligonucleotides suggested that the nanostructures remain intact for at least 48h after cellular uptake. Chang and co-workers utilized a six-point-star motif icosahedral DNA nanostructure, see Figure 60, with a conjugated DNA aptamer specific for MUC 1 (a tumor marker expressed in epithelial cancer cells) and intercalated DOX as a targeted therapeutic.[1404] The NP showed specific internalization into epithelial cancer cells and subsequent cytotoxicity.[1404] This approach specifically exploits DOX's natural affinity for DNA to noncovalently label the conjugate. Pilo-Pais used DNA origami templates to tailor the location of seed AuNPs, functionalized with complementary ssDNA.[1405] The seed AuNPs were then enlarged and fused by electroless deposition of Ag to produce a variety of unique

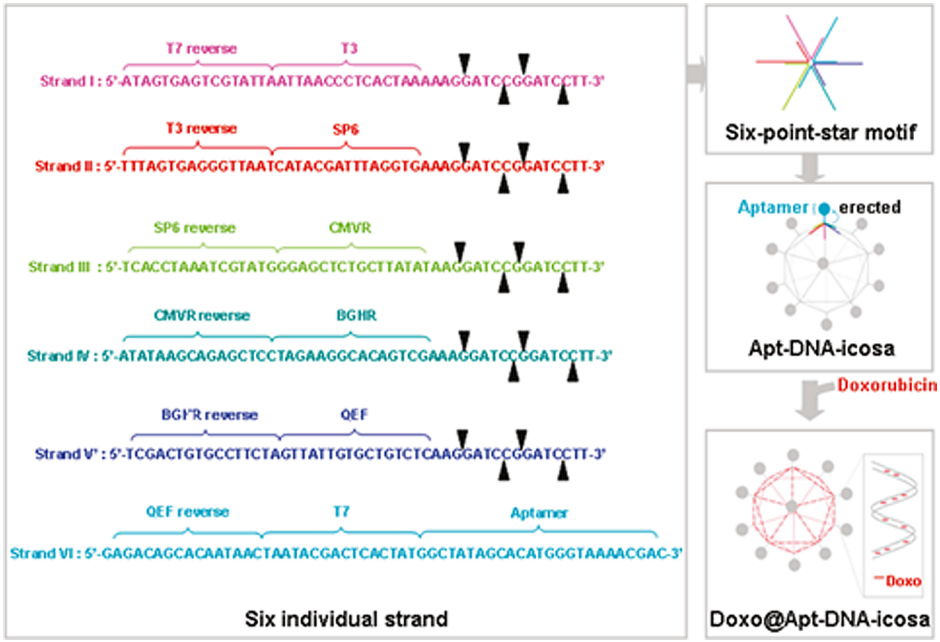

Figure 60. DNA NMs functionalized with DOX. Six DNA strands assemble into sticky-ended six-point-star motifs. These then further assemble into Apt-DNA-icosa. DOX is intercalated into both DNA-icosa and Apt-DNA-icosa. The DOX-intercalated area is highlighted (black triangles). Reprinted with permission from ref 1404. Copyright 2011 American Chemical Society.

metallic structures including rings, pairs of bars, and "H" shapes with potential applications in electronics and plasmonics. Rajendran and co-workers proposed a photo-cross-linking strategy using 8-methoxypsoralen to improve the thermal stability of DNA origami structures above 55 °C, which will be useful for many electronic and photonic devices, although it may not be useful for many biomedical applications.[1406] While clearly in the early stages of development, oligonucleotide-based NMs offer unique benefits including highly programmable nanostructure generation and access to a wide variety of modification chemistries and a wide range of potential applications still waiting to be explored. The ability to obtain the DNA monomer(s) that generate these structures with site-specific modifications (e.g., thiol, amine, azide) facilitates almost all bioconjugation strategies. Moreover, the monomer can undergo many modification steps prior to assembly of the final structure, thus placing the bioconjugated species on the DNA exactly where and when desired. Given this, it is important to note that no other NM can currently provide the same level of site-specific, quantitative, and controlled bioconjugation as these materials. However, one continuing issue with such NP constructs is the limited number of techniques available to characterize the structure and confirm correct modifications along with assembly efficiency.[1407]

4.2.3. Proteins and Polypeptides. Protein and polypeptidyl-based NMs come in a variety of forms, as outlined in Table 12, and comprise either naturally self-assembling protein units or subunits or engineered nanoscale materials prepared using a variety of synthetic techniques otherwise typical of polymer NP formation. As with the polysaccharide-based NMs, a number of the protein-based NPs have previously been used in larger, micrometer-particle size formats. Gelatin, a popular derivative of collagen, and albumin represent some of the more common NMs typically prepared from harvested precursors; however, polypeptide materials that self-assemble represent a wider and more utilized class of materials. For example, certain proteins or peptide sequences, such as silk proteins (combined with surfactants), casein,[1414,1415] and various charged or amphiphilic peptides[1445−1448] will self-assemble to form nanoscale materials under mild or physiological conditions. Another class of polypeptide NMs that are increasingly being proposed for bioapplications are protein cages, which have distinct interior and exterior regions that can be exploited for differential functionalization.[1435,1449] Common protein cages include heat shock proteins (Hsp), ferritin, and viral-coat materials; the latter two are discussed in the next sections.

As apparent from Table 12, protein-based NMs find current application in drug delivery applications, where their biocompatibility, biodegradability, and generally low toxicity are considered key attributes.[1450−1452] One of the driving motivations used by researchers investigating protein NMs is their ability to undergo chemical and genetic modification leading to NPs with highly functional surfaces that can be further conjugated to therapeutics, targeting ligands, or imaging or contrast agents. The ability to use different approaches in functionalizing protein-based materials also lends itself to the generation of multifunctional NMs.

4.2.3.1. Targeting Natural Amino Acids. Targeting the natural amino acid side chain functionalities present in a protein NM is one of the simplest methods for achieving biofunctionalization and borrows from almost all the techniques developed for standard protein modifications.[80] In an illustrative example, Xu and co-workers utilized both cysteine and lysine residues on human serum albumin (HSA) to generate targeted albumin NPs for delivery of DOX.[1408] First, HSA was labeled at a cysteine residue with maleimide−Alexa Fluor 488 to facilitate imaging, then a maleimide−PEG−NHS cross-linker was conjugated to a number of the free amines on the surface of HSA. The maleimide on the PEG−HSA was then conjugated to the cysteine residue on a cyclic targeting peptide. Additional amine residues on the HSA surface were then functionalized with thiol−DOX via the cross-linker sulfo-LC-SPDP. The increase in hydrophobicity of the HSA, due to the loss of a number of exposed primary amines as well as conjugation to DOX, drives the auto assembly of the final construct

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Table 12. Common Proteins and Polypeptides Formulated as Nanoparticles

material	source and details	nanoparticle applications	references
albumin	serum albumin most abundant plasma protein in mammals; ~66–67 kDa molecular weight protein; human (HSA) and bovine (BSA) most common sources	drug delivery, imaging	228, 1408–1413
casein	protein found in milk; major casein subunits designated as $\alpha s1$-, $\alpha s2$-, β-, and κ-caseins distinguished by electrophoresis; relatively hydrophobic, forms micelle structures held together by calcium ions and hydrophobic interactions; bovine milk most common source	drug delivery	1414–1418
collagen	group of proteins found in mammals and is the main component of fibrous tissues; while many types of collagen have been identified, types I–IV represent the most abundant; extracted from the boiled bones, connective tissues, organs, etc. of cattle, pigs, and horses	drug delivery	1419
ferritin	protein cage comprising 24 protein subunits; functions primarily as an intracellular iron-storage protein; produced by bacteria, algae, higher plants, and animals.	drug delivery, imaging, *in vitro* diagnostic assays	1420–1425
gelatin	protein produced from the hydrolysis of collagen (see above); type A generated from acid hydrolysis and type B generated from alkaline hydrolysis	drug delivery	1426–1429
gliadin	glycoprotein extracted from wheat and other cereals; four types, α-, β-, γ-, and ω-gliadin; primarily involved in the formation of gluten; responsible for gluten sensitivity in humans	drug delivery	1430, 1431
heat shock proteins (Hsp)	group of related proteins named according to molecular weight (10–100 kDa; e.g., Hsp60); expression is increased when cells are exposed to increased temperatures or other stress factors; produced by nearly all living organisms	drug delivery	1432–1436
protamine	group of nuclear proteins rich in Arg amino acid residues; typically short proteins comprising 50–110 amino acids; found in the sperm of animals (humans, mice, fish, bulls, etc.)	drug delivery	1437–1439
silk proteins	sericin and fibroin are the two main proteins in silkworm silk; fibroin forms the structural core of silk and is surrounded by sericin; a common source is silkworms	drug delivery	1440–1443
whey protein	mixture of proteins extracted from whey; created as a biproduct of cheese production; typically a mixture of β-lactoglobulin, α-lactalbumin, and serum albumin; bovine milk most common source	drug delivery	1444

into 30 nm NPs, which demonstrated cytotoxicity when exposed to human melanoma cells. Naczynski and co-workers developed rare earth metal encapsulating albumin NPs that could be used to image cells with near-IR excitation.[228] The amines of the resulting albumin NPs were then functionalized with cRGD peptides via sulfo-LC-SPDP to successfully target cell lines expressing $\alpha_v\beta_3$ integrin receptors.

Kommareddy and Amiji functionalized thiolated gelatin type B NPs with PEG chains via primary amine (gelatin)-succinimidyl glutarate chemistry.[1453] Compared with their non-PEGylated counterparts, the PEG−gelatin NPs showed increased circulation potential and passive tumor targeting via the EPR effect, when systemically dosed to breast cancer-bearing mice. As an alternative to relying on the passive uptake of NPs in tumors, EGFR-targeted gelatin NPs have also been demonstrated for lung and pancreatic cancers.[1428,1429] Magadala and Amiji encapsulated plasmid DNA encoding GFP in type B gelatin NPs using a solvent displacement method.[1428] The primary amine groups on the gelatin NPs were then modified with succinimidyl-PEG or succinimidyl-PEG−maleimide and the resulting maleimide group was used to graft cysteine-containing EGFR-targeting peptides. Pancreatic cancer cell studies demonstrated successful targeting and GFP gene transfection. Tseng and co-workers, in contrast, adsorbed cisplatin onto preformed gelatin NPs before converting the primary amines of the gelatin to thiols using 2-iminothiolane.[1429] These were then exposed to maleimide-activated NeutrAvidin before addition of biotinylated-EGF. Lung cancer bearing mice were treated with various cisplatin formulations via aerosol delivery, with EGFR-targeted NP formulations achieving the highest cisplatin dosage.

4.2.3.2. Mutagenesis and Genetic Fusion. Mutagenesis and genetic fusion represent the most popular methods for introducing site-specific amino acids, small peptides, or even large polypeptides into such NMs. These methods are particularly popular for modification of viral capsids and cage proteins and are often used in combination with additional chemical modification or affinity methods. For example, unique cysteines are commonly inserted into either the interior or exterior of the protein and are the least likely to influence the subsequent protein-cage folding and assembly. It is also common to insert small-to-large polypeptide sequences and even whole proteins into protein cage structures. Uchida and co-workers engineered a Hsp from *Methanococcus jannaschii* to display two unique features: (1) a glycine residue displayed on the protein interior was replaced by a cysteine residue to facilitate labeling with Cy5.5 or fluorescein−maleimide dyes for imaging or cell sorting, and (2) the C-terminus, exposed at the exterior of the assembled Hsp, was modified with a LyP-1 peptide residue (CGNKRTRGC) that targets tumor-associated lymphatic vessels and macrophages.[1436] The resulting dual-labeled Hsp NPs demonstrated potential as targeted imaging agents for the diagnosis of atherosclerosis, the underlying pathology in the majority of cardiac and vascular diseases. The Douglas and Young groups have been actively involved in the use of Hsps for cell-targeted imaging and therapeutic applications,[1432,1433] expanding later to study biodistribution in mice.[1434] Genetic or chemical addition of targeting species to cysteine Hsp mutants has also been demonstrated.[1433] Genetic fusion of the RGD-4C targeting peptide, which specifically binds certain integrins expressed in tumor vasculature, showed specific binding of the Hsp to C32 melanoma cells. Chemical conjugation of monoclonal antibodies specific for CD4 was then achieved by reacting partially reduced antibodies with Hsp cages, whose

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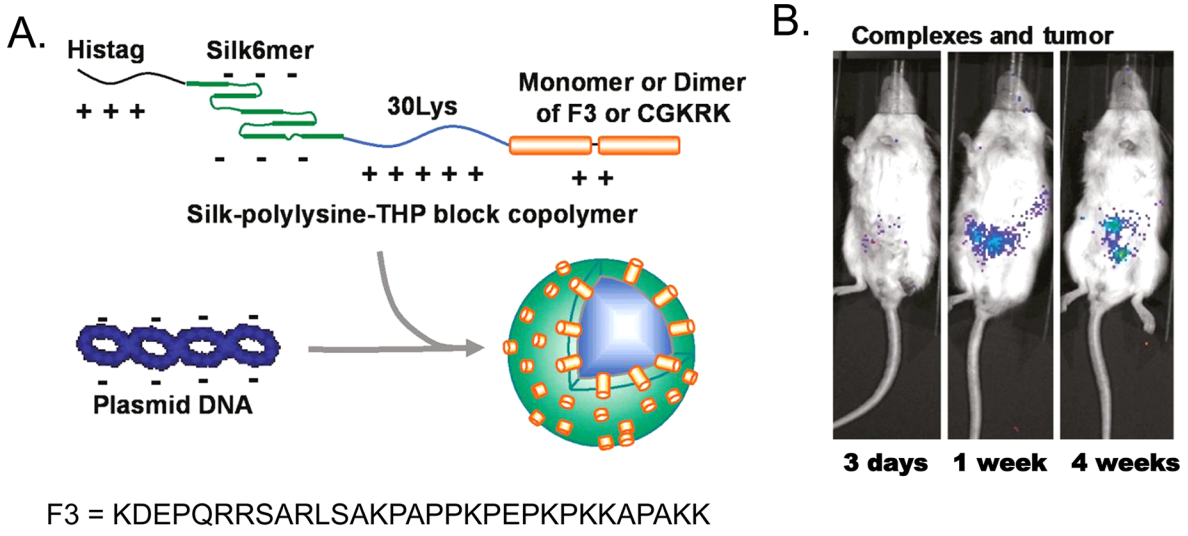

Figure 61. Self-assembling silk protein NPs. (A) Schematic of formation of pDNA complexes with recombinant silk proteins, namely, the silk-poly(L-lysine)-THP block copolymers. (B) *In vivo* transfection results after loading pDNA complexes in MDA-MB-231 tumor cells in mice (a,b,c). Bioluminescent images of 3 days, 1 week, and 4 weeks of resulting luciferase expression in mice. Reprinted with permission from ref 1441. Copyright 2011 American Chemical Society.

primary amines had been converted to maleimides using sulfo-SMCC. The resulting antibody−Hsp bioconjugates demonstrated specific binding to CD4 positive lymphocytes.

Recombinant techniques have also been used on noncage proteins. Numata and co-workers, for example, developed a recombinant spider silk protein that acted as a gene carrier for tumor cell-specific delivery, which comprised three components: (1) six contiguous copies of a spider silk repeat unit derived from the dragline protein MaSp1 of *Nephila clavipes*; (2) a 30 lysine repeat unit for DNA condensation; (3) a tumor homing peptide sequence (either F3 or CGKRK), see Figure 61.[1441] Plasmid DNA encoding luciferase protein was encapsulated into these materials and generated 150−250 nm diameter globular NPs. Subsequent *in vitro* cell studies and *in vivo* mice studies confirmed DNA transfection and luciferase expression in tumors. Silk elastin-like polypeptides (ELPs) are a related class of nanocomposite materials with similar properties, which find use in therapeutic delivery.[1454]

4.2.3.3. Noncovalent Adsorption and Encapsulation. Probably one of the best known protein-based drug delivery systems is NP albumin-bound PAX (nab-P; Abraxane; Abraxis BioScience Inc.), which is an FDA approved treatment for metastatic breast cancer.[1409,1410] Hydrophobic PAX is mixed with HSA, and high-pressure homogenization is used to prepare albumin-loaded NPs of approximately 130 nm in size.[1409,1410] The albumin NP not only encapsulates and protects the drug but is also thought to facilitate tumor cell targeting through albumin binding to the cell surface gp60 receptor glycoprotein as well as the extracellular matrix glycoprotein SPARC (secreted protein acid and rich in cysteine). Albumin NPs have also been used to encapsulate DOX,[1411] rare earth NPs,[228] and the PDT drugs mTHPP [5,10,15,20-tetrakis(*m*-hydroxyphenyl)-porphyrine] and mTHPC [5,10,15,20-tetrakis(*m*-hydroxyphenyl)chlorin].[1412,1413] Similarly, collagen NPs have been used for the transdermal delivery of hydrophobic 17β-estradiol-hemihydrate.[1419] Here, the estradiol was absorbed into preformed collagen NPs and then incorporated into a hydrogel matrix for transdermal delivery to postmenopausal women in whom enhanced estradiol absorption was observed. Further, gelatin NPs have been investigated for the delivery of natural polyphenols, compounds extracted from green tea with potential anticancer properties, which were loaded into the preformed gelatin NPs.[1426] Model proteins such as BSA have also been encapsulated into recombinant human gelatin NPs; the BSA was mixed with the gelatin prior to NP formation.[1427] Wu et al. used natural ELPs to generate pH-responsive biopolymer nanocarriers for DOX.[1443] Here, the ELP and DOX solutions were premixed, and NPs were prepared using an electrospraying technique.

Self-assembling proteins such as casein have been proposed for the encapsulation and delivery of hydrophobic anticancer drugs.[1416,1417] Natural casein micelles involve the complex interaction of four phosphoproteins, αs1-, αs2-, β-, and κ-caseins, and they can be purified from fresh cow's milk and are found to readily adsorb the hydrophobic drug curcumin, which originates from the spice turmeric and which have anti-inflammatory and analgesic properties.[1416] Others have specifically investigated the self-assembly of β-caseins,[1417,1418] with Shapira and co-workers demonstrating PAX and vinblastine sulfate encapsulation for oral delivery applications.[1417] Micellar nanostructures for carrying the hydrophilic and hydrophobic drugs insulin and PAX, respectively, were prepared using the natural silk glue protein sericin in combination with various surfactants and demonstrated a subsequent reduction in MCF-7 breast cancer cell growth.[1440] Anumolu and co-workers also demonstrated incorporation of plasmid DNA and fluorescein into various recombinant silk-elastin-like NPs that were fabricated, purified, and characterized using electrospray differential mobility analysis (ES-DMA).[1442]

Lastly, a protein or polypeptide is sometimes engineered into a NP vector from the top-down, because this can impart some useful properties. A prime example is the manufacture of insulin NPs. Zn-insulin is known to be poorly water-soluble, and NP dispersions of these materials have been investigated to overcome this issue.[1058] The Liversidge group used wet milling technology to process Zn-insulin powder with a mean size >16 μm into NPs <150 nm and then tested the NP efficacy in hyperglycemic rats with subcutaneous and intraduodenal doses.

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They found that, along with keeping the drug in a stable and biologically active state for long periods of time, this NP formulation was as effective as soluble insulin.

4.2.4. Ferritin-Based Bioconjugates. Ferritins are a family of conserved cage-like protein complexes that function to sequester, store, and transport metabolic Fe in the body, specifically binding Fe(II) and storing it as Fe(III) in an iron "core".[1455] These proteins are prevalent in almost every human cell type. Apoferritins are the empty or non-Fe containing forms, whereas holoferritins contain the Fe(III) core. These complexes consist of both 19 kDa light (L) and 21 kDa heavy (H) subunits that aggregate into a large (~450 kDa) symmetrical 24-mer protein complex, see Figure 62. Within the protein interior, the Fe interacts with OH^- to form a ferrihydrite. It is estimated that each ferritin complex can sequester or carry a "cargo" equivalent to 4500 Fe(III) ions. Due to its capacity for mineralization, ferritin is also found in the shells of mollusks, where it functions to control distribution and local concentrations of Fe with concomitant impact on color and morphology. Ferritin has also found some industrial application as a precursor material for making FeNPs.[1456−1458]

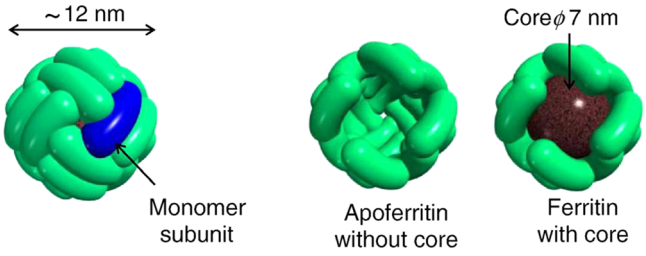

Figure 62. Schematic representation of 24-mer apoferritin with and without the iron core.

There are numerous characteristics of apoferritin that make it an excellent candidate for use as a bioconjugable nanoplatform. The symmetrical protein cage is about 12 nm in diameter with a hollow center of about 7−8 nm (Figure 62). The residues on the surface of the protein cage are also highly amenable to bioconjugation with peptides and other biomolecules for further functionality. The protein can be re-engineered using standard molecular biology techniques for a variety of customized applications. Because the apoferritin center is designed for holding and oxidizing inorganic Fe and providing a site for biomineralization, this also makes it an ideal space for carrying or synthesizing inorganic NPs *in situ*. This has been extensively reviewed in refs 1456−1458. Materials can also be directly encapsulated in ferritin cages in two ways: dissociation of the protein cage at low pH levels and reassembly at basic pH levels, allowing for encapsulation of materials by simple pH changes, or alternatively, encapsulation through hydrophilic and hydrophobic channels capable of channeling a wide variety of materials to the core.

As with many other NP materials that have an intrinsic "cargo" capacity and potential for bioconjugation, engineered ferritins are currently being investigated as targeted drug delivery platforms. Many tumors express receptors for ferritin, which can potentially allow for augmented and targeted drug delivery using these constructs.[1459] Additionally, other targeting molecules can be attached to the apoferritin surface to further enhance targeting efficiency, stability, imaging, or efficacy (see below). With these considerations in mind, Yang et al. utilized both of the methods described above to encapsulate the chemotherapeutics cisplatin and carboplatin.[1459] Both constructs were more effective than the free drug at comparable concentrations in decreasing the viability of PC12 cells in culture. Conjugates formed via the channel method were the most effective due to the higher amount of drug per protein; however the conjugates formed by reassociation contained purer cisplatin.[1459]

Methods similar to those described above have been used to create numerous other apoferritin nanoconjugates with a variety of labels or cargos. These include the anticancer drug daunomycin,[1460] Eu,[1461] Gd,[1462] and $CaCO_3$[1463] using dissociation and reassociation; and Pd,[1464] Cu, and Cu/Fe analogs of Prussian Blue,[1465] $Pb_3(PO_4)_2$,[1466] along with Au,[539,1467] $Cd_3(PO_4)_2$,[1468] and $LuPO_4$[1451] using the channel method. These constructs were designed for a variety of applications, including nanoreactors,[1463−1465,1467] drug delivery,[1451,1459,1460] biological imaging,[539,1461,1462] and *in vitro* biosensing.[1466,1468,1469] If necessary, the apoferritin protein cage structure can also be retained in place or easily removed after synthesis or chemical modification to release the interior contents, adding to its functional versatility. This last point suggests specific utility in synthesizing biotemplated NP materials of a fixed size that conform to the ferritin core diameter, where internally displayed residues can also help dictate which materials are included by preferential interactions with target molecules.

The solvent-exposed amine groups at the surface of apoferritin are particularly popular sites for modification since they permit facile conjugation with NHS chemistry. This allows for the direct one-step addition of a variety of functional (bio)molecules on the protein surface. The most popular technique is to attach NHS−biotin to the surface for further SA/biotin attachment of peptides. *In vitro* biosensors using this conjugation method are currently being developed and include a Cd−apoferritin sandwich immunoassay for tumor necrosis factor-α.[1469] Crich et al., however, have taken the technology one step further and demonstrated *in vivo* targeting using Gd-loaded apoferritin conjugated with C3d peptide.[1462] The C3d peptide binds the neural cell adhesion molecule, which is overexpressed during human angiogenesis. The Gd was loaded via dissociation−reassociation, which resulted in approximately 8−10 Gd chelates per apoferritin. The conjugate was then biotinylated at accessible amine groups with NHS−LC−biotin, resulting in approximately five biotins per apoferritin. The Gd−apoferritin−biotin complex was then incubated with SA and a biotinylated C3d, resulting in the final construct shown in Figure 63. The C3d conjugates bound to tumor endothelial cells *in vitro*, while unconjugated material did not. SA and Gd−apoferritin−biotin, with and without conjugated C3d, were also injected into mice previously implanted with a subcutaneous bolus of human tumor endothelial cells expressing neural cell adhesion molecules. In mice that received the injection of biotinylated C3d, there was a >30% increase in MRI signal at the xenograft site. The enhancement was only 3−5% in the control mice without the biotinylated C3d injection.[1462] The primary benefit of this approach is that the SA−biotin conjugation method is simple and well understood. Further, the strategy was not hard to implement, and all reagents were easily obtainable. Although this approach can allow the creation of a variety of biofunctionalized apoferritin NPs, the liability would be that many of the resulting conjugates would be heterogeneous and poorly defined.

Aside from encapsulating materials, apoferritin can be engineered to display surface/functional biomolecules. The protein itself is slightly negatively charged and can bind to positively charged surfaces electrostatically.[1470] Alternatively, because it is a protein, the DNA coding sequence can be mutagentically modified to express peptides or sequences on the protein surface in a predesigned

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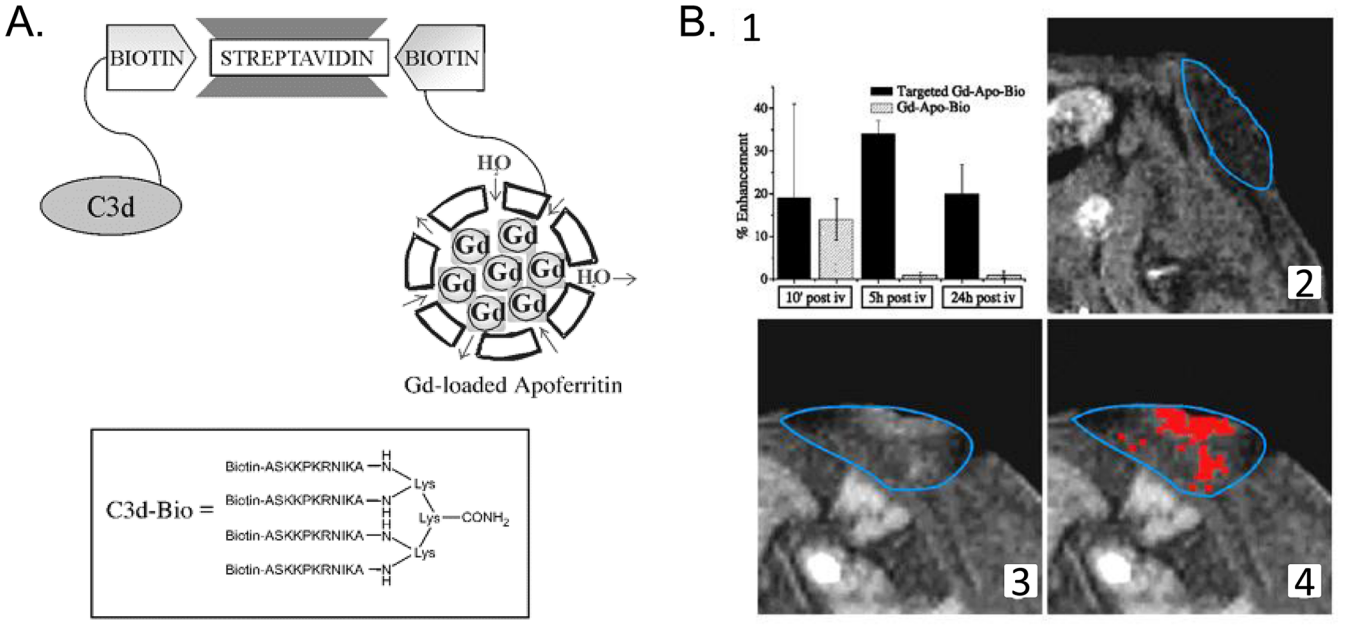

Figure 63. Gd-loaded apoferritin probes. (A) Schematic of Gd-loaded apoferritin with biotin−SA−biotin attachment to C3d neural cell adhesion molecule recognition peptide. (B) MRI images before (B-2) and after (B-3) injection with construct. (B-4) Regions of >30% enhancement are marked in red, and quantitation is shown in panel B-1. Figure adapted from ref 1462. Copyright American Association for Cancer Research.

manner. Yamashita et al. demonstrated this by engineering a Ti-binding peptide onto the N-terminus of the L-subunit of horse-spleen ferritin; these were arranged to be displayed on the exterior of the apoferritin cage after assembly. This mutant ferritin manifested a 25-fold increase in adhesive force to a Ti substrate but not a Si substrate, demonstrating specificity.[1471] Given the intense interest in selecting peptides that bind a variety of metal and other (bio)molecular substrates,[103] similar strategies could allow a large variety of targeting peptides or other functional peptide motifs, such as CPPs or intracellularly cleaved sequences or enzymatic substrates to be engineered onto the NP surface to promote combined therapeutic and multimodal diagnostic use. The Douglas and Young groups have also applied a mutagenic approach to a member of the ferritin superfamily. In this case, they used LiDps (DNA-binding protein from starved cells of the bacterium *Listeria innocua*, a member of the ferritin superfamily) to generate Janus (meaning biphasic) NPs for sensing applications.[1423,1424] The LiDp protein cages were genetically engineered to include a cysteine residue on each of the 12 subunits and then immobilized on beads where the exposed side of the protein cages was modified with a biotin−maleimide and subsequently SA.[1424] The SA−LiDps were then released from the beads and functionalized with fluorescein−maleimide and biotin-labeled antibodies specific for the microbial pathogen *Staphylococcus aureus* allowing the resulting constructs to be used in diagnostic flow cytometry assays.

Mutagenic approaches have also yielded a variety of other ferritin-based biosensors. Shapiro and co-workers prepared ferritin cages genetically fused with either of the kinase inducible domains, KID or KIX.[1420] Addition of protein kinase A to a premixed substrate solution containing these two ferritin fusions caused aggregation due to phosphorylation that could be measured using dynamic light scattering and MRI. Such sensors have potential to be used for real time detection of protein kinase A activity. Jaaskelainen and co-workers fused a scFV fragment to ferritin before incorporating Eu(III) ions and using the resulting construct in a bioaffinity assay for thyroid stimulating hormone.[1421] Lee and co-workers fused specific antigenic epitope regions of glutamate decarboxylase to ferritin (derived from heavy chains only) and used the resulting nanoscale construct in immunoassays for ultrasenstive detection of autoantibodies specific for type I diabetes.[1422] Kim and co-workers used an engineered ferritin-based NP that combined the C-terminus of the human ferritin heavy chain with the N-terminus of eGFP or red fluorescent protein (DsRed) via a flexible glycine-rich spacer.[1425] The resulting fluorescent ferritin NPs, functionalized with a total of 24 eGFP upon self-assembly of the protein cages, were then labeled by attaching an aminated DNA aptamer to a mutagenically inserted cysteine residue present in the eGFP using SMCC. The DNA aptamer−eGFP−ferritin nanocomplexes were used as fluorescent probes in aptamer-based sandwich assays for platelet-derived growth factor B chain homodimer (PDGF-BB), see Figure 64, where they demonstrated enhanced sensitivity compared with DNA aptamer−eGFP or DNA aptamer−Cy3 fluorescent probes.

Perhaps the most elegant use of a genetically modified ferritin was recently reported by Kim's group.[1472,1473] They engineered a system called "InCell SMART-I (intracellular supramolecular assembly readout trap for interactions)", which is geared toward directly visualizing intracellular dynamic molecular interactions using a specific bait and prey strategy. As shown in Figure 65, the system consists of a ferritin-derived NP that expresses an FK506 binding protein−monomeric red fluorescent protein (FKBP−mRFP) fusion. The ferritins also express the FKBP-rapamycin binding domain (FRB) of the mammalian target of rapamycin (mTOR) protein, and this domain is fused to eGFP (FRB−eGFP). FKBP and FRB are known to form heterodimers in the presence of the cell-permeable anticancer drug rapamycin. Expression of these constructs on the N-terminus of ferritin in HeLa cells led to a diffuse pattern of both green and red fluorescence across the cytosol. In the sensing scenario, addition of rapamycin to the

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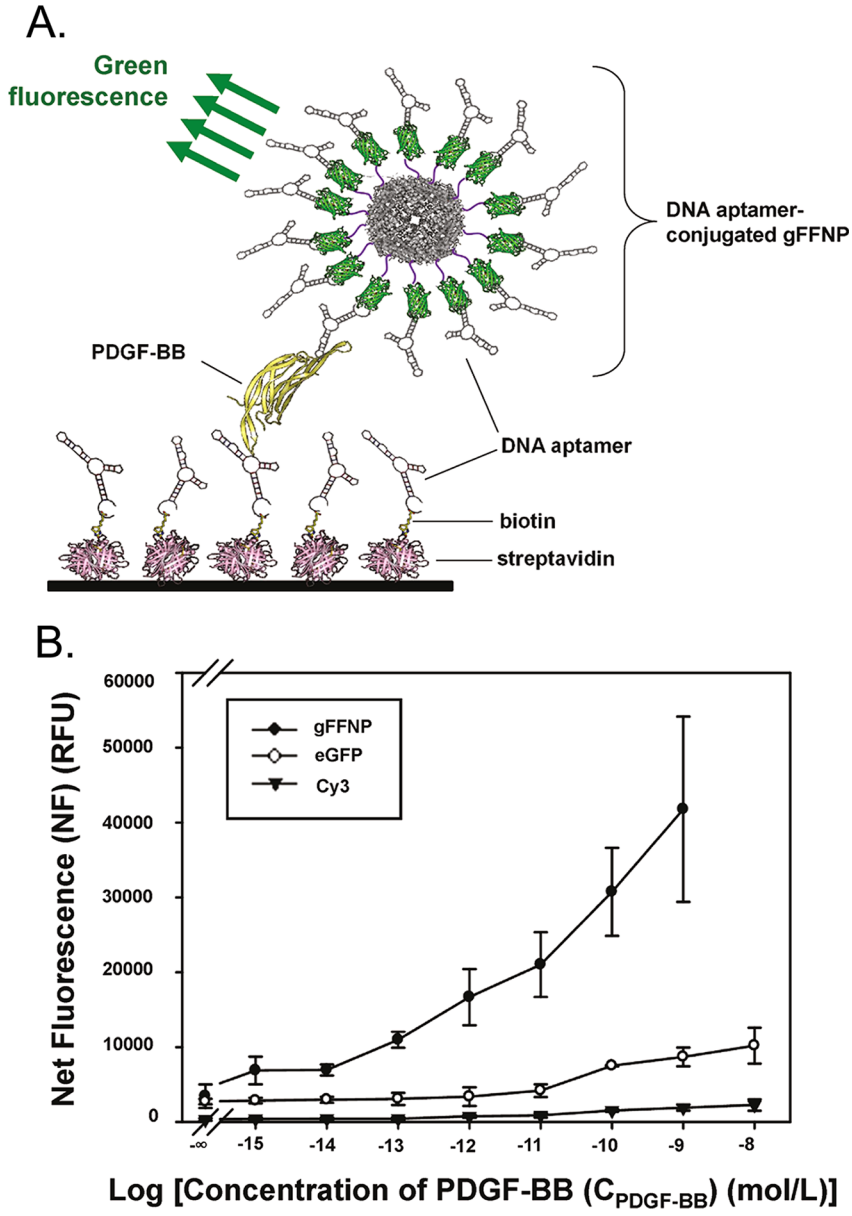

Figure 64. Multifunctional ferritin-based NPs as fluorescent probes. (A) Schematic illustration of a DNA-aptamer-based assay of PDGF-BB using DNA-aptamer-conjugated gFFNPs. (B) Assay results using DNA-aptamer-conjugated gFFNPs, DNA-aptamer conjugated eGFP, and DNA-aptamer-conjugated Cy3 as reporter probes in the detection of PDGF-BB. Reprinted with permission from ref 1425. Copyright 2011 American Chemical Society.

growth media was followed by rapid cellular uptake and subsequent heterodimerization of the intracellular proteins, which rapidly drove the formation of discrete punctate agglomerates within the cell, see Figure 65. The authors were able to build upon this initial sensing mode and design more complex sensors that could capture both rapamycin and tumor necrosis factor-α in a three fluorescent protein−three color system. Although just an initial demonstration, this genetically expressed and modifiable NP bioconjugate and its unique sensing strategy clearly have much to offer for monitoring intracellular molecular interactions.[1473]

Overall, apoferritins are a very a promising tool in the nanobiotechnology and NP "toolbox". As a naturally occurring protein in humans, recombinant versions can be considered at the very least "biocompatible", which may help mitigate toxicity concerns. The biggest benefit is derived from how easily apoferritin can be functionalized both within its core and on its surface, allowing for a plethora of multifunctional constructs with an inherent payload capacity that ranges from small discrete drug molecules up to and including other NPs.

4.2.5. Virus-Derived Structures. Viral capsids and virus nanoparticles (VNPs), sometimes termed bionanoparticles, offer the exciting possibility of functioning as programmable nanoscale protein platforms with a variety of predicted applications under development including (1) antigen display vectors for vaccine generation, (2) platforms for targeted gene and drug delivery, (3) data storage and molecular electronic devices, (4) platforms for biosensing and bioimaging, and (5) ordered arrays for tissue engineering.[1449,1474−1485] As naturally occurring macromolecular protein complexes, VNPs are isolated from a variety of sources including plants, fungi, insects, bacteria, and even humans. Almost all VNPs are amenable to recombinant

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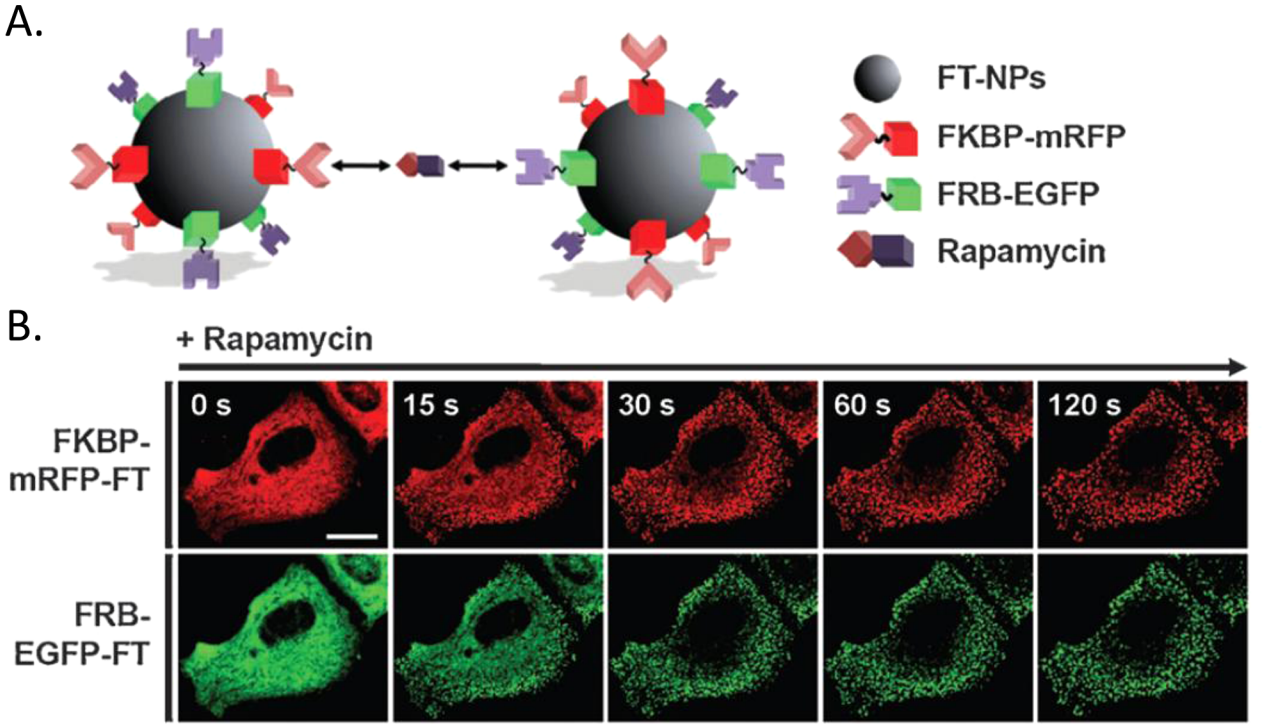

Figure 65. SMART-i nanosensors. (A) Schematic of the InCell SMART-i for visualizing small-molecule−protein interactions inside living cells. (B) InCell SMART-i detecting interactions of rapamycin with FKBP and FRB using ferritin-derived nanoparticles (FT-NPs). Time-lapse images of nanocluster formation. Scale bar = 20 μm. Figure reprinted from ref 1472 with permission. Copyright 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

engineering. The topic of viral capsid structures, their subsequent chemical modification, and different applications have been the subject of several recent reviews.[1449,1474−1481] Table 13 highlights a representative set of examples, where a variety of VNPs originating from different sources have undergone various chemical modifications in pursuit of new applications or materials. While there are extensive families of viruses, covering a range of sizes and shapes, the viruses of interest for these purposes have in common that their protein shells or capsids consist of various coat proteins that reproducibly self-assemble into the final three-dimensional architecture and encapsulate their genomes. Of particular interest is that the coat proteins coassemble into the structure with symmetrical subunits repeatedly displayed around the surface. For example, cowpea mosaic virus (CPMV) consists of 60 copies of the small (S) and large (L) subunit coat proteins that arrange themselves into the final icosahedral particle.[1486] Figure 66 highlights how these viruses all retain strong symmetrical architectures even though their structures can be quite different.[1449] Viruses are classified into "enveloped" and "nonenveloped" varieties, where envelopes cover the protein capsid and are typically derived from phospholipids and proteins that originate from the host cell along with, in some cases, glycoproteins of viral origin. Envelopes help the virus enter the host cells during the infective process. Virus-like particles (VLPs) typically comprise the same self-assembled coat proteins but lack the encapsulated viral genetic material making them inherently safer for potential *in vivo* applications. The VNPs and VLPs described here are mostly nonenveloped due to the extra layers of complexity that this adds to any bioconjugation strategy.

Naturally occurring capsid VNPs have a number of inherent advantages compared with their synthetic NP counterparts, which make them of particular interest for bionanotechnological applications. First, they can be produced in relatively large milligram to gram quantities in the laboratory either directly in their natural hosts, for example, as in the case of plant viruses, or recombinantly in bacteria or other expression vectors. As shown in Figure 66, the viral capsids utilized for these purposes are typically icosahedral, quasi-circular, or rod-shaped. Filamentous or rod-shaped particles can reach up to 2 μm in length while icosahedral capsids range from 18 to 500 nm in size. Because the principle role of the capsid is to encapsulate and protect the viral genome, they tend to be robust and relatively rigid and are generally more resistant to pH, temperature, and solvents than typical cytosolic proteins. When fully assembled, the different capsid structures are symmetrical, discretely shaped, and monodisperse in size, the latter a feat far beyond the reach of current NP synthetic procedures. More pertinently, the capsids are inherently biocompatible and biodegradable. Because capsids are protein-based, they display selected amino acids in a polyvalent geometrically repeated manner. Thus recombinant engineering allows the placement of specific residues and even small peptides on the particle surfaces in a discretely controlled manner. The ability to repeatedly display multiple moieties around the virus lends itself directly to potentiating high-avidity interactions. Moreover, extensive genetic and crystallographic studies have revealed the exact number and placement of amino acids available on capsid surfaces. These residues are critical for subsequent chemical modification, whether modifying only a single type of residue in one or multiple steps or using multiple labeling chemistries to target different residues. Note that similar to ferritin, viral capsids can be modified on both their exterior and their interior surfaces and can encapsulate cargos in the cavity normally occupied by genomic material. Cumulatively, this can provide access to a variety of designer nanoscale structures with highly programmable surface functionalities, defined sites for the polyvalent display of one or more (bio)molecular ligands,

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Table 13. Representative Examples of Viral Nanoparticle Bioconjugates

virus	source/description	viral surface ligand targets[a]	conjugated species	chemistries applied	intended use	refs
cowpea mosaic virus (CPMV)	plant RNA icosahedral virus, 30 nm diameter, nonenveloped	lysine, cysteine, glutamic and aspartic acid COOH's, $(His)_6$	NHS−nanogold, −PEG, −dyes, −biotin, −DNA; COOH−QDs, COOH−SWCNTs, COOH−$(His)_6$; peptide−QDs, alkyne- or azide-modified sugars, peptides, PEG polymers, folic acid−PEG, Gd-DOTA and Tf; arylhydrazone or hydrazone biomolecules, PEG, fluorescein, and targeting peptide; maleimide−dyes, −gold, −NeutrAvidin, −DNA; antibodies; gold colloids; methyl(aminopropyl)viologen; Ni−NTA−nanogold	NHS-ester; EDC/NHS; click chemistry; hydrazone ligation; Au/S interaction maleimide; EDC/NHS; Ni−NTA/$(HIS)_6$ affinity	imaging, biosensors, drug delivery; DNA microarrays; electron mediators	25, 33, 1476, 1487−1496
tobacco mosaic virus (TMV)	plant RNA rod-shaped virus, 18 nm diameter, 300 nm long, nonenveloped	tyrosine (exterior), COOH (interior), cysteine, His_6	biotin or PEG-modified alkoxyamines, azides, alkynes; various amine-functionalized species including fluorescein derivatives; maleimide−fluorophores	diazonium salts; EDC/NHS; maleimide; genetic modification	light harvesting	1497−1500
cowpea chlorotic mottle virus (CCMV)	plant RNA icosahedral virus, 28 nm diameter, nonenveloped	glutamic acid, lysine	iron oxide (magnetic) nanoparticles; NHS−PEG−fluorescein	electrostatic; NHS	drug delivery, catalysis	1501, 1502
potato virus X (PVX)	plant RNA rod-shaped virus, 13 nm diameter, 515 nm long, nonenveloped	lysine, COOH's	NHS−biotin, 3-azidocourmarin, amine−biotin	NHS; click chemistry; EDC/NHS	drug delivery	1503
bacteriophage MS2	bacterial RNA icosahedral virus, 27−34 nm diameter	tyrosine (interior), lysine (exterior), cysteine, inserted *p*-aminophenyl-alanine-(paF)	fluorescein; NHS−PEG; gadolinium−isothiocyanate (ITC) derivatives; peptides; maleimide−porphyrin, maleimide−AlexaFluor 488; *N,N*-diethylphenylene diamine-substituted DNA	diazonium salts; NHS, ITC; sodium periodate	targeted therapy, MRI contrast agents	1504−1508
bacteriophage Qβ	bacterial RNA icosahedral virus, 27−30 nm diameter	lysine; mutants, methionine analogs	Gd−(DOTA)−alkyne chelates; PEG; COOH-modified C_{60} buckyballs; alkyne- or azide-functionalized proteins and DNA	click chemistry; EDC/NHS; genetic modification	biomedical photosensitizers and inflammation treatment	1509−1513
hepatitis B virus (HBV)	human DNA icosahedral virus, 42 nm diameter, enveloped	protein A−HBV coat protein chimeric mutant	antibodies, anti-troponin I	Fc domain/protein A affinity	diagnostic assays	1514
flock house virus (FHV)	insect RNA icosahedral virus, 30 nm diameter, nonenveloped	lysine, ANTXR2 VWA chimeric mutant	aminooxy-derivatized maltose binding protein (MBP); MBP-modified with anthrax protective antigen (PA) domain 4	aniline-catalyzed oxime ligation; protein affinity	vaccine development	1515
Sulfolobus islandicus rod-shaped virus 2 (SIRV2)	*Sulfolobus* DNA rod-shaped virus, 23 nm diameter, 900 nm long	COOH, lysine, carbohydrate	biotin−hydrazide; NHS−biotin, NHS−AlexaFluor 568	EDC, NHS; hydrazone linkage	various	1516
Moloney murine leukemia viruses (MoMLVs)	RNA icosahedral virus, 100 nm diameter, enveloped retrovirus	sialic acids present on the retrovirus producing cell surface	aminooxy-AlexaFluor 488 and aminooxy-biotin	*p*-anisidine catalyzed oxime ligation	targeted delivery	1517

[a]Here, COOH refers to the carboxyl groups on glutamic and aspartic acid residues.

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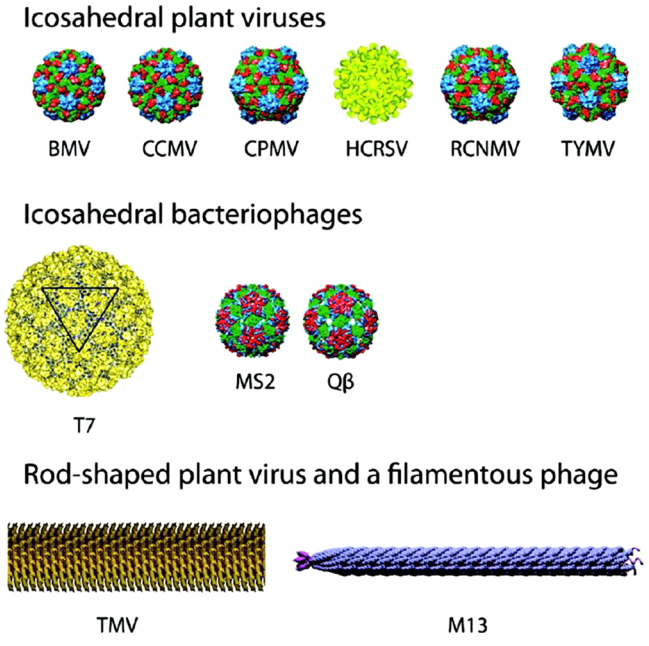

Figure 66. Overview of the structure of some representative viral NP structures that have been developed for materials science and medicine. Icosahedral plant viruses: brome mosaic virus (BMV), cowpea cholorotic mottle virus (CCMV), cowpea mosaic virus (CPMV), hibiscus cholorotic ringspot virus (HCRSV), red clover necrotic mottle virus (RCNMV), turnip yellow mosaic virus (TYMV). Icosahedral bacteriophages: T7, MS2, and Qβ. Note that T7 is a head–tail phage, but only the head is shown. Rod-shaped and filamentous viruses: tobacco mosaic virus (TMV) and phage M13. For size comparison, the CPMV has a diameter of ~27 nm. Reprinted with permission from ref 1474. Copyright 2011 American Chemical Society.

intrinsic cargo capacity, and excellent potential for being redesigned into new types of functional NP materials, see also Table 13.[1449,1474−1483]

In comparison to almost all the other types of synthetic NPs described here, VNPs allow for perhaps the widest range of modifications to be applied in their bioconjugation. This includes direct chemical labeling of their internal or external surfaces, use of functional cross-linkers, noncovalent assembly, mutagenesis, incorporation of non-natural amino acids, encapsulation, and, more recently, combinations of multiple orthogonal chemistries that allow several different, usually sequential, modifications to be made to the capsid. Critically, many of these chemistries can be applied to the monomeric coat proteins before assembly of the final macromolecular structure. Moreover, in some cases, wild-type viruses come with intrinsically expressed cell binding motifs, as exemplified by adenovirus, which displays 12 RGD integrin binding sequences extending away from the capsid surface on polypeptide loops.[1480] Each of these chemical approaches are briefly reviewed below.

4.2.5.1. Targeting Natural Amino Acids. One of the simplest and first strategies applied to bioconjugating VNPs involves targeting the side chain functional groups of existing amino acid residues displayed on the VNP surface for direct chemical modification using common protein-labeling chemistries. Numerous examples of this approach abound including EDC chemistry to attach dyes and redox-active methyl viologen to CPMV,[1518] NHS-activated PEG, PEG–fluorescein, biotin, and dyes to label amines on CPMV,[1487,1490,1491] NHS-activated luminescent Tb and EDC–biotin–dyes to functionalize amines and carboxyls on turnip yellow mosaic virus,[1519,1520] and FITC to modify amine groups on bacteriophage T4 NPs.[1521] All of these couplings achieved high labeling efficiencies where, for example, in the case of the latter T4, up to 1.9×10^4 dyes were estimated to be attached per NP.[1521] Such a highly localized display of dyes is not always desirable because it can lead to fluorescence self-quenching and quite often requires scaling back on the dye/NP ratios for optimal emission during imaging applications. However, in some cases, such a high-labeling efficiency is actually desired. Anderson et al. modified MS2 bacteriophage with Gd–DTPA–ITC at 500 sites (92% of available lysine residues) to increase MRI contrast,[1506] and further colabeling with FITC provided a potential bimodal fluorescent/MRI contrast agent.

VNPs have proven to be an advantageous platform for applying direct and multistep chemistries that utilize the CuAAC reaction. Bruckman et al. utilized a diazonium salt derived from 3-ethynylaniline to label tobacco mosaic virus (TMV) with alkyne groups at the *ortho* position of tyrosine phenolic rings.[1497] The alkynes were subsequently coupled to a variety of azido compounds that ranged from dyes to PEGs and polypeptides. Reaction with bis-azidomethylbenzene allowed subsequent modification with a second CuAAC step, providing access to the display of different (bio)molecules using the CuAAC in two "reversed" steps, see Figure 67.[1497] CPMV and bacteriophage Qβ have also been modified with an NHS-ester azido compound that targeted lysine residues to allow subsequent labeling with Gd–DOTA via CuAAC in pursuit of high-contrast viral MRI agents.[1522] A water-soluble, sulfonated bathophenanthroline ligand was shown to accelerate Cu(I) catalysis on VNPs even in the presence of a lower concentration of reactants.[1523] When applied to CPMV, efficient coupling of complex sugars, peptides, polymers, and Tf was possible, especially where the conjugation site had shown previous resistance to modification. Combining a similar CuAAC labeling scheme with direct modification by an NHS ester derivative allowed the display of biotin, dyes, and PEGs on potato virus X.[1503]

In an example that highlights a combination of multiple labeling chemistries targeting the same VNP substrate, Li and colleagues extensively screened the reactivity of three different functional groups displayed on the rod-shaped M13 phage P8 coat protein by targeting surface lysine, aspartate, glutamate, and tyrosine amino acid residues, see Figure 68.[1524] The amine and carboxylic acids groups from these residues were probed using NHS-activated fluorescein or TAMRA dyes or EDC/NHS activation for the addition of rhodamine B amine. The authors found that the NHS chemistry could load ~1600 dyes/phage, although this needed to be decreased to ~400 to mitigate self-quenching. In contrast, only ~150 carboxyl groups could be labeled. The tyrosine residues were modified with alkyne groups using a diazonium coupling reaction, and the alkyne was modified with azido derivatives of coumarin or biotin using CuAAC. This method yielded ~340 coumarin and ~400 biotin labels per particle. Understanding the surface chemistry allowed dual modification by coupling the lysine residues with NHS–fluorescein and the tyrosine residues with FA–azide. The FA-modified M13 NP showed relatively good binding affinity for human KB cancer cells displaying FA receptor.[1524] Similar to the above example, Steinmetz and colleagues applied three different chemistries to site-specifically address a *Sulfolobus islandicus* rod-shaped virus 2.[1516] This hyperthermophilic and acid-resistant archaeal virus was isolated from a volcanic spring in Iceland, and its extreme acidothermophilic

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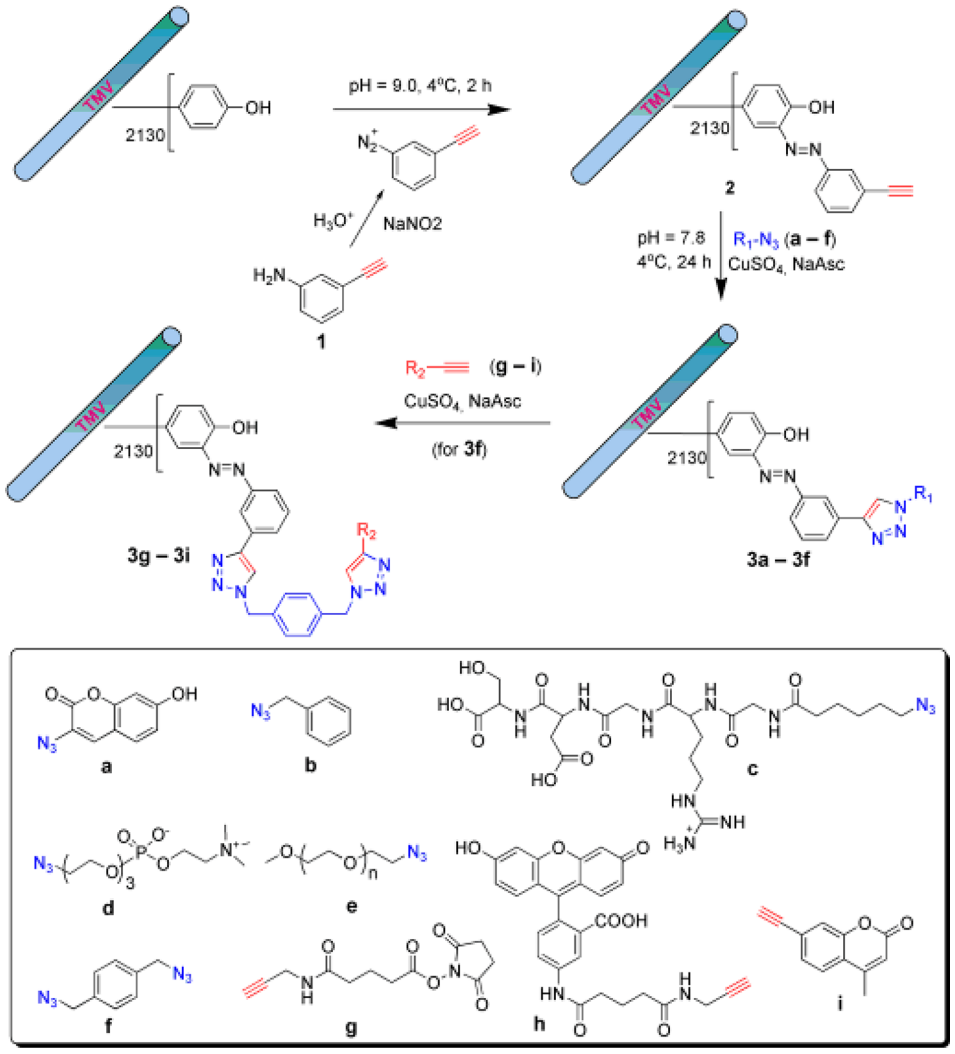

Figure 67. Schematic showing the bioconjugation of TMV by means of differentially implemented CuAAC reactions. Reprinted from ref 1497 with permission. Copyright 2008 Wiley-VCH GmbH & Co. KGaA, Weinheim.

tolerance (~pH 2.5 and 88 °C) suggests that it may be extremely rugged and even more stable as a VNP building block. The virus was biotinylated at carboxyl groups using EDC chemistry, at amine groups using NHS-activated biotin, and at carbohydrate moieties using mild oxidation with sodium meta-periodate followed by reaction with biotin–hydrazide.[1516] The latter converted hydroxyl groups to aldehydes, which then formed a hydrazone bond upon reaction with biotin–hydrazide. Beyond those examples described here, there are many others that target the natural amino acids of different viral platforms. For example, see ref 1480 for an extended discussion of adenovirus labeling.

4.2.5.2. Mutagenesis. The fact that all viruses are genetically encoded makes them directly amenable to recombinant engineering and confers the ability to display residues that are different from those found in the parent capsid. This can include more or less of a particular existing residue or, alternatively, the introduction of a new residue such as cysteine, which can provide the capsid with unique thiol handles. It is important to note that introduction of such a (single) mutation into a coat protein monomer yields multiple symmetrically displayed cysteines arrayed around the final self-assembled VNP; this in essence can be considered a multiplicative display effect that is another unique property of such viral materials. Recombinant modification of proteins is usually not, in and of itself, considered a "classical" bioconjugation technique. It is usually applied to proteins to allow their subsequent coupling to NPs via site-specifically incorporated residues or reactive or affinity polypeptide motifs. However, given that the capsid represents the functional NP, mutagenesis can be an important and indispensible step in conjugate preparation for this class of NMs.

Cysteine mutants are perhaps the most common recombinant alteration of VNPs because these are not normally expressed in an exposed or available form on capsids. In an example of multiplicative display, a recombinant GGCGG loop placed between positions 98 and 99 of the CPMV large protein yielded 60 accessible cysteine thiols symmetrically displayed around the final assembled capsid.[1525] Subsequent dual modification of these cysteines with ~40 copies of maleimide-activated Cy5 dye and NeutrAvidin allowed further assembly of biotinylated DNA and yielded signal-amplified fluorophores for visualization of DNA arrays.[1494] The same CPMV mutant was amine-labeled with dyes, and subsequent use of bifunctional GMBS allowed further coupling of antibodies to cysteine residues yielding a potentially customizable immunoassay tracer.[1496] The Francis group labeled cysteine residues introduced onto TMV coat protein monomers with three-different maleimido dyes.[1500] These were then self-assembled into stacks of disks and rods that approached hundreds of

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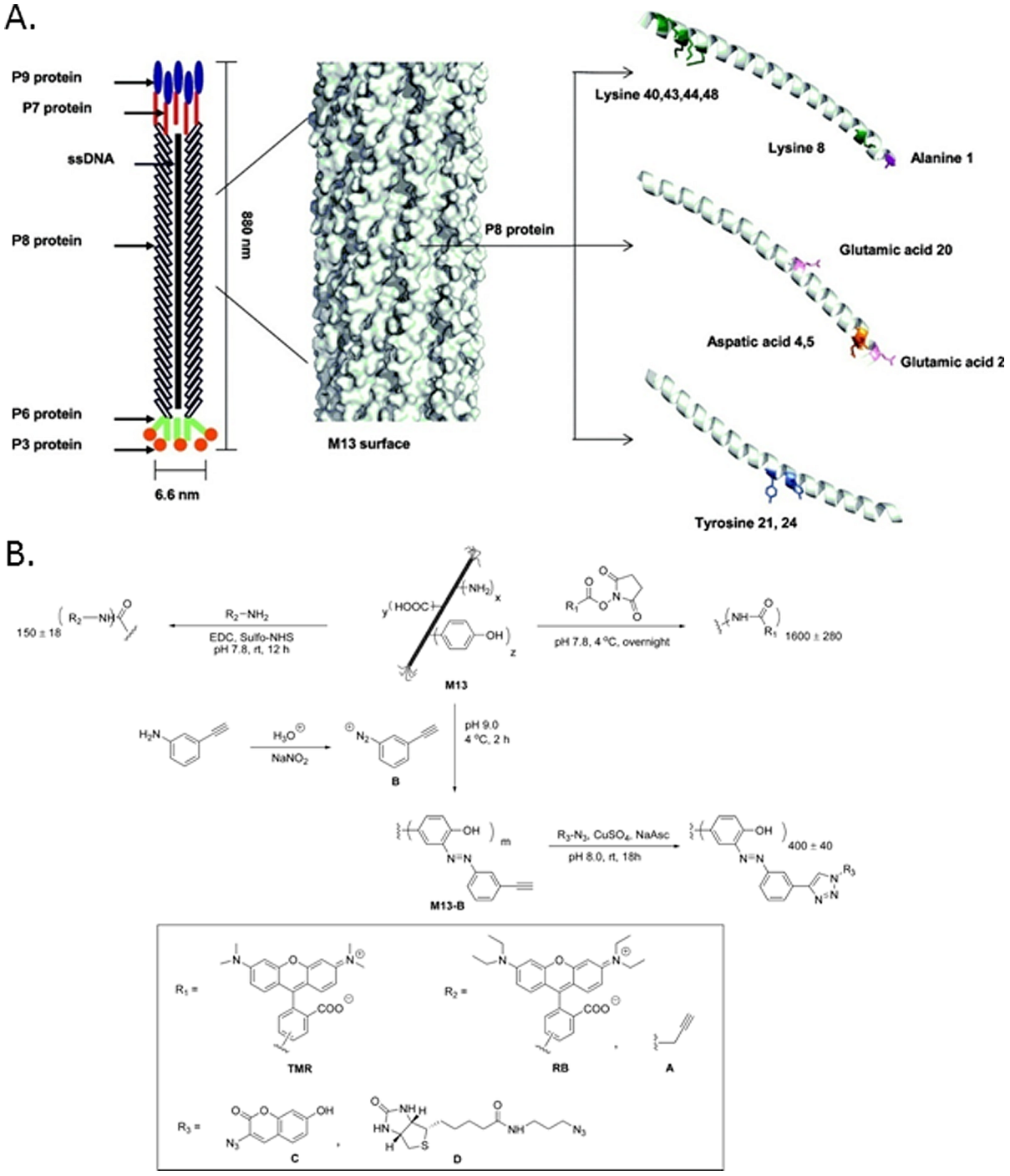

Figure 68. Structure and modification of M13: (A) left, schematic depiction of M13 bacteriophage; middle, surface crystal structure of M13 bacteriophage; right, the structure of M13 P8 protein subunit with target amino acid residues highlighted; (B) schematic showing the chemical modification of the three different side chain functionalities. Reprinted with permission from ref 1524. Copyright 2010 American Chemical Society.

nanometers in length and demonstrated complex energy transfer properties including multiple donor/single acceptor and three-chromophore relay systems as depicted in Figure 69. Interestingly, introduction of cysteine residues tends not to grossly cross-link or bridge the VNPs, which is a problem quite often encountered when introducing two or more thiols to smaller proteins. The outcome here is probably a result of strong interparticle repulsion, which maintains a lack of direct contact between the thiols on different particles.

His_6 tags have also been commonly incorporated into viral protein monomers to facilitate purification following expression; however, these residues can also serve to modify capsid physiochemical properties. The Johnson group inserted His_6 tags at five different sites throughout the CPMV small and large subunits.[1488] They found that the mutant particles showed differential affinities for binding to Ni(II) and that their electrostatic properties could be controlled by the His_6 protonation state allowing for selective capsid modification with nanogold Ni-NTA.

Early attempts at displaying heterologous proteins and peptides on viral surfaces focused on modifying native lysine and mutant cysteine residues with bifunctional cross-linkers. Johnson's group derivatized the cysteine residues on the

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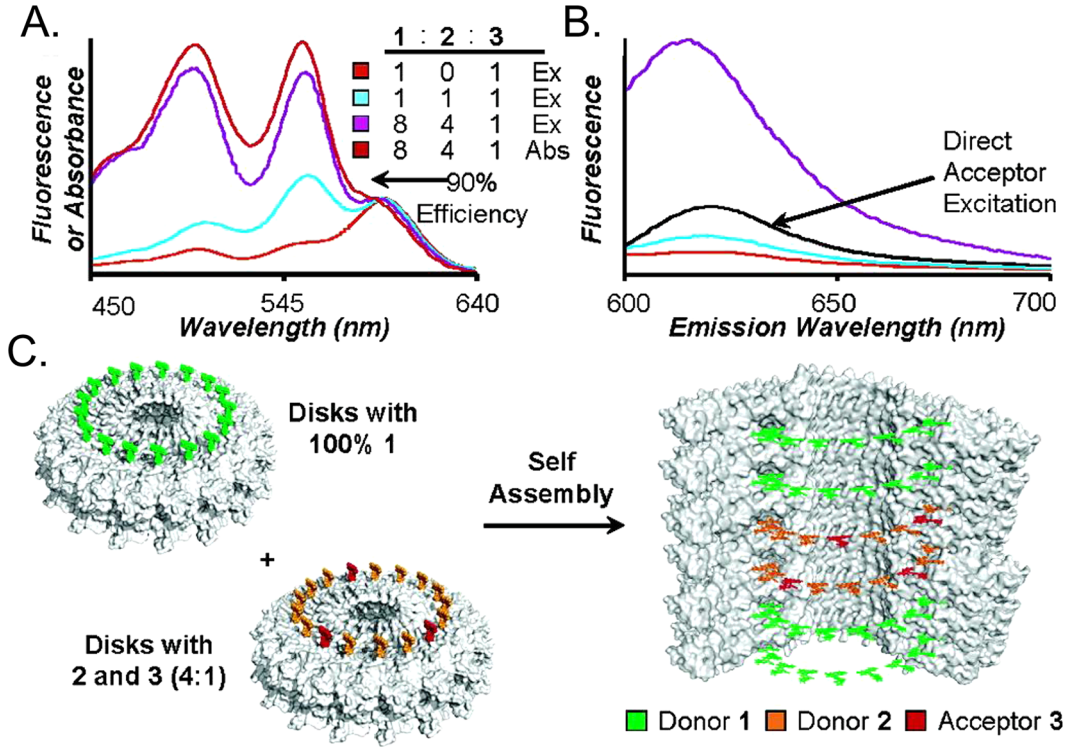

Figure 69. Three-chromophore systems for broad-spectrum light harvesting. TMV protein monomers labeled with **1** (Oregon Green 488), **2** (tetramethylrhodamine), or **3** (Alexa Fluor 594) using maleimido chemistry were combined in the ratios indicated. (A) Fluorescence excitation spectra (Ex), normalized at the acceptor excitation at 597 nm, indicated light harvesting over a wide range of wavelengths. The absorbance spectrum (Abs) for the 8:4:1 system is shown in red. (B) The antenna effect for each spectrum (λ_{ex} = 495 nm) is shown relative to the sample's acceptor emission by direct excitation (λ_{ex} = 588 nm). (C) Spatial distribution of chromophores for the 8:4:1 system. Reprinted with permission from ref 1500. Copyright 2007 American Chemical Society.

leucine-rich repeat domain of internalin B, T4 lysozyme, and the intron 8 gene product of the HER2 tyrosine kinase receptor with both homo- and heterobifunctional linkers for subsequent conjugation to targeted lysine and cysteine residues associated with CPMV.[1488] In contrast to this, genetic engineering allows direct expression of chimeric protein and peptide sequences on the capsid surface. For example, flock house virus (FHV) was engineered to display 180 copies of the protective antigen (PA)-binding von Willebrand A (VWA) domain (~181 amino acids in length) to produce a potential high-avidity anthrax antitoxin that inhibited lethal toxin action in both *in vitro* and *in vivo* models of anthrax intoxication.[1485] Exposure of rats to the FHV−VWA particles bound with PA was found to produce a potent protective immune response that was likely due to the multivalent nature of the FHV−VWA−PA complex. Viral-based anthrax vaccines were also developed by Phelps who genetically modified CPMV to contain 60 copies of a 25-amino acid peptide sequence derived from the antigenic region of PA.[1484] Hepatitis B virus genetically modified with staphylococcal protein A produced chimeric viral capsid NPs with high affinity for the Fc domain of IgG antibodies. These were used to bind to antibodies, and their application in immunoassays for troponin I, a biomarker important for detecting acute myocardial infarction, yielded diagnostic assays with a 10^6−10^7-fold improvement in sensitivity compared with conventional ELISAs.[1514]

Mutagenic changes to VNPs are also leading the development of newer NMs. The Francis group created a circular permutant of the TMV capsid protein that repositioned the N- and C-termini to the center of the rod assemblies.[1526] Disks assembled from this protein were stable over a significantly larger pH range than the parent protein and allowed for the further controlled functionalization on the inner disk pore. These disks could also be assembled with unmodified monomers yielding similar rod geometries with the ability to be functionalized both internally and externally. This was translated in practice to an energy transfer configuration where the pore of each ring displayed, on average, a single FRET acceptor that interacted with a ring of 17 surrounding donors. Bruckman et al. found that His_6 insertion into a TMV coat protein allowed the subsequent self-assembly of disks, hexagonally packed arrays of disks, stacked disks, rods, fibers, and elongated rafts.[1498] In a related example, Xiao and colleagues created a 24 nm × 30 nm ellipsoid protein NP by re-engineering the core protein of the bacteriophage ϕ29 DNA packaging motor.[1527] Adding N-terminal peptides stabilized the structure, while proteolytic cleavage of these extensions controllably reversed the structure to a dodecamer subunit.

Recombinant approaches are also yielding new materials that originate from viral components. For example, Hilvert redesigned the natural capsid-forming lumazine synthetase enzyme from *Aquifex aeolicus*, a rod-shaped chemolithotrophic bacterium, to allow encapsulation of specific guest proteins *in vivo*.[1528] The luminal surface was engineered to display negatively charged residues to promote host−guest interactions with positively charged "cargo" molecules, and the initial demonstration sequestered a toxic protease in the cytosol of *E. coli*. More recent work showed that the same approach can be extended outside the cell.[1529] In this case, encapsulation of ~100 positively supercharged GFPs could be accomplished either

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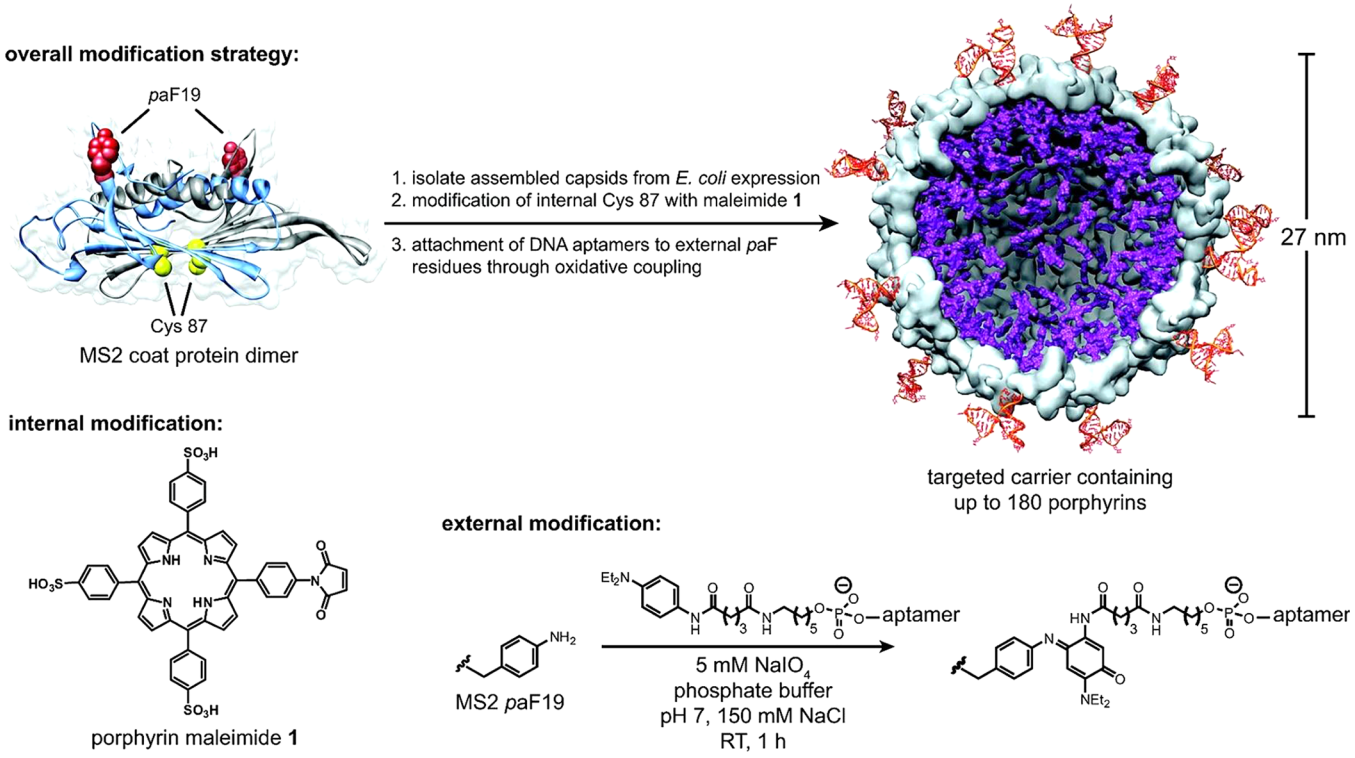

Figure 70. Construction of a multivalent cell-targeted PDT vehicle using recombinant bacteriophage MS2. Cysteine residues on the capsid interior were modified using porphyrin maleimide 1 (purple), enabling the generation of singlet oxygen upon illumination at 415 nm. Exterior *p*-aminophenylalanine (*p*aF) residues, introduced using an amber suppression technique, were coupled to phenylene diamine modified DNA aptamers that bind to tyrosine kinase 7 receptors. About 20 aptamers were installed on each capsid surface. Reprinted with permission from ref 1507. Copyright 2010 American Chemical Society.

starting with empty intact capsids or from mixing capsid fragments with GFP cargo in buffer. They also found that the packing density directly reflected the host/guest mixing ratio.

4.2.5.3. Incorporation of Non-natural Amino Acids. Genetic manipulation of viral genes coupled with recombinant expression systems makes them tractable to translational incorporation of non-natural amino acids. Using a methionine replacement strategy in a methionine auxotrophic *E. coli* strain originating from the Tirrell Laboratory, the Finn group were able to specifically incorporate homopropargylglycine and azidohomoalanine into hepatitis B virus (HBV) and bacteriophage Qβ coat proteins.[1530] They found that these substitutions did not alter particle self-assembly into icosahedral structures; however, subsequent CuAAC to alkyne-modified fluorescein decomposed the HBV particles, while the Qβ structure remained intact. This finding highlights how complex macromolecular structures can be vulnerable to chemical modifications in an unpredictable manner. Modified Qβ structures were further coupled in a similar manner to cognate-derivatized biotin, Tf, and a Gd complex. Subsequent studies demonstrated interior surface labeling of the Qβ structure with a Gd-complex using the same chemistry and found that the modified-VNP plasma clearance rates in Balb/c mice were substantially increased by acylation of surface exposed amines during the first chemical step.[1509] This could be alleviated by reintroducing surface amines with NHS ester azides and subsequent coupling to propargylamine. Azidohomoalanine and homopropargylglycine residues have also been incorporated into Qβ and bacteriophage MS2 using cell-free protein synthesis, and interestingly, this significantly increased the final VNP yield.[1512] Subsequent CuAAC chemistry coupled three-different azide- or alkyne-containing proteins, nucleic acids, and PEG to the modified VNPs in a single step with control over relative ratios and surface abundance.

The Francis group utilized an amber stop codon suppression system to introduce *p*-aminophenylanine (*p*aF) at position 19 of the MS2 bacteriophage for a site-specific oxidative coupling reaction.[1508] An amine-terminated DNA sequence was acylated with an NHS-ester precursor and then reacted to the *p*aF−MS2 in the presence of sodium periodate. The DNA functioned as a cell targeting aptamer sequence by recognizing tyrosine kinase receptors on Jurkat leukemia T cells. Cysteine mutations introduced to the interior of the same phage were also labeled with maleimide dyes that functioned as a model cargo while providing fluorescence contrast.[1508] The same oxidative coupling strategy was used to modify *p*aF on the MS2 surface with the targeting aptamer, while the phage's interior cysteine residues were coupled to a porphyrin maleimide as depicted in Figure 70.[1507] In this case, the porphyrins were exploited to generate cytotoxic singlet oxygen and selectively killed more than 75% of target Jurkat leukemic cells with only 20 min of illumination, amply demonstrating the potential of multivalent VNPs as a targeted PDT platform.

4.2.5.4. Encapsulation. Analogous to the natural role of a virus during an infection, that is delivering a genetic "cargo" to a targeted cell, the concept of exploiting viral capabilities to encapsulate and deliver a designer cargo for imaging, contrast, or therapy in a similar manner has been under consideration for some time.[1474,1478,1480] For example, incorporating other heterogeneous magnetic or fluorescent NP materials inside the VNPs was stimulated by earlier demonstrations that these cargos could be grown within the virus *in situ*.[1501] Cumulatively, this led to investigations of encapsulating strategies as an active form of VNP

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bioconjugation. Encapsulating materials within the viral interior is usually achieved by two approaches: (1) infusion into the intact virus or (2) cooperative self-assembly between the coat protein subunits and species to be encapsulated.[227,1531−1533]

In an example of the first approach, Lockney and co-workers allowed DOX to simply infuse into red clover necrotic mosaic virus during an overnight incubation.[227] Subsequently, an average of >100 cell targeting peptides, which specifically recognized N-cadherin on cancer cells that had undergone the epithelial-to-mesenchymal transition associated with metastasis, were attached to the VNPs with a sulfo-SMCC linker. This allowed them to be delivered to human cervical cancer HeLa cells, where a specific cytotoxic effect was observed. Exploiting the second approach, Jung and co-workers used the negatively charged near-IR chromophore indocyanine green (ICG) as a nucleating agent to drive the self-assembly of coat protein subunits derived from brome mosaic virus (BMV).[1534] It is generally accepted that RNA and other anionic materials can act as a nucleating agent to drive the assembly of coat proteins into intact virions, and in this case, the authors surmise that the negative ICG provided a similar activity. The ICG-functionalized BMV particles were then used to image human bronchial epithelial (HBE) cells with no apparent cytotoxicity observed. The strong negative charges of encapsulated polystyrene sulfonate polymers have also been used to both nucleate and stabilize the structure of cowpea chlorotic mottle virus (CCMV) particles displaying PEG modifications on exterior lysine residues.[1502]

Kwak and colleagues specifically utilized nucleation to create VLPs that were templated by DNA micelles.[1532] This approach began by modifying DNA with lipids and poly(propylene oxide) blocks to create amphiphiles that formed micellar structures followed by addition of CCMV coat proteins that encapsulated the micelle. Other hydrophobic moieties, such as the fluorophore pyrene, could be absorbed into the micelle core, while hydrophilic rhodamine-labeled DNA could be hybridized to the original DNA, thereby demonstrating a controlled loading of chemically diverse cargos by alternate methods including DNA complementarity. Brasch and co-workers utilized both infusion and self-assembly methods to encapsulate phthalocyanine, a chromophore with strong medical, photonic, electronic, and energy conversion properties, within CCMV VNPs, see Figure 71A.[1535] The authors noted the formation of pthalocyanine stacks instead of dimers during encapsulation, which was ascribed to increased confinement at high concentrations. The phthalocyanine−CCMV capsids were then used as photosensitizers for PDT treatment of RAW 264.7 macrophages, where almost complete cell death was noted only in the directly illuminated areas.

It is also possible to encapsulate full-sized proteins in VLPs as amply demonstrated by Cornelissen's group.[1531,1533] In one approach, eGFP expressing an N-terminal His_6 tag was engineered to also display a C-terminal coiled coil motif, while a CCMV capsid protein was engineered with an N-terminal coiled coil.[1533] Following purification of the separate components, the eGFP−capsid was allowed to preform, via the coil−coil interactions, into a trimer that, when mixed with wild-type capsid protein, formed a full capsid with up to ∼15 encapsulated eGFPs, see Figure 71B. A subsequent study demonstrated encapsulation of the enzyme lipase B in the same CCMV capsid.[1531] In this case, assays indicated that the overall enzyme reaction rates increased upon encapsulation. This result was attributed to the extremely high confinement molarity (∼1 mM), which led to very rapid formation of the enzyme−substrate complex. The latter example also highlights the potential of these materials for creating highly efficient biomimetic nanoreactors.

The Finn group recently described an elegant and somewhat related recombinantly driven approach for fluorescent protein packaging in Qβ capsids.[1513] In this strategy, T7 expression vectors drive the production of capsid protein, Rev-tagged cargo proteins, and a bifunctional RNA. The Rev-tag on the cargo protein binds to an α-Rev aptamer on one end of the RNA, while the Qβ genome packaging hairpin motif on the other end binds to the interior of the capsid protein monomer. This results in tethering of the cargo to the interior of the Qβ VLP where it is also protected from proteases and other hydrolytic enzymes, see Figure 71C.[1513] This procedure was used to produce VLPs, encapsulating 10−15 appropriately modified eGFP per particle that were indistinguishable from the wild-type VLPs. The fluorescent VLPs were further subjected to external acylation on amine groups to attach a short alkyne linker that was then itself modified by CuAAC to a sialoside (Figure 71D), which provided for specific binding to the $CD22^+$ receptor on CHO cells. It will be fascinating to see which other proteins and "cargoes" can be packaged into this and similar VLPs, using this approach for labeling and delivery applications.

4.2.5.5. Chemoselective Ligation. The backbone protein structure of all VNPs also makes them amenable to a variety of chemoselective ligation chemistries originally developed for peptide and protein synthesis.[279] With this in mind, Brunel et al. utilized an aniline-catalyzed reaction between aldehydes and hydrazides to assemble a multifunctional CPMV for cell imaging and tumor targeting purposes;[1495] ca. 280 of the 300 CPMV surface exposed lysine residues were initially modified to display aldehyde groups using a highly reactive, water-soluble, 4-formylbenzoyl-sulfoNHS ester. Dye-labeled peptide ligands containing a PEG or a vascular endothelial growth factor receptor 1 specific peptide and displaying terminal hydrazido groups were synthesized for the subsequent ligation chemistry. Judicious control of ligand ratios relative to the number of CPMV-displayed benzaldehyde moieties during the ligation reactions allowed sequential labeling with both ligands at different ratios. In application, the modified particles recognized and provided imaging of vascular endothelial growth factor receptor 1 on endothelial cell lines and a tumor xenograft in a mouse model.[1495]

Schlick et al. demonstrated a dual surface modification of TMV that incorporated oxime formation.[1499] In this format, interior glutamate residues were targeted for amide bond formation using EDC in the presence of 1-hydroxybenzotriazole, which minimized formation of stable EDC adduct side products. Exterior tyrosine residues were modified with a diazonium salt to provide ketone groups. The ketones acted as sites for further conjugation via oxime formation following the addition of alkoxyamine-modified substrates such as biotin and several differentially sized PEG. Coupling efficiencies approaching 60% were achieved with this oxime approach. Venter and colleagues recently developed two strategies for multivalent and oriented site-specific presentation of whole proteins on FHV, see Figure 72.[1515] The first strategy depended on an engineered FHV chimera displaying genetically inserted anthrax toxin receptor 2 (ANTXR2) protein on its surface. This chimeric protein assembly (termed VNI) could then noncovalently bind target proteins expressing a terminal portion of the protective antigen sequence in a high-affinity manner, allowing the target proteins to be arrayed around the VNP surface. The second strategy incorporated aniline-catalyzed oxime ligation in a far

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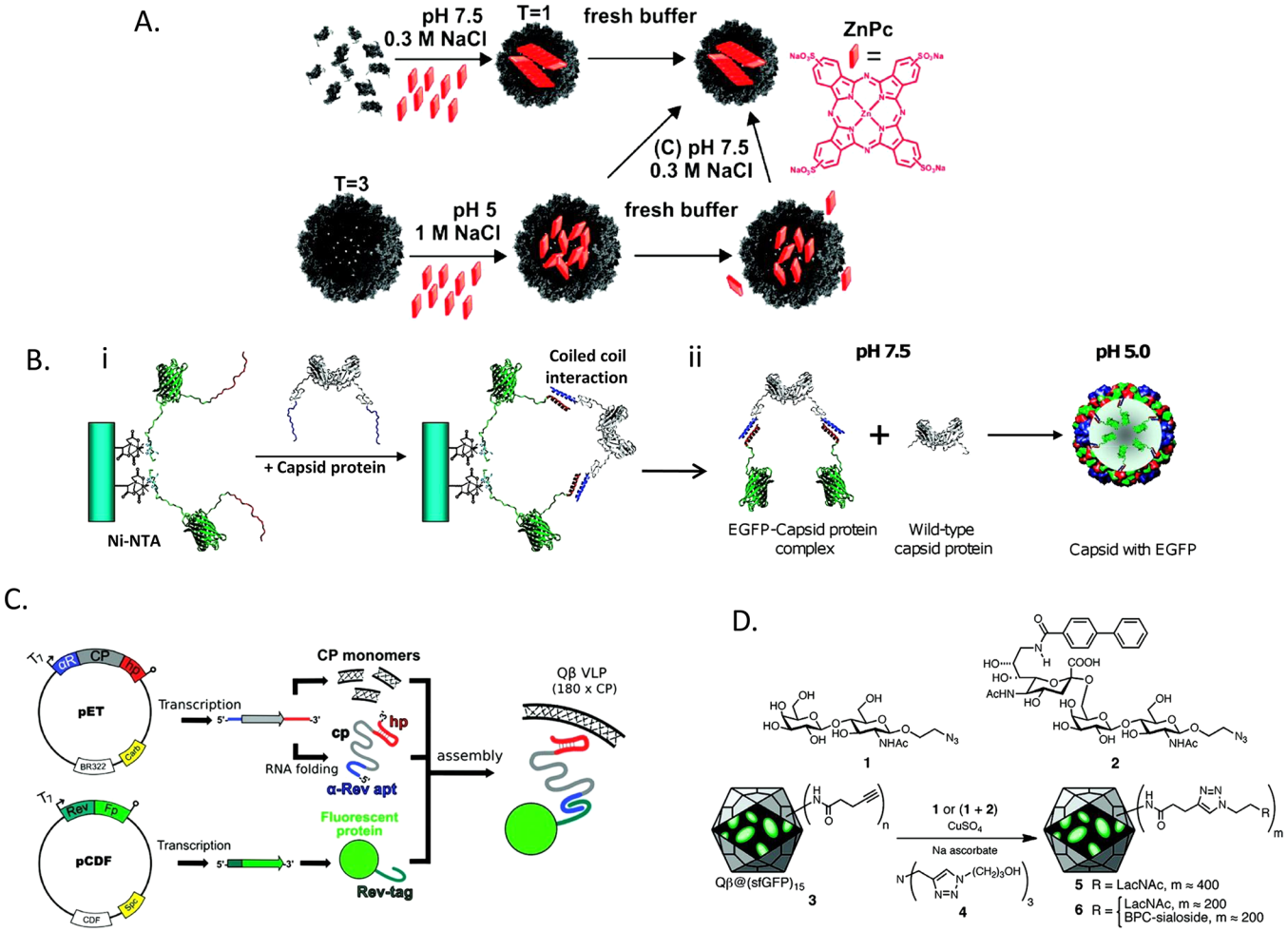

Figure 71. Viral modification and utility. (A) Schematic representation of the encapsulation of ZnPc stacks into CCMV VLPs. The length and arrangement of the stacks within the capsids in the cartoon are tentative. The top method uses coassembly while the bottom relies on infusion. (B) (i) Purification of the eGFP−capsid protein complex. Bacterial lysate containing the eGFP is added to a Ni−NTA column. Only eGFP binds to the Ni−NTA with its N-terminal histidine tag. A wash step removes all other proteins that lack the histidine tag. The lysate containing the capsid protein is then added. The capsid protein binds to the C-terminal coiled coil (red) of the eGFP with its N-terminal coiled coil (blue). After another wash step, the entire complex is eluted from the column using an excess of imidazole. (ii) Schematic representation of eGFP encapsulation. The eGFP−capsid protein complex is mixed with wild-type capsid protein at pH 7.5 and subsequently dialyzed to pH 5.0 to induce capsid formation. (C) Schematic of the technique used to package protein inside Qβ VLPs. Compatible T7 expression vectors drive expression of capsid protein (CP), Rev-tagged cargo, and bifunctional mRNA. The Rev-tag binds to the α-Rev aptamer (apt), and Qβ genome packaging hairpin (hp) binds to the interior of the CP monomers, tethering the cargo to the interior of the VLP. (D) Derivatization of Qβ with encapsidated GFP by glycan ligands LacNAc (using **1**) and a derivative of sialic acid (using **2**) with CuAAC. Reprinted with permission from refs 1513, 1533, and 1535, Copyright 2009, 2011 American Chemical Society.

more sophisticated bioconjugation protocol. Here, the target protein was expressed with a sequential intein tag and chitin binding domain, the latter of which allows for immobilization on a chitin affinity column during purification. Intein-mediated thiolysis released the target protein from the column, leaving a C-terminal thioester group that was coupled, via NCL, to a heterobifunctional peptide linker that also provided a terminal aminooxy group. This latter group then underwent an aniline-catalyzed oxime ligation to FHV displaying benzaldehydes on engineered lysine residues introduced for just this modification. In the reduction to practice, approximately 100 MBP were covalently coupled per FHV. The resulting bioconjugates produced a significantly higher anti-MBP response in immunized mice relative to monomeric MBP and FHV−MBP bioconjugates formed with noncovalent chemistry.

There have been other examples of chemistries that, although applied to VNPs, were not originally developed for peptide ligation. Nonetheless, many of these can still be described as a chemoselective ligation. For example, the Francis group incorporated a modified hetero-Diels−Alder reaction into a four-step, 4 h process for site-selectively labeling the interior surfaces of hollow viral capsids.[260] Capsids of bacteriophage MS2 were used in this study since they display 32 pores, each 1.8 nm in diameter, allowing chemical access to the interior volume. The capsids were first emptied of the RNA genome using an alkaline hydrolysis step. The 180 native tyrosine residues on the interior surface of the capsids were then selectively and efficiently modified via diazonium-coupling reactions, and the resulting azo bond was further reduced with $Na_2S_2O_4$ followed by $NaIO_4$ oxidation to yield a corresponding number of *o*-imino-quinone groups. The reactivity of this group was then verified by treatment with acrylamide, a dienophile that formed a covalent bond via the hetero-Diels−Alder reaction at room temperature. This functionalization approach left the

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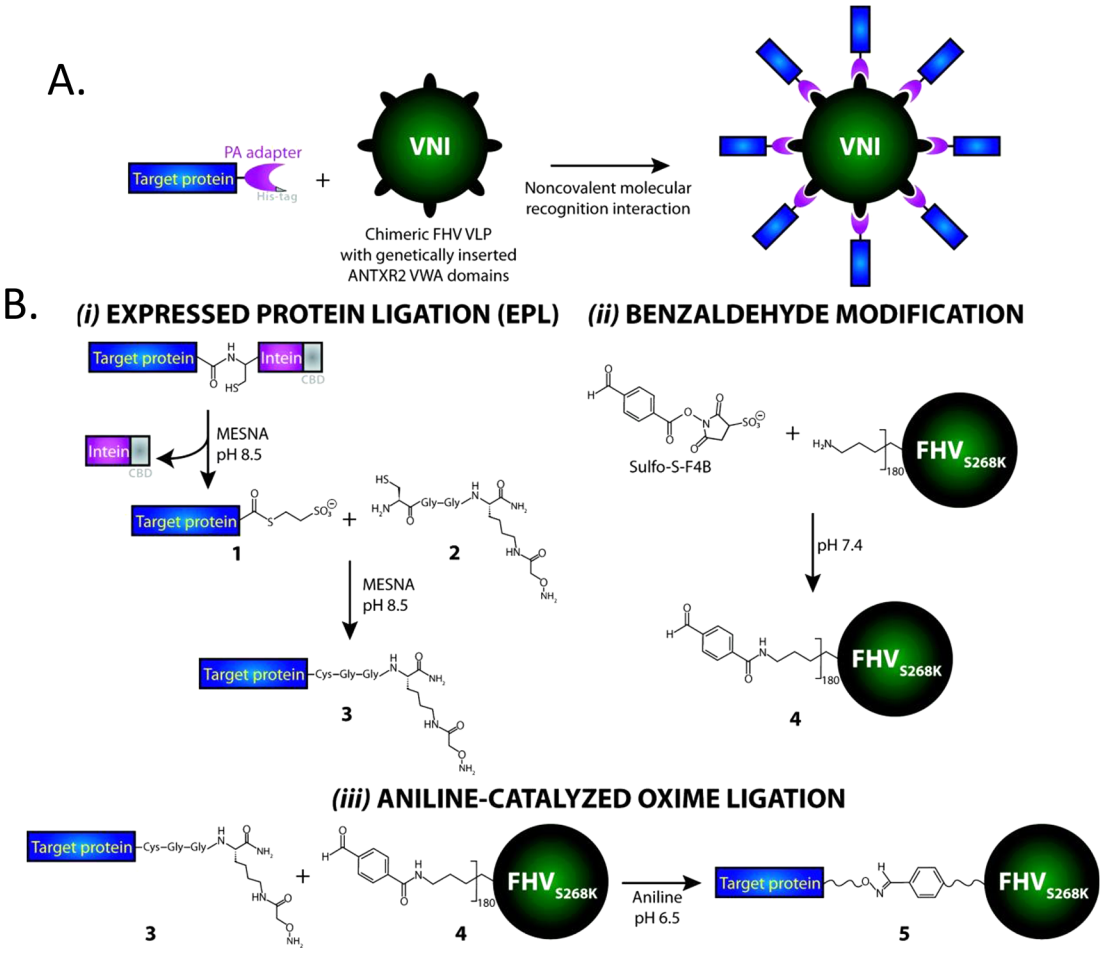

Figure 72. Modification strategies allowing orientation- and site-specific presentation of whole proteins on FHV-based NPs. (A) Noncovalent attachment: Target protein is expressed as a fusion with a fragment of protective antigen (PA) required for binding to ANTXR2. A C-terminal His_6 allows purification via Ni-affinity chromatography. Multivalent display is achieved by binding of the fusion protein to VNIs, which display 180 copies of ANTXR2 on their surface. (B) Covalent attachment: Target protein is expressed with an intein tag and immobilized on a chitin affinity column via a chitin-binding domain (CBD). Subsequent to intein-mediated thiolysis, the target protein is released from the intein with a C-terminal thioester group **1**, which is then conjugated via NCL to a heterobifunctional peptide linker **2** to generate an aminooxy-derivatized product **3**. Coupling of this product to FHV requires benzaldehyde groups on derivatized FHV mutant S286K containing 180 surface-exposed lysines, **4**. An aniline-catalyzed oxime ligation reaction between products **3** and **4** yields FHV-based NPs displaying multiple copies of the target protein **5**. Reprinted with permission from ref 1515. Copyright 2011 American Chemical Society.

cysteine residues located on the capsid's exterior surface available for further functionalization.[260]

4.2.5.6. Modifying Viruses with Other Nanoparticle Materials. The symmetrical, three-dimensional nanoscale architectures inherent to viral capsids has stimulated strong interest for achieving programmable and templated electronic devices at the sublithographic regime by exploiting the virus as a molecular scaffold.[1475,1486] This goal, in turn, initiated interest in displaying inorganic, metallic, and carbonaceous NPs on the viral templates due to the inherent quantum-confined optical, electrical, or contrasting properties these materials can provide to the resulting bioconjugate. Similar to the history of VNP bioconjugation, the approaches began with utilization of available protein-derived covalent-labeling chemistries and are now evolving to incorporate encapsulation and far more complex coupling strategies.

In perhaps the simplest approach, the Johnson group utilized commercial NHS-ester activated ~1.4 nM Nanogold AuNPs to site-specifically label the lysine residues groups on CPMV.[1489] Monomaleimido-functionalized Nanogold AuNPs were similarly coupled at near stoichiometric ratios to the thiols in the recombinant GGCGG loop placed into CPMV.[1525,1536] EDC chemistry is another commonly utilized approach. Portney et al. utilized EDC to attach carboxylated SWCNTs and QDs to FHV and CPMV, respectively.[1537] For both of the latter materials, control over labeling stoichiometry was not an issue since the goal was to investigate the formation of cross-linked networks rather than monodispersed conjugates of controlled valency. Cheon's group utilized sulfo-SMCC linkers to couple lysine residues on adenovirus to the thiols on dimercapto-succinic acid stabilized manganese-doped magnetism-engineered iron oxide (MnMEIO) NPs, see Figure 73A.[43] Use of a large stoichiometric excess of MnMEIO NPs helped bypass virus-to-virus cross-linking issues during the reactions. Antibody-mediated recognition is another approach that has been exploited for VNP display. For example, biotin moieties introduced on the *Sulfolobus islandicus* rod-shaped virus 2 surface using direct EDC chemistry were subsequently decorated using gold-labeled anti-biotin antibodies.[1516] This approach mitigates potential cross-linking issues if gold-labeled quadravalent avidin had been used.

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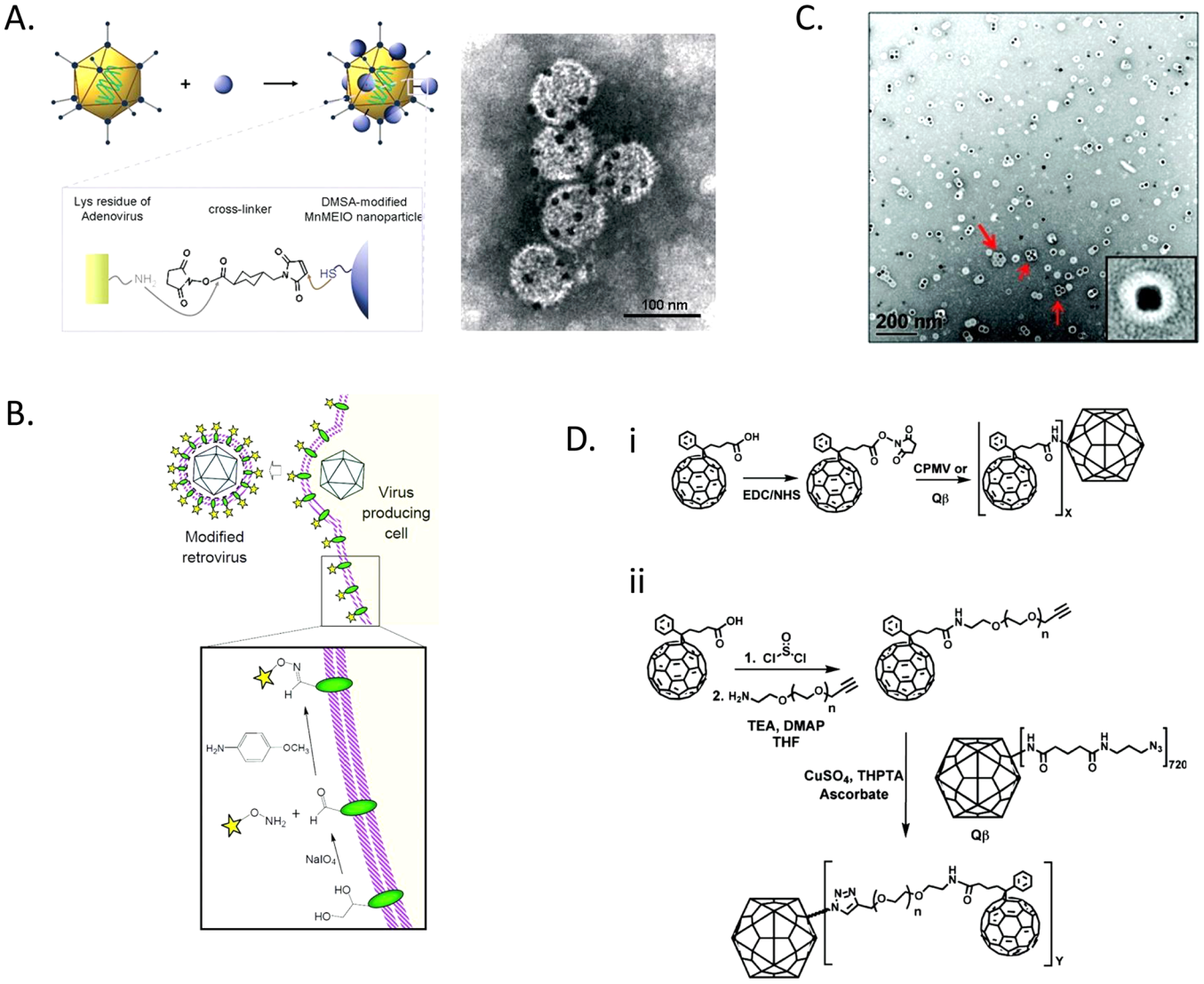

Figure 73. Hybrid virus-NP materials. (A) Schematic of the formation of adenovirus−MnMEIO hybrid NPs using sulfo-SMCC. Reprinted from ref 43 with permission. Copyright 2007 Wiley-VCH GmbH & Co. KGaA, Weinheim. (B) Production of functionalized retrovirus by bioorthogonally modifying virus producing cell surface whose sialylated glycoproteins (green) on the phospholipid cell membrane (purple) are oxidized to generate aldehyde groups using sodium periodate. Aminooxy-functionalized molecules (yellow) are conjugated with aldehyde groups via oxime linkages in the presence of *p*-anisidine (catalyst). (C) Negatively stained TEM image of VNPs formed by self-assembling BMV proteins around the 18.6 nm cubic NPs coated with HOOC-PEG-PL. The dark spots are IONPs. Light rings around NPs are BMV shells including the HOOC-PEG-PL shells. The red arrows illustrate possibly preassembled VNP clusters. Inset shows a higher magnification image of a single VNP. (D) Derivatization of CPMV and Qβ with C_{60}. Derivatization of Qβ with propargyl-O-PEG−C60; THPTA = tris(3-hydroxypropyl-4-triazolylmethyl)amine as an accelerating Cu-binding ligand. Panels B−D Reprinted with permission from refs 1511, 1517, and 1540. Copyright 2009, 2011 American Chemical Society.

AuNPs can also be directly chemisorbed to the surface of cysteine-modified VNPs. Blum et al. utilized three different cysteine-modified CPMV mutants and examined the ability of 2 and 5 nm AuNPs to self-assemble to the thiolated capsids over time and yield different functionalization patterns as visualized by TEM.[1492] This approach also formed the basis for functionalizing a CPMV scaffold with AuNPs that were subsequently connected with thiol-terminated organic molecules to provide a three-dimensional conductive network capable of sensing biotin−avidin interactions. Once the conducting network was assembled, the lysine groups on the CPMV surface were modified with biotin, and the resulting nanosensors were assembled onto electrode substrates. Binding of the biotin binding proteins, avidin, NeutrAvidin, or streptavidin, resulted in a perturbation of the network and a measurable change in the conductance of the system.[1538]

Similar approaches have also been used to functionalize VNPs with semiconductor QDs. His_6-expressing CPMV was immobilized on a NeutrAvidin-coated surface using a biotin−NTA linker. Anionic and biotinylated QDs were electrostatically attached to an avidin coating on the upper surface of the CPMV or, alternatively, via biotin−avidin binding, respectively.[1539] A derivative of this technique used EDC to attach short His_6-appended peptides to lysine residues on the CPMV surface; the exposed His_6-motifs were then able to directly coordinate to the Zn-rich surface of CdSe/ZnS QDs.[623,1493] The latter approach overcame the inability of His_6-motifs directly expressed on the viral surface to accomplish the same conjugation, a result ascribed to lack of accessibility and bulk repulsion effects.

Functionalization of VNPs with magnetic NPs can facilitate separation and purification during bioconjugate preparation and enhance contrast in MRI applications. For example, Wong and

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Kwon began with Moloney murine leukemia viruses cultured in 293-GPG cells, which displayed vesicular stomatitis virus glycoprotein (VSV-G) as an envelope protein.[1517] Sialylated glycoproteins on the (live) host cell membrane were modified by oxidation to aldehydes with sodium periodate. Aminooxy-biotin was added in the presence of a *p*-anisidine catalyst to complete the oxime ligation, and excess reactants were washed away. Virion formation and detachment from these cells resulted in membrane incorporation and display of the desired functional groups on the retroviral envelope, see Figure 73B. Anti-biotin magnetic particles were then attached to the virus allowing ∼90% purification efficiency. Using a different approach, Huang et al. demonstrated that ∼19 nm cubic IONPs could be efficiently functionalized with phospholipids containing PEG tails and terminal carboxyl groups to allow for subsequent self-assembly of BMV capsid protein shells, yielding hybrid encapsulated structures with a mean diameter of ∼37 nm.[1540] The authors noted that the VLP maintained a quasi-spherical appearance despite its predominantly cubic cargo, and this was attributed to a combination of the capsid's rigid exterior surface and the flexibility of the termini of the constituent proteins as they interacted with the Fe cube in the interior, see Figure 73C. In preliminary experiments, the functionalized particles demonstrated deep penetration in plant leaves along with good MRI contrast.

More recently, Steinmetz and co-workers used two covalent methods to attach C_{60} to the surface of CPMV and the bacteriophage Qβ.[1511] Both methods start with a carboxylic acid derivative of C_{60}, [6,6]-phenyl-C_{61}-butyric acid, that was either activated by EDC/NHS and coupled to the capsid lysine residues or converted to an alkyne as propargyl-O-PEG−C_{60}, where the CuAAC reaction was used to couple to NHS−azide-modified viral capsids, see Figure 73D. Click chemistry was found to provide superior C_{60} loading on bacteriophage Qβ capsids with ∼30−50 per virus compared with ∼3 per virus for EDC chemistry. Such conjugates have potential as photosensitizers for treatment of inflammation, and preliminary experiments confirmed that they could be delivered to HeLa cells. This is a remarkable demonstration of how two very different NPs can function together in a biological environment. This example also highlights Qβ capacity to carry insoluble cargos in particular since C_{60} has almost no inherent aqueous solubility.

Overall, the bioconjugation of viruses and development of modified VNPs will continue to expand. The complexity of the structures assembled will directly reflect the capabilities of the chemistries utilized. Three areas will see the most development in the near term: (1) modification of viruses and capsids with other NPs; (2) the development of combined contrast agent and drug delivery (i.e., theranostic) platforms based on VNPs; and (3) the development of new materials of viral origin. This prediction is strengthened by recent examples of VNPs delivering complex cargos to cells, as well as the application of new conjugate chemistries, such as an atom transfer radical polymerization that builds from CuAAC-modified sites on viruses to create virus-supported polymers.[1505,1510] It is also clear that these biological nanoplatforms are capable of far more complex and multipronged chemical modifications than has been achieved to date. However, one continuing limitation, that currently affects the widespread use of viral capsid NP technology, is the apparent lack of commercial sources for the capsid materials. Most researchers in the field either synthesize their own material, including mutant varieties, or collaborate closely with research groups equipped to produce such capsid materials in bulk. Furthermore, despite their many architectural advantages, the inherent biological nature of the capsid still predisposes them to many of the natural limitations exhibited by other biological systems: temperature, solvent sensitivity, and degradation by proteases being among the most prominent. That said, only a very small subset of known viruses have been extensively studied, and the recent discovery of thermophilic viruses may address some of these concerns.[1516]

4.2.6. Light Harvesting Complexes. The structural diversity and functional complexity found in the superfamily of plant, bacterial, and algal light harvesting protein complexes is beyond the scope of this review. However, several have found extended use as bioconjugable fluorophores, and their large physical size and high molecular weight essentially qualify them as protein-based NPs. For brevity and illustrative purposes, we only focus on the more common and widely available phycobiliproteins, including β-phycoerythrin (β-PE; MW ≈ 240 kDa; ε_{max} = 2 410 000 cm^{-1} M^{-1}; QY = 0.98), *R*-phycoerythrin (MW ≈ 240 kDa; ε_{max} = 1 960 000 cm^{-1} M^{-1}; QY = 0.82), and allophycocyanin (MW ≈ 104 kDa; ε_{max} = 700 000 cm^{-1} M^{-1}; QY = 0.68).[170] These highly soluble fluorescent proteins originate from algae and cyanobacteria where they function to collect light and direct it to the photosynthetic reaction center.[1541,1542] Fluorescence arises from linearly arranged tetrapyrrole chromophores that are covalently linked to the protein backbone, which minimizes effects of external changes in pH or salt. Evolutionary pressure has maximized the intracellular synthesis of phycobiliproteins, which can reach 40% of the total cell protein content, and also imbued the phycobiliproteins with extended stability and impressive absorbance and fluorescence properties.[170,1542] This combination, along with their amenability to (bio)conjugation, has led to their systematic use as an extremely bright fluorophore that can be stably conjugated to various other biologicals, using standard chemistry, for use in bioassays. From a functional and structural perspective, phycobiliproteins can be described as a protein platform analog of a dye-doped or dye-impregnated NP.

Early work by Glazer at Berkeley extensively characterized these protein complexes and further confirmed that they could be recombinantly expressed and engineered to prevent dissociation along with amenability to different types of bioconjugation.[1543] This included, for example, a phycocyanin fusion with a 114-residue biotin carboxyl carrier protein, allowing for intracellular biotinylation during expression, and an N-terminal His_6 sequence, which could also be site-specifically removed with tobacco etch virus protease. Currently, a wide variety of conjugated phycobiliprotein derivatives are commercially available (primarily from Life Technologies) encompassing standard avidin, NeutrAvidin, and SA functionalized β-PE, *R*-phycoerythrin, and allophycocyanin complexes, along with various antibody linked versions that target primary antibodies. Further, phycobiliprotein constructs modified with longer wavelength fluorescent acceptor dyes are also available and can allow for multiplexing because they exploit FRET to shift emissions to the red. An activated *R*-phycoerythrin pyridyldisulfide derivative can also be obtained and allows direct linkage to thiols on antibodies or other molecules by disulfide exchange.[170] The most widespread utility is in immunoassays where, for example, Sun et al. used *R*-phycoerythrin−SA to quantitate aldosterone and testosterone by conjugation to the cognate antibodies in multiplexed formats.[1544] Goldman et al. used the same conjugate to visualize biotinylated phage-displayed single domain antibodies that were specific for the biothreat agents ricin

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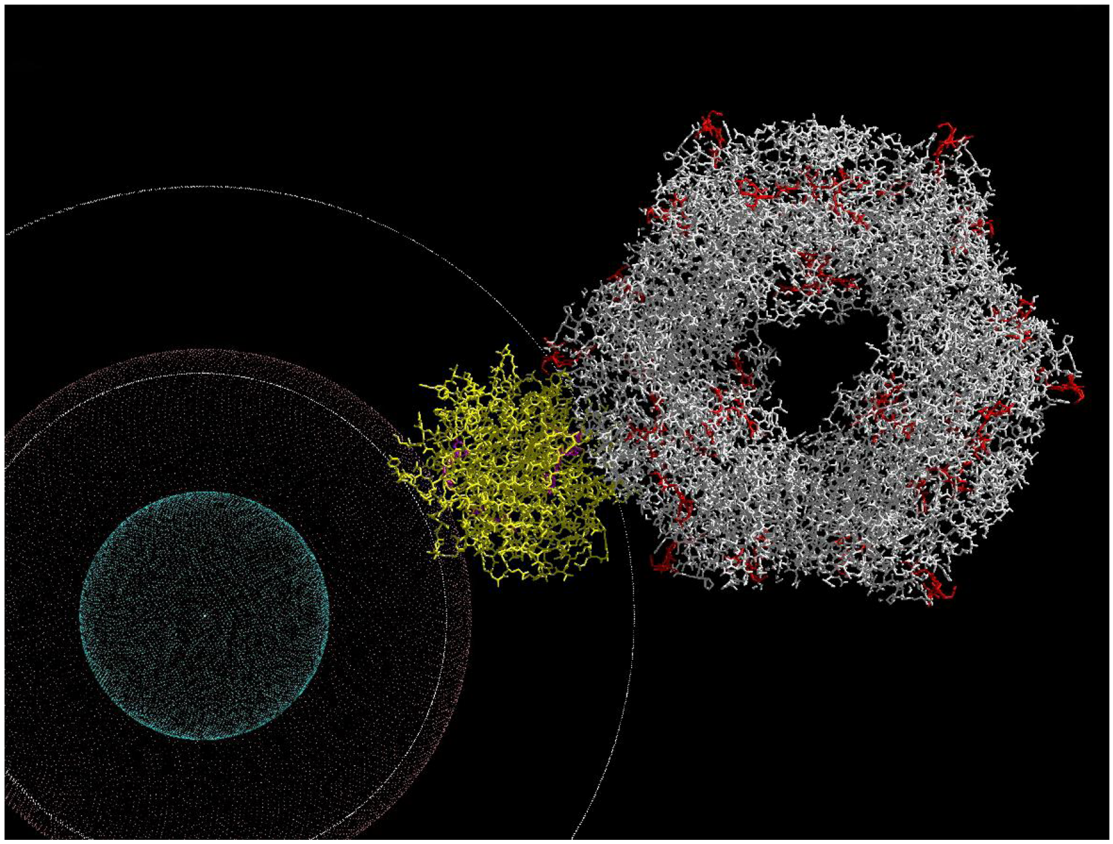

Figure 74. Model of the QD−β-PE conjugate structure in an extended conformation. The central QD with a radius of ∼29 Å shown in blue is surrounded by a crimson shell of ∼25 Å thickness representing the DHLA−PEG−biotin ligand solubilizing layer. The intermediary SA protein is shown in yellow with biotin binding sites highlighted in purple. The β-PE ring structure is shown in white, with the multiple chromophores highlighted in red. The inner concentric white circle corresponds to the predicted 53 Å Förster distance (R_0) for this QD donor/B-PE acceptor pair. The second outer white circle is a visual distance marker set at ∼95 Å from the QD center and represents the closest predicted approach of the β-PE to the QD. Reprinted with permission from ref 199. Copyright 2009 American Chemical Society.

and botulinum neurotoxin A.[1545] Similar chemistries have also been used to visualize biotinylated DNA in array formats.[1546]

To date, only a few reports have appeared where these protein complexes have been coupled to other NP materials. SA−β-PE was coassembled onto QDs functionalized with biotin-terminated PEG ligands along with His_6-appended CPP for cellular delivery.[99,198] In this demonstration, the QDs were able to facilitate cellular uptake of a protein cargo that exceeded 10^3 kDa in a relatively nontoxic manner. The separate QD and β-PE emission allowed tracking of both entities and confirmed that the assembly was still intact following delivery. The FRET between the QD−biotin (donor) and β-PE (acceptor) within the conjugates was also extensively probed, and estimates of the separation distances allowed modeling of the overall assembly structure. Figure 74 shows a comparison of the size of β-PE to the QD and also highlights the large distances over which viable FRET was observed.[199] The extended range of FRET observed in these conjugates resulted almost exclusively from the enormous extinction coefficient of the β-PE, which is 20−30 times larger than that of most organic dyes. Although clearly amenable to EDC chemistry, the formation of β-PE bioconjugates in this manner appears to be much rarer. This is probably due to a combination of the wide availability of avidin conjugates and the vast number of amine and carboxyl groups present on β-PE, which can promote heterogeneous, nonfunctional orientations, and cross-linking in an EDC reaction. Similar bioconjugation strategies should be generally applicable to other light-harvesting protein complexes. The principle caveats being that the proteins remain stable, soluble, and strongly fluorescent and do not dissociate at low concentrations, although mild chemical cross-linking may help alleviate the last issue.

4.3. Synthetic Polymer Nanoparticles

The field of polymeric NPs is large and complex and encompasses both classical polymeric materials and dendritic-type structures. Once again, the driving force behind development of these NPs has been their application for drug or gene delivery, which has been the subject of a number of excellent reviews.[1547−1552] In general, polymer NP-based drug delivery systems are designed to improve the solubility, stability, bioavailability, release, and other pharmacokinetics of a drug while concurrently reducing its toxic effects through lowering the required dose or specifically targeting the therapeutic site. In contrast to many of the other NP materials reviewed here, the unique capabilities of polymer NPs are not intrinsically unique to the selection of a material (c.f. metal or semiconductor NPs) but are rather derived from the chemical and structural diversity possible through different monomers, copolymerization, block modularity, and other classic organic synthetic techniques. This includes tailoring the NP to be biodegradable or hydrophobic or hydrophilic in nature, as well as introducing stimulus-responsive mechanisms, such as swelling, dissociation, or other responses to temperature, pH, or enzyme activity, along with other suitable environmental

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triggers for drug release. In general, polymer NPs have an advantage over lipid NPs in that they can be designed to be structurally stable even at dilute concentrations. While linear and branched polymers have been used for drug and gene delivery,[1552] NP formulations typically offer higher drug loading capabilities, greater protection from the surrounding physiological environment, and more control over sustained release. The vast scope of this field and its inherent complexity allow for only a few representative chemistries to be presented highlighting contemporary approaches. Given the flexible chemistry of these materials, it is also important to note that almost any bioconjugation strategy is potentially feasible by incorporation or interconversion to provide appropriate starting or reactive groups.

4.3.1. Classic Polymer Nanoparticles. Polymer NPs can be prepared from a range of monomer and polymer starting materials, both hydrophobic and hydrophilic, using a range of techniques, as recently reviewed.[1549,1553] Commonly selected hydrophobic materials include polyesters such as poly(lactic acid) (PLA), poly(glycolic acid), or poly(lactic-*co*-glycolic acid) (PLGA). Other common materials include poly(vinyl chloride) (PVC) and polystyrene (PS). Grafting carboxylic acids to their structures improves hydrogen bonding and results in some water solubility, especially at low MW. It is important to note that PLGA, along with several other related materials, are already in certain medical devices that have received FDA approval. Other common hydrophilic materials for NPs include PEI, poly(amido amine), poly(lysine), poly(*N*-isopropylacrylamide) (pNIPAm), poly(methyl methacrylate) (PMMA), poly(alkylcyanoacrylate), and PEG. While polymer NPs represent the most common structure used to date, polymeric nanotubes, nanorods, and nanofibers have also been prepared and have numerous potential applications in biomedicine.[1554−1556] One significant advantage inherent to polymeric NP development is the commercial availability of many monomer and polymer starting materials.

4.3.1.1. Hydrophobic Polymer Nanomaterials. NPs formed from hydrophobic polymers are typically prepared using either precipitation of preformed polymers or monomer polymerization. Preformed polymer precipitation methods include emulsion/solvent evaporation, solvent displacement, salt-induced desolvation (salting out), supercritical fluid carbon dioxide technology, and supercritical antisolvent precipitation.[1158] Monomer polycondensation reactions carried out in an aqueous phase emulsion have also been used.[1549] Surfactant molecules are often added to facilitate NP formation and to provide subsequent stability in aqueous solutions.[1549] Such NP materials alone can be directly used as contrast agents in ultrasound imaging techniques[1557] and Raman spectroscopy.[1558] Liu and co-workers used PLA NPs to directly image breast cancer cells with ultrasound.[1557] The PLA NPs were functionalized with an anti-HER2 antibody immobilized onto the surface carboxylic acids via EDC/NHS chemistry. HER2 positive cells demonstrated high staining with these antibody-modified NPs when imaged using high-resolution ultrasound. The inherent hydrophobic nature of these NPs has led to them being routinely used to encapsulate hydrophobic drugs and dyes within their core.[1549,1559,1560] In most cases the drugs or dyes are incorporated into the organic phase, with the preformed polymer or monomer, and then encapsulated during the subsequent synthetic steps. For example, a lipophilic fluorescent Bodipy dye,[1559] DOX,[1561] and the photosensitizer *meso*-tetraphenylporpholactol[1562] have all been encapsulated into PLGA NPs, while incorporating chromoionopores and ionophores into PVC NPs provided for ion-selective nanosensors.[1563] Helle and co-workers studying model drug encapsulation in PLA NP cores found that encapsulation efficiency was dependent on the hydrophobicity of the drug with the more hydrophilic model drugs (salbutamol sulfate and sodium cromoglycate) less efficiently encapsulated than the hydrophobic model drug beclomethasone dipropionate.[1560] Meena et al. encapsulated atorvastatin-Ca in PLGA NPs using a cosolvent emulsion−diffusion−evaporation method with two stabilizers, didodecyl dimethyl ammonium bromide and vitamin E tocopheryl poly(ethylene glycol) 1000 succinate (Vit E-TPGS).[1156] They observed that the NP formulations had similar efficacy as the nonparticulate formulation, albeit with a 66% lower dosage in hyperlipidemic Sprague−Dawley rats that were fed a high-fat diet. Results were based on decreases in blood glucose, triglyceride, and low-density lipoprotein (LDL) levels and increased high density lipoprotein (HDL) levels. Furthermore, plasma creatine and blood urea nitrogen levels, indicative of muscle and kidney toxicity and renal failure, respectively, were elevated in rats treated with commercial avorstatin but not in rats treated with the NP drug formulation. This indicates that NP formulations of avorstatin may mitigate some toxicity associated with this drug.[1156]

Lipid-based molecules and copolymers are commonly used to further modify the surface of polymeric NPs and introduce additional functionalities, such as PEG groups[1564] or PVC,[1563] to improve biocompatibility or, alternatively, reactive-ester groups that mediate antibody immobilization via amine targeting.[1565] Andersson and co-workers used a triblock Pluronic F-108 copolymer [$(PEO)_{129}$−$(PPO)_{56}$−$(PEO)_{129}$], modified with a pyridyl disulfide group to functionalize polystyrene NPs with cysteine-terminated RGD peptides.[1559] Here, the central poly(propylene oxide) (PPO) block of the triblock copolymer was firmly coupled to the PS surface by hydrophobic interactions. Although studies were performed with micrometer-sized PLGA particles, Ratzinger and co-workers demonstrated that care must be taken when using these triblock pluronic copolymers to stabilize particle surfaces; too high a surfactant concentration directly affected the ability for future coupling of antibodies to the PLGA when EDC chemistry was used.[1566] Griset and co-workers prepared cross-linked NPs based on hydrophilic acrylate monomers whose hydroxyl groups were masked by an acid-labile protecting group rendering the monomers hydrophobic in nature.[1567] Drug-loaded NPs were then synthesized using mini-emulsion polymerization, with PAX and the monomer present in the organic phase. Exposure to a mildly acidic environment (pH ≈ 5), for example, in tumor cells, resulted in cleavage of the hydroxyl protecting group on the polymer generating a hydrophobic-to-hydrophilic transition in the NP, which caused the polymer to expand and release the entrapped drug. In a derivative approach, Park and co-workers functionalized DOX-loaded PLGA NPs with a gold half-shell to generate a combined thermo-chemotherapeutic agent, which then provided rapid thermally induced drug delivery to exposed HeLa cells.[1561]

4.3.1.2. Hydrophilic/Hydrophobic Polymer Nanomaterials. In some instances, a combination of hydrophobic and hydrophilic monomers are used for NP preparation.[1568−1570] Lu and co-workers encapsulated fluorescein during the synthesis of poly(styrene−acrylamide−acrylic acid) NPs using soapless emulsion polymerization.[1570] The NPs contained terminal carboxylic acid and amide functional groups. The amide group was first converted to a hydrazide, then to an acyl azide, which allowed it to act as a unique handle to functionalize the NPs with proteins such as BSA or an anti-human chorionic gonadotropin antibody. Alternatively, the precipitation of hydrophobic

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PLA and hydrophilic PEG chains was found to be an efficient method of encapsulating insulin[1571] and also prostaglandin E_1, which is effective in the treatment of peripheral vascular diseases.[1572,1573]

4.3.1.3. Hydrophilic Polymer Nanomaterials. Hydrophilic or semihydrophilic polymers are often favored over their hydrophobic counterparts since aqueous solubility reduces the need for organic solvents, which may induce structural denaturation or degradation to labile biomolecules. NP synthesis from hydrophilic starting materials can occur by cross-linking preformed polymer chains using chemical or physical processes, polymerization of monomers, or self-assembling of block copolymers.[1547,1549,1550,1553,1574,1575] Chemical cross-linking of either preformed polymers or larger monomers are popular methods for preparing polymer NPs and networks of cross-linked polymers that swell in the presence of water, commonly referred to as hydrogels.[1550] Polyacrylamide hydrogels represent a popular class of polymeric NPs and have been used to encapsulate a range of fluorescent dyes (both pre- and postpolymerization),[1576,1577] drugs,[1578] and proteins.[1579] Geo and co-workers produced biodegradable NPs based on acrylamide monomers copolymerized with biodegradable glycerol dimethacrylate cross-linker monomers.[1577] Propidium iodide dye was further included in the microemulsion-based chain transfer polymerization synthetic reaction, and the resulting materials rapidly degraded when microinjected into the cytoplasm of mouse macrophages releasing the encapsulated dye, as visualized through the resulting nuclear staining. The Lahann group have produced some unique biphasic NPs using side-by-side electrified cojetting of mixtures of two aqueous polymer solutions, poly(acrylamide-*co*-acrylic acid) and PAA, followed by thermal cross-linking via imidization.[1576,1580] The resulting biphasic materials, known as Janus particles, have two distinct hemispheres that can be independently modified with biomolecules or fluorescent dyes.[1576,1580] Fluorescent dyes such as propidium iodide, FITC−dextran, or rhodamine-B-dextran, the ligand biotin−dextran, and annexin V protein were all encapsulated into the polymers by incorporating them into the aqueous polymer solution prior to the cojetting process. The biphasic NP nature was confirmed by confocal laser scanning microscopy and flow cytometry, and the binding of TRITC-labeled SA to biotin-modified hemispheres was also demonstrated.[1576] Core−shell-type structures can also be readily synthesized using similar polymer materials. For example, Blackburn used acrylamide-based hydrogel NP cores as seeds for the addition of a hydrogel shell comprising acrylamide monomers and aminopropyl methacrylate.[1581] The amine groups in the polymer shell were converted to maleimide-functional groups by EDC-coupling with ϵ-maleimidocaproic acid. Cysteine-containing Eph2A receptor (overexpressed in ovarian cancer cell lines) targeting peptides were then attached to the NP surface with standard maleimide−thiol chemistry. siRNA was then encapsulated into the peptide-functionalized NPs using a "breathing-in" method, in which the polymer NPs are first lyophilized and then rehydrated in a volume of water containing the siRNA, which is almost completely imbibed as the NPs swell. The construct-mediated delivery of the siRNA successfully down-regulated the expression of EGF receptors on the target cell surface.

Acrylamide polymer shells have also been used to coat Au- or AgNPs with fluorescent dyes to generate water-soluble, plasmonically active fluorescent NMs, which were further functionalized with glucose and TAT-peptide for imaging and detection applications.[1582] Sexton and co-workers coated nonporous silica templates with various peptides and proteins, which were then encapsulated in a disulfide cross-linked polymethacrylate polymer using a LbL process.[1583] The silica-templated NPs were subsequently dissolved resulting in protein or peptide functionalized hydrogel capsules with strong potential for vaccine delivery applications. In addition to chemical-based cross-linking, physical-based methods have also been used, and in fact, the electrostatic interaction between plasmid DNA and heparin or positively charged polymers, such as poly(lysine), poly(β-amino) esters, and poly(urethane-*co*-ester), is a common method of producing such conjugated NPs.[148,1584−1586] Biodegradable DNA−polymer constructs are typically preferred for gene delivery applications due to their inherent ability to facilitate gene release.[1584−1586] Plush and co-workers prepared nanoassembled capsules in which Gd−DOTP chelates were first assembled with various cationic polymers, including polyallylamine, poly(L-arginine) and poly(L-lysine), and the resulting constructs were stabilized by the addition of negatively charged SiO_2 NPs.[1587] The resulting capsules could be varied in size from 200 nm to 5 μm, although the smaller sizes were found to provide the best MRI contrast. Although not explicitly demonstrated here, the silica shell could be further modified with functional groups via silane chemistry allowing additional functionalities to be attached.

Interestingly, polymer NPs have also been proposed as plastic antibody mimics using imprinted polymer techniques. Hoshino and co-workers used a range of acrylamide monomers added to the target protein prior to polymerization to generate imprinted polymer NPs selective for the biotoxin melittin.[1588] Optimization of the monomer composition was found to play a critical role in developing high target selectivity in the resulting NPs and offers an alternative to natural antibodies. Another potential use for polymeric NMs is the magnetically triggered release of drugs. For example, Hoare and co-workers developed an ethyl cellulose membrane containing both SP magnetite NPs and thermosensitive pNIPAm nanogels (prepared using polymerization of a combination of acrylamide monomers).[1589] The resulting nanogels were designed to swell at physiological conditions (37 °C) resulting in a drug-impermeable membrane. Application of an oscillating magnetic field caused the magnetic NPs to inductively heat the surrounding membrane causing the nanogels to shrink and the drug to readily travel through the membrane.

4.3.1.4. Self-Assembling Polymer Nanomaterials. One of the most active fields of polymer NP research is that encompassing polymeric micelles. These core−shell structures form through the self-assembly of amphiphilic copolymers and have been extensively reviewed.[1547−1549,1553,1574,1590,1591] Typical di- or triblock copolymers used for micellar NP formation have a hydrophilic region (e.g., PEG) and a second region that forms the NP core through hydrophobic/amphiphilic, electrostatic, metal complexation, or hydrogen bonding interactions.[1574] Given optimal conditions, these copolymers will spontaneously self-assemble to form micelle or vesicle structures in aqueous solution.[1553,1591,1592] Although micellar structures appear more popular in the literature, vesicular structures have the added advantage of containing a hydrophilic reservoir at their core, allowing encapsulation of both hydrophilic (core) and hydrophobic (shell) species.[1592−1594] Popular examples of diblock copolymers include PEG−polypeptide and PEG−polyester derivatives, mainly due to their biocompatible and biodegradable nature.[1547,1553,1574] In one

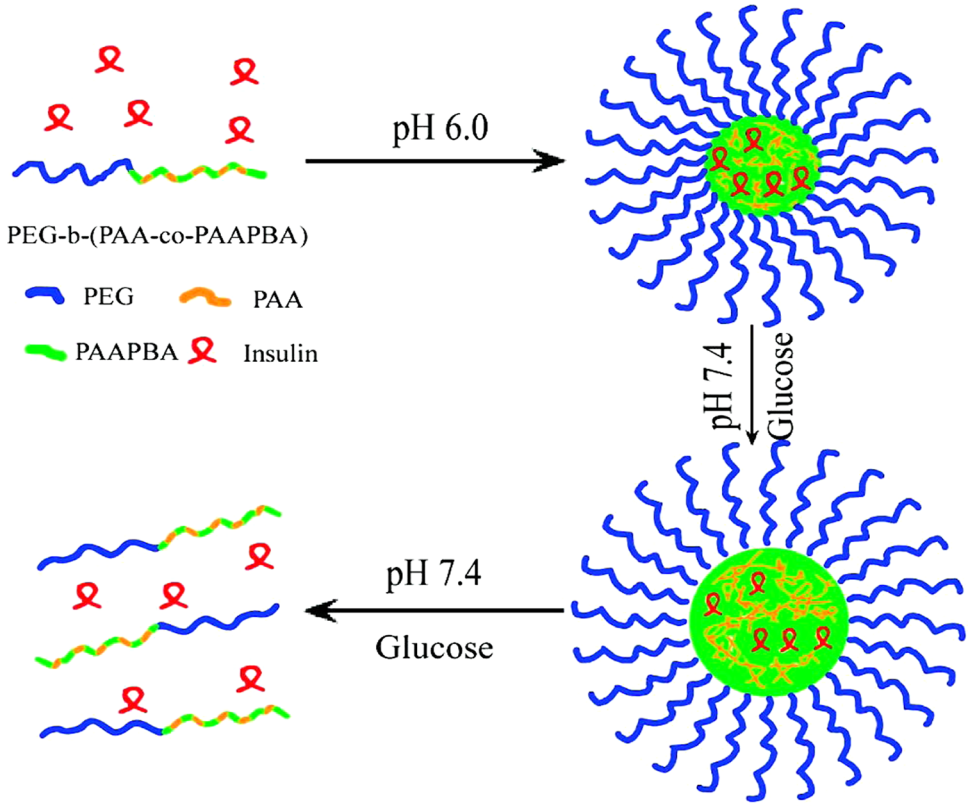

Figure 75. Schematic illustration of the formation, swelling, and disaggregation of insulin-loaded micelle and release of insulin from the micelle in response to glucose. Reprinted with permission from ref 1600. Copyright 2010 American Chemical Society.

promising example, Shi and co-workers demonstrated that PEG–PLA-based block copolymer (BCP) micelles were effective in the repair of spinal cord injuries in a rat system.[1595] Their approach focused on quickly sealing damaged axonal membranes with the self-assembled micelles. Injured spinal tissues treated with intravenously injected micelles were effectively repaired and recovered locomotive function without adverse effects and with minimal toxicity.

Due to the modular nature of BCPs, different functionalities can also be incorporated into the final NP design. For example, Vetvicka and co-workers designed PEO–*block*–poly(allyl glycidyl ether) diblock copolymers in which the hydrophobic poly(allyl glycidyl ether) core was functionalized with DOX covalently attached by a pH-labile hydrazone bond.[1596] The PEO outer layer of the resulting micelles extended circulation time allowing tumor penetration via the EPR effect, as demonstrated in model murine studies, and once inside the tumor, the lower pH facilitated subsequent DOX release. Amir and co-workers designed a stimulus-responsive block copolymer that was enzymatically triggered to self-assemble through conversion from a hydrophilic–hydrophilic to a hydrophilic–hydrophobic BCP.[1597] Song and co-workers prepared bright, stable phosphorescent BCP NPs for use in bioassays.[1598] Poly(vinyl chloride-*co*-ethyl acetate-*co*-maleic acid) and the phosphorescent platinum(II) tetra-*meso*-fluorophenylporphine (Pt-TMPFP) complex were mixed in water to form the NP. The surface carboxylic acid groups were then modified with C-reactive protein monoclonal antibodies using EDC chemistry to yield tracers for bioassays.

In other examples, Sun and co-workers designed a polymeric NP comprised of the triblock [monomethoxy poly(ethylene)]-*block*-[poly(ε-caprolactone)]-*block*-[poly(2-aminoethyl ethylene phosphate)] ([mPEG]-*b*-[PCL]-*b*-[PPEEA]).[1599] The central hydrophobic PCL block self-assembled to form the micelle core, leaving the hydrophilic mPEG and PPEEA blocks exposed to the aqueous environment. The mPEG block improved the biocompatibility of the resulting micellar NP, while the cationic PPEEA block interacted with siRNA by electrostatic interactions generating materials capable of gene delivery. Wang and co-workers designed glucose-responsive micelles for insulin delivery by incorporating a boronic acid functionality into a PEG-*block*-PAA diblock polymer.[1600] The PAA block was first modified with 3-aminophenylboronic acid using standard EDC chemistry, and the resulting self-assembled micelles comprised a PAA-phenylboronic core with a PEG hydrophilic shell. Insulin was incorporated into the acidic solution and drove the micellization process of these block copolymers. Addition of glucose caused a swelling of the core structure resulting in insulin release, the rate of which was dependent on the glucose concentration, see Figure 75 for a schematic.

As a functional alternative to having NPs systemically distributed throughout the body to accumulate in targets, some researchers have proposed polymeric micelle coatings on devices implanted in the direct vicinity of the therapeutic site as a method of controlled drug delivery.[1601−1603] Kim and co-workers loaded the anti-inflammatory drug diclofenac and the chemotherapeutic drug PAX into preformed BCP micelles of poly(2-vinyl ethylpyridinium)-*block*-poly(ε-caprolactone) (P2VEP-b-PCL), where the hydrophobic PCL block comprised the core.[1602] LbL assembly was then used to coat test surfaces with alternating layers of cationic drug-loaded micelles or the biodegradable polymer poly(β-amino ester) and anionic therapeutic

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agents such as heparin or dextran sulfate. The rate of degradation of these tetrafunctional surfaces was governed by the structural polymer layer of poly(β-amino ester), leading to controlled release of the drug-loaded micelles and the anionic macromolecules. As an alternative to a biodegradation mechanism of controlled release, Zhu and co-workers used temperature and sodium chloride to induce the release of pyrene from poly(*N*-vinylpyrrolidone)-*block*-poly(*N*-isopropylacrylamide) block copolymer micelles immobilized onto surfaces via LbL assembly with PMAA.[1603] Because micelle structures are dynamic and their formation and stability is governed by the critical micelle concentration, one of the main advantages of polymeric micelles over their lipid and other smaller surfactant counterparts is their much lower critical micelle concentration, which offers better stability upon dilution.[1553]

Researchers have employed various techniques to enhance the stability of micellar polymeric NPs. It is increasingly popular to introduce covalent bonds between polymer chains to enhance particle stability, although this can affect the structure and overall properties of the final construct, such as drug release kinetics.[1604] Song and co-workers found that phosphorescent NPs made from the joint self-assembly of poly(vinyl chloride-*co*-ethyl acetate-*co*-maleic acid) and poly(vinyl chloride-*co*-vinyl acetate-*co*-expoxy monomer) copolymers and further cross-linked via their terminal carboxylic acid and expoxy functional groups, respectively, were more stable in the presence of organic solvents and common surfactants than the non-cross-linked materials.[1598] Kakwere and co-workers used poly(ethyl acrylate)-*block*-[poly(hydroxyethylacrylate-*co*-*N*-acryloxysuccinimide)] to form micellar NPs that could be cross-linked by their succinimide shell with hexamethylenediamine, resulting in structurally stable NPs that could also be dissolved.[1605] When also functionalized with thiocarbonyl groups, reduction resulted in micellar NPs that could be further coupled to maleimide derivatives of fluorescent dyes and biotin. Pan and co-workers also used diamine cross-linking to stabilize PS-*block*-PAA polymer micelles using carbodiimide chemistry.[1606] The PS core contained radio-opaque elements such as Bi or I, which act as contrast agents, and residual surface carboxylic acid groups, which were functionalized with either biotin hydrazide or fluorescein thiosemicarbazide using EDC chemistry. Click chemistry[1607] and disulfide bonding[1608] have been similarly employed.

4.3.1.5. Polymer Nanofibers. Polymeric nanofibers have demonstrated great potential as next generation tissue engineering and regeneration scaffolds, as nanocomposite membranes for controlled drug release in wound dressings and implanted localized therapies, and as sensing surfaces.[1555,1556,1609−1615] For tissue engineering, in particular, these synthetic 3D polymeric nanofiber networks are designed to mimic the fibrous architecture of the native extracellular matrix, which is essential for the adhesion, growth, and functioning of connective tissue cells, such as bone, cartilage, vascular tissue, neural tissue, and dermis, and as such, this has been the topic of a number of excellent reviews.[1555,1556,1609−1611] Briefly, nanofibers can be prepared using a number of techniques including self-assembly, electrospinning, phase separation, and chemical etching.[1555,1556,1609,1611,1616] Although the use of natural protein polymers, such as collagen, is desired due to enhanced cytocompatibility, a number of synthetic polymers including poly(glycolic acid), PLGA, PLA, and PCL have also been investigated as nanofibrous scaffolds.[1556,1609] While the nanofibrous architecture typically promotes enhanced protein adsorption and subsequent cell adhesion, compared with smooth surfaces, it is not uncommon to attempt to mimic some of the natural components of the extracellular matrix. Such approaches can encapsulate progenitor cells such as chondrocytes or osteoblasts into the fiber network, along with growth factor proteins and other proteins such as fibronectin that promote cell adhesion, growth, migration, and differentiation, although the exact components depend on the specific tissue under study.[1555,1556,1609,1611] Yan and co-workers studied BSA and nerve growth factor encapsulation in poly(L-lactide-*co*-ε-caprolactone) electrospun nanofibers.[1617] Using BSA, they found that mixed electrospun nanofibers produced a rapid burst release, whereas coaxial electrospun nanofibers released the BSA in a slower and more sustained manner, demonstrating control over release profiles. Dong demonstrated the encapsulation of multiple proteins within an electrospun fiber.[1618] Here, two sets of PVA NPs encapsulating either BSA or EGF were first prepared and then mixed with a carrier polymer matrix, PLGA or polyurethane, prior to electrospinning. The electrospun PLGA or polyurethane fibers contained distinct regions comprising either encapsulated BSA−PVA or EGF−PVA NPs within a single fiber, allowing for the possibility of control over the release of multiple species at distinct rates depending on the design. While still very much in the early stage of development, such nanofiber-based scaffolds clearly have enormous potential for biomedical applications, especially as synthetic techniques are developed to scale fiber sizes down to the nanoregime in a controlled manner.

4.3.2. Dendritic Structures. Dendritic architectures represent a class of polymer materials that are repeatedly branched or tree-like and can be further subdivided based on the molecular weight of the "as-synthesized" macromolecule.[1619] Hyperbranched polymers are random in structure and possess polydisperse molecular weights, whereas dendrimers and dendrons represent highly ordered structures with monodisperse molecular weight.[1619] Other dendritic architectures, such as dendrigrafts, graft-, brush-, and star-structures, typically have narrower molecular weight polydispersities and fall somewhere between the hyperbranched and dendrimer extremes.[1619,1620] Of these structures, dendrimers are most commonly conjugated with biologically relevant materials. Diagnostics and therapeutics dominate the applications of dendrimers, as outlined in several excellent reviews.[1590,1621−1627] The basic dendrimer structure is comprised of three components: (1) the core; (2) the interior branching shells, which are described by the generation number (G) that represents the number of successive branching points emanating from the central core; (3) the outer shell, which consists of the terminal or peripheral groups, which are typically the primary sites for bioconjugation.[1619,1622−1624] The ability to tailor the physical and chemical properties of each of three components, and therefore the overall properties of the final dendrimer structure (size, shape, charge, flexibility, reactivity, valency) has been key to their successful application in numerous fields.[1619,1622−1624,1628,1629] Interestingly, dendrimers are also commonly utilized to encapsulate, stabilize, and direct the delivery or activity of many other organic and inorganic NPs, and this utility is extensively covered in a recent review from Bronstein and Shifrina.[842]

Dendrimers can be synthesized from a wide range of monomer branching units; however, PAMAM, polypropyleneimine, poly(phosphorhydrazone), polyaryls, polyethers, and polyesters represent some of the more popular species used for biological applications.[1622,1624,1629−1632] Peptide-derived dendrimers are also becoming increasingly popular due to their potential applications as protein mimics and as biodegradable

scaffolds for drug or gene delivery. Likewise, Y-shaped DNA oligonucleotides have been used to derive dendrimer structures.[1633−1636] Carbohydrate-modified dendrimers, glycodendrimers, and especially glycopeptide dendrimers are a rapidly expanding area of research due to potential applications in drug and vaccine design, targeted imaging, scaffolds for contrast, and therapeutic agents, along with acting as useful materials for studying cell adhesion interactions or bacterial adhesion prevention.[1630,1631,1637−1640] While carbohydrates have been used as dendrimer cores and within interior branching structures, they are more commonly attached to the peripheral groups on the dendrimer scaffold, where their multivalent presentation can improve therapeutic efficacy and binding affinity.[1630]

The various synthetic strategies for dendrimers have recently been reviewed but are briefly summarized as either divergent methods, convergent methods, hybrid convergent−divergent methodology, or the more recent CuAAC click chemistry.[1623,1628] There are structural limitations associated with the formation of larger dendrimer structures, and these are dependent on a number of factors, including the core multiplicity, branching multiplicity, and branch length. PAMAM-based dendrimers still remain the most popular in the literature, due in part to the ability to purchase them commercially. The terminal groups, which largely control the dendrimer properties and the selection of bioconjugate chemistry, can be charged (e.g., amine, carboxyl, or phosphonate groups), neutral (e.g., PEG, acetyl, glycidyl), or zwitterionic (e.g., amino acids).[1622,1628] While symmetric dendrimer structures are more common, convergent synthesis has also led to asymmetric structures such as bow-tie dendrimers, which present two regions with differing terminal modifications.

Drug delivery represents one of the major driving forces behind dendrimer development and is the subject of many reviews.[1622,1623,1625,1641] Their uniform size, monodispersity, and highly reproducible nature, coupled with programmable chemical functionality, make dendrimers favored over their polymer counterparts, where product consistency can be problematic.[1622,1623,1625,1641] Drug molecules are either encapsulated within the dendrimer interior or interact with the dendrimer surface through electrostatic or covalent interactions, as extensively reviewed.[1622,1623,1625,1641,1642] Hu and co-workers recently demonstrated that the interaction between dendrimers and drugs was strongly dependent on the surface charge of the dendrimer, as well as the charge and structure of the drug.[1643] Patri and co-workers compared methotrexate encapsulated in a dendrimer against methotrexate covalently bound to the dendrimer surface, both in the absence and in the presence of FA as a targeting ligand, and found that the covalently conjugated drug−dendrimer complexes were superior for intracellular delivery.[1644] In this study, amine-terminated PAMAM dendrimers were first modified with glycidol, where the oxirane group converts the dendrimer primary amines to secondary amines with β- and γ-hydroxyl groups. The hydroxyl groups were then modified with DCC-activated methotrexate in dimethyl sulfoxide to form an ester-linked drug−dendrimer complex.[1644] An ester linkage was also used by Perumal and co-workers to conjugate the model drug methyl prednisolone to hyperbranched polyol and G4-OH functionalized PAMAM dendrimers, see Figure 76.[1645]

PEG modification of dendrimers has been key to their drug delivery development and has been shown to improve solubility and circulation time in mice, thereby improving the chance of the drug reaching the desired target.[1646] Drug release from the dendrimer structure is another critical issue. In some instances, the dendrimer is made biodegradable through the selection of monomers such as polyesters or polypeptides, where degradation in physiological environments facilitates drug release. Ideally though, the drug should be released only upon reaching the desired target, and drug release based on relative GA levels is a promising technique in this regard.[1647] To achieve GA-mediated release, amine-terminated PAMAM dendrimers were modified with a thiol-containing antioxidant and anti-inflammatory agent, *N*-acetyl-L-cysteine (NAC), using SPDP, which forms a disulfide linkage between the drug and dendrimer. Intracellular GA levels are much higher than extracellular levels, and potentially more so in cancer cells, such that, upon intracellular delivery, the higher GA levels increased the rate of thiol exchange with the NAC-modified dendrimer (i.e., reduction of the original disulfide) resulting in NAC release.[1647]

Aside from drug delivery, dendrimers have been modified with a variety of biomolecules and contrast agents using a variety of covalent and noncovalent methods.[1648,1649] Froehlich, for example, recently reported on the noncovalent interaction of HSA with a range of dendrimers of varying compositions.[1650] Probably the most common noncovalent modification of dendrimers, however, involves the electrostatic interaction of nucleic acids with cationic (lysine)-terminated dendrimers (PAMAM, polylysine, polypropyleneimine, and amine-modified triazine dendrimers) for application in gene therapy. Not surprisingly, the biological performance of these vectors is found to be closely linked to the surface charge, size, and morphology of the resulting complex.[1552,1622,1624,1651−1654] Despite the success of dendrimers as drug and gene delivery vectors, it is more frequently the case that biomolecules are covalently attached to the dendrimer surface. Given the controlled architecture and defined chemical groups available for modification, standard bioconjugate reactions are quite common. Singh et al., for example, investigated several reactions for coupling various proteins to carboxyl- and amine-terminated dendrimers.[1655]

Given the commercial availability of PAMAM dendrimers, the utilization of amine-reactive chemistries for dendrimer modification is extremely popular. For example, NHS- and isothiocyanate-activated fluorescent dyes,[1656,1657] carbohydrates,[1640] isothiocyanate-modified Gd−DOTA and DTPA metal chelates,[1658,1659] NHS−PEG,[1660] and NHS−biotin[1661] have all been conjugated directly to dendrimers displaying surface amines. Carboxyl-modified molecules are also commonly attached to dendrimer surface amines using carbodiimide activation chemistry, as demonstrated for a carboxyl-modified cryptate and NIR fluorophore with a G3 polyester dendrimer[1660] and FA with modified PAMAM dendrimers.[1622,1644,1657,1658] Other coupling reagents commonly used in solid phase peptide synthesis have also been found useful for amide coupling reactions between amine and carboxyl groups associated with dendrimers, as demonstrated for the attachment of ligands that target various cell surface receptors[1662] and Gd-chelating agents.[1663] This approach, however, is limited to materials stable in the common organic solvents used, such as dimethyl sulfoxide (DMSO) or dimethylformamide (DMF), which may preclude some proteins.

Often times, the dendrimer surface amines are first converted to an alternative functionality, such as a carboxylic acid, thiol, or maleimide, prior to modification. For example, Humenik and co-workers modified PAMAM dendrimers with the bifunctional linker succinimidyl-[(*N*-maleimidopropionamido)-di(ethylene glycol)] converting terminal amines to maleimides, which were then used for conjugation to a cysteine mutant of esterase 2.[1664]

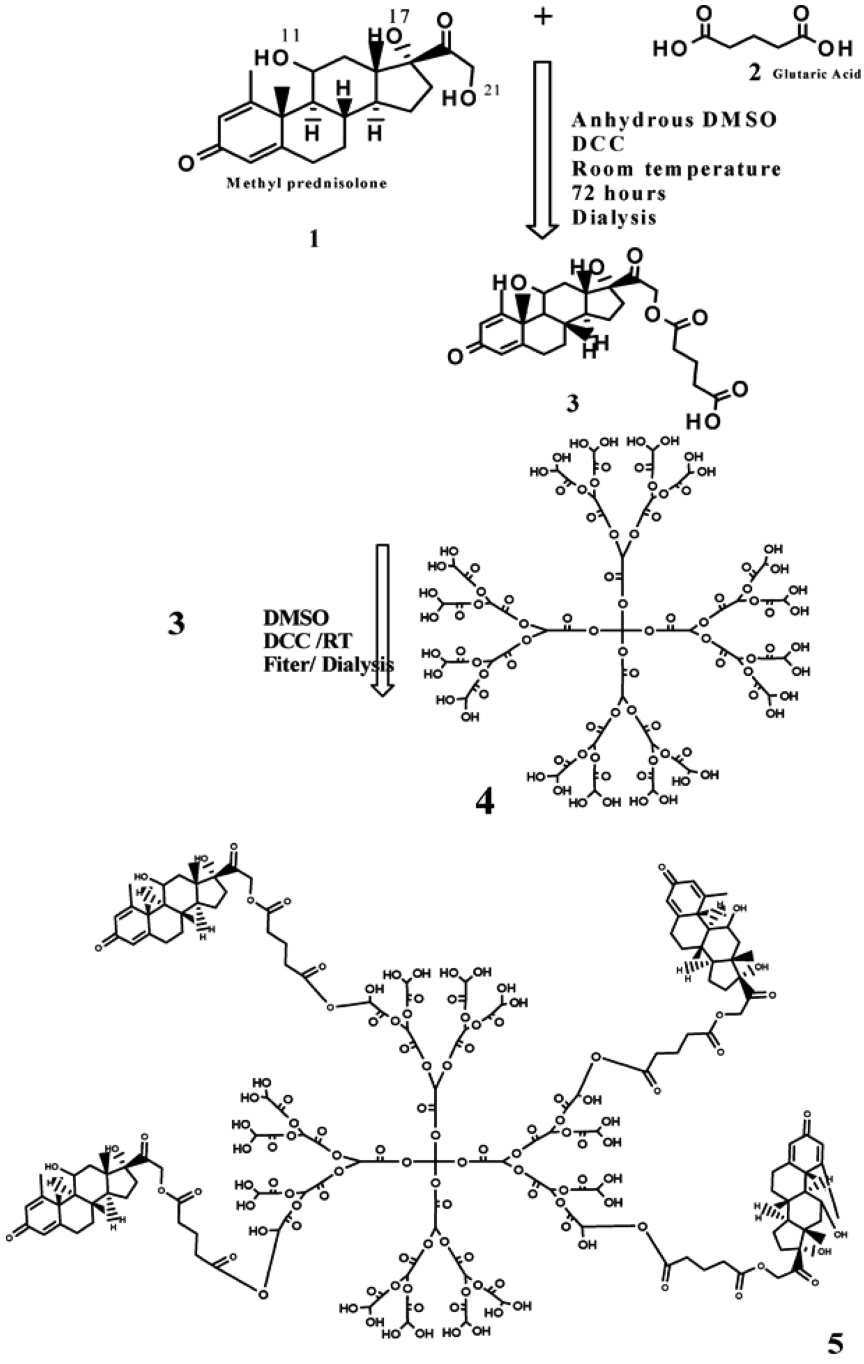

Figure 76. Synthetic scheme for deriving a methylprednisolone−glutaric acid−polyol dendrimer conjugate. Numbers represent reactants as shown or modification steps. Reprinted with permission from ref 1645. Copyright 2009 American Chemical Society.

The resulting dendrimer contained multiple enzymes and was used for electrochemical biosensor signal enhancement. A similar modification procedure was used to attach HER2 specific antibodies to PAMAM dendrimers. Here, thiol groups were introduced to the dendrimer surface amines via sulfo-LC-SPDP while sulfo-SMCC was used to convert antibody-lysine residues to maleimido-functional groups.[1656] The resulting dendrimer was also labeled with a fluorophore and used to target and image HER2 expressing cancer cells.[1656] In another example, Myc and co-workers modified amine-terminated dendrimers with NHS−iodoacetate to produce an iodoacetyl derivative that then formed a thioether bond upon reaction with thiol-modified BH3 fusion peptides; these were found to induce apoptosis in cells.[1655,1657]

In an elegant example from the Fréchet group, radioactive labels were placed at the core of a PEG-functionalized dendrimer structure to prevent *in vivo* dehalogenation while the terminal amine groups were converted into carboxylic acids by reaction with glutaric acid. Carbonyldiimidazole was then used to couple a lysine-modified cyclic RGD peptide to the carboxyl groups for cell targeting purposes, and the construct functioned as a positron-emitting nanoprobe specific for integrins in angiogenic muscles, see Figure 77.[1665]

Disulfide bond formation and maleimide chemistry are also commonly used for the modification of thiolated dendrimers. For example, following TCEP reduction of a PAMAM dendrimer with a cystamine core, maleimide-functionalized ssDNA

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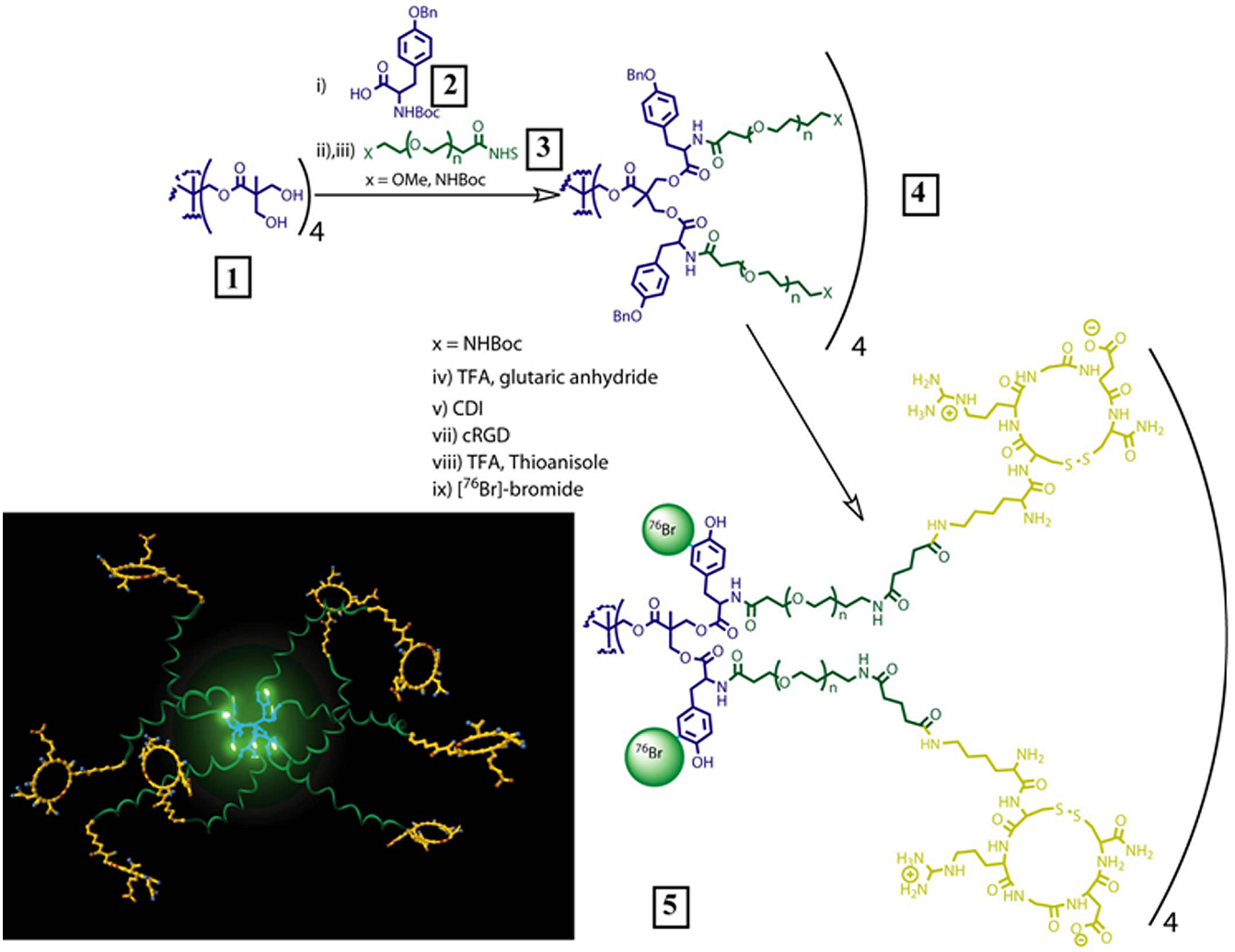

Figure 77. Preparation of the PET nanoprobes targeted to integrin receptors. Numbers represent reactants as shown or modification steps. Figure reproduced from ref 1665 with the permission of the National Academies of Sciences, U.S.A.

was attached, and the resulting dendron, which was also functionalized with esterase 2, was used to detect hybridization events in electrochemical biosensors.[1664,1666] Hassane and co-workers used disulfide bond formation to couple thiol-modified PNA to thiol-terminated peptide dendrimers, and the resulting dendrimers were found to readily transfect HeLa cells.[1632]

CuAAC click chemistry is increasingly growing in popularity for functionalizing dendrimers, and this approach has already been applied with peptide[1667] and carbohydrate[1668] modifications.[1669−1671] Yim and co-workers generated cyclic octreotide peptide dendrimers using CuAAC between alkyne-terminated dendrimers and azido-peptides.[1667] Octreotide is a somatostatin analogue that has a high affinity for G-protein coupled somatostatin receptors (SSTRs) overexpressed in a number of human cancers. Again, peptide synthetic protocols are also sometimes applied to attach relatively short peptide sequences to dendrimers, as demonstrated for the preparation of collagen-mimicking peptide−dendrimer complexes using *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU)/1-hydroxy-7-azabenzotriazole (HOAt) coupling.[1672] EDC/DCC activation of carboxylic acids, while commonly used for reaction with amines, has also been used to form esters with hydroxyl-containing species, as demonstrated for the modification of carboxyl-terminated dendrimers with carbohydrates and hydroxyl-terminated dendrimers with biotin.[1673]

One of the more attractive features of dendrimers is the ability to have them mono-, bi-, or trifunctionally conjugated to combinations of drugs, DNA, enzymes, or contrast agents, typically in conjunction with targeting agents such as peptides, carbohydrates, antibodies, other proteins, or FA. Baker and co-workers, in particular, have developed multifunctional dendrimers modified with drugs,[1644] therapeutics,[1657] or contrast agents[1658] that are targeted to cancer cells using FA. Additionally, Alumtairi and co-workers covalently encapsulated a NIR fluorophore into a G3 polyester dendrimer. Here, the polyester dendrimer amine groups were *tert*-butyloxycarbonyl (Boc)-protected for this initial dye conjugation but were later deprotected to allow reaction with NHS−PEG, which improved the *in vivo* circulation time of the final complex.[1660] In a later study, dendritic branches of a trifunctional nanoprobe were modified with tyrosine allowing the attachment of radiohalogens as imaging agents.[1665] The dendritic branches were further functionalized with PEO to improve biocompatibility and a cyclic RGD peptide to target $\alpha_v\beta_3$ integrin, which is overexpressed during angiogenesis. Dendrimers and dendrons have also shown the ability to self-assemble into an array of discrete and supramolecular structures, including micelles or

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vesicles, nanofibers, nanoclusters, and extended aggregate or liquid crystal arrays, expanding the scope of potential applications and demonstrating improvement over current drug loading capabilities.[1619,1674−1679]

4.3.3. Hyperbranched Polymers and More Complex Dendritic Structures. Similar to their dendrimeric relatives, hyperbranched polymers also have NP formulations that have been modified with a variety of molecules for biomedical applications.[1680] While they possess more random structures and polydisperse molecular weights, hyperbranched polymers are typically cheaper to produce and easier to modify, and analogous to dendrimers, covalent and noncovalent binding are used. For example, Khan and co-workers developed an amino-modified, PEG-based, hyperbranched polymer that bound RNA through electrostatic interactions producing NP structures of 60 ± 10 nm in size and simultaneously protected the RNA from enzymatic degradation.[1681] Kim and co-workers also developed hyperbranched polysiloxysilane NPs with terminal carboxylic acid and quaternary ammonium groups to encapsulate various hydrophobic two-photon dyes used for fluorescent imaging.[1682]

Bioconjugation chemistry can also be implemented with more complex versions of these materials. For example, Hamilton and Harth cross-linked linear hydrophilic benzyl acrylate to form 5−10 nm sized polymer NP cores, then functionalized them with a maleimide−PEG−hydrazide linker via the carboxyl groups on the polymer chain.[1683] The maleimide was subsequently reacted with the thiol focal point of a dendritic molecular transport molecule, producing a dendronized NM. Prior to this step, some of the polymer carboxylic acids were activated with chloroformate and converted to protected primary amines using *N*-trifluoroacetyl (Tfa)-ethylenediamine. After deprotection, the amines were modified with an NHS-functionalized fluorescent dye. A disulfide linker was also introduced into the polymer chain by a two-step reaction with chloroformate-activated 3-(2-pyridyldithio)propionic acid addition at the deprotected primary amines, followed by disulfide exchange with the cysteine residue of fluorescein-labeled peptides. Further, Reuter and co-workers modified a range of dendritic polymeric structures using isothiocyanate-functionalized sialic acid, which reacted with both primary and secondary amines, and studied the ability of the modified dendrimers to inhibit viral cell adhesion: comb-branched and dendrigraft structures were most effective in preventing viral infection of cells.[1684] Tao and co-workers synthesized four-armed poly(*N*-isopropylacrylamide) star polymers with protected maleimides that could be conjugated to the available thiol groups of lysozyme.[1685]

4.3.4. Lipid Nanoparticles. Since their first description over 50 years ago, lipid NPs have been extensively researched for chemical and biomedical applications and are again especially prominent in drug delivery.[1686−1688] There have been tremendous strides in the field marked by key milestones including the first successful genetic transfection trials in the early 1980s,[1689−1691] development of stealth PEGylated liposomal carriers in the early 1990s,[1692] and the approval of the first lipid-based drug-carrier system (Doxil) in 1995. Since then, many more lipid-based systems have been approved by the FDA to treat a wide range of afflictions.[1693]

The sustained interest in developing lipid NP carrier systems for bioactives has been fueled by the diverse advantages these systems offer. Foremost among these is that lipids are naturally biocompatible and well-tolerated by the body.[1694−1696] Additionally, since many lipid NPs are composed of amphiphilic components, they have the capability to solubilize various forms of bioactives, concurrently in some cases, regardless of hydrophilicity. Hydrophobic therapeutics can thus be loaded at high densities in the lipid regions of the NP while water-soluble bioactives may be conjugated or loaded to hydrophilic regions. The unique configuration of such lipid systems also allows for the protection of the loaded bioactive from harsh environmental conditions, both *in vitro* and *in vivo*. Lipid encapsulation can further limit systemic exposure, which is beneficial in many instances where the therapeutic payload may have undesired cytotoxic side effects.

Other advantages of lipid NP systems include their ease of production and modification, their ability to be specifically targeted through conjugation of targeting ligands, and the ability to control the release rate of the therapeutic payload through various means including compositional alterations or changes in the processing conditions. Given these unique advantages, lipid NP systems are heavily employed as delivery vehicles for a wide range of bioactives, including pharmaceuticals,[1697] peptides and proteins,[1698,1699] contrast agents for imaging,[1700] food additives,[1701] genetic material,[1702] and cosmetics.[1703] There are many comprehensive reviews that cover almost all aspects of lipid NPs including synthesis, constitutive components, and major applications.[158,1697−1702,1704−1713] The goal in this section is to provide a descriptive overview of lipid NPs with a specific emphasis on available bioconjugation methods. We point out that in describing how many of these NPs are assembled, we also describe the route to obtaining the most common bioconjugate: encapsulated bioactive materials.

4.3.4.1. Synthesis and Noncovalent Encapsulation. Lipids are generally classified into eight main subtypes: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides.[1714] As stated previously, the amphiphilicity of specific lipid subtypes (e.g., glycerophospholipids) is highly exploitable for drug delivery due to their ability to self-assemble in aqueous environments and their capability to solvate both hydrophobic and hydrophilic species concurrently. There are numerous geometries and distinct architectures that may be created simply by changing the lipid type, lipid concentration, or ratio of lipids employed. The main architectures that are used for the delivery of bioactives include emulsions or swollen micelles, liposomes, solid lipid NPs (SLNs), and lipoproteins, see Figure 78. The transition between a micellar solution and an emulsion generally occurs as the ratio of dispersed phase to surfactant is increased beyond a certain specific point.[1709] For the purposes of this review, we treat emulsions and swollen micelles as synonymous.

4.3.4.1.1. Emulsions. A lipid emulsion is conventionally defined as a thermodynamically stable assembly consisting of oil and water that is stabilized by some form of surfactant, see Figure 78.[1699,1709,1715] The two main categories of emulsions are microemulsions and nanoemulsions. Although there is ambiguity in categorizing emulsions into either category, it is generally accepted that nanoemulsions, also called Windsor system microemulsions or miniemulsions, are less than ∼200−300 nm in size and are only kinetically stable, as opposed to microemulsions, which are thermodynamically stable and may range up to ∼1 μm in size.[158,1709,1716−1718] Lipid emulsions are also categorized based on phase-type starting from the core. The vast majority of formulations are either oil-in-water (O/W), where the inner phase is oil and the outer phase is water, or water-in-oil where the reverse is true. Research has also been conducted on multiple phase emulsions where smaller emulsions are encased or incorporated into larger emulsions, for example,

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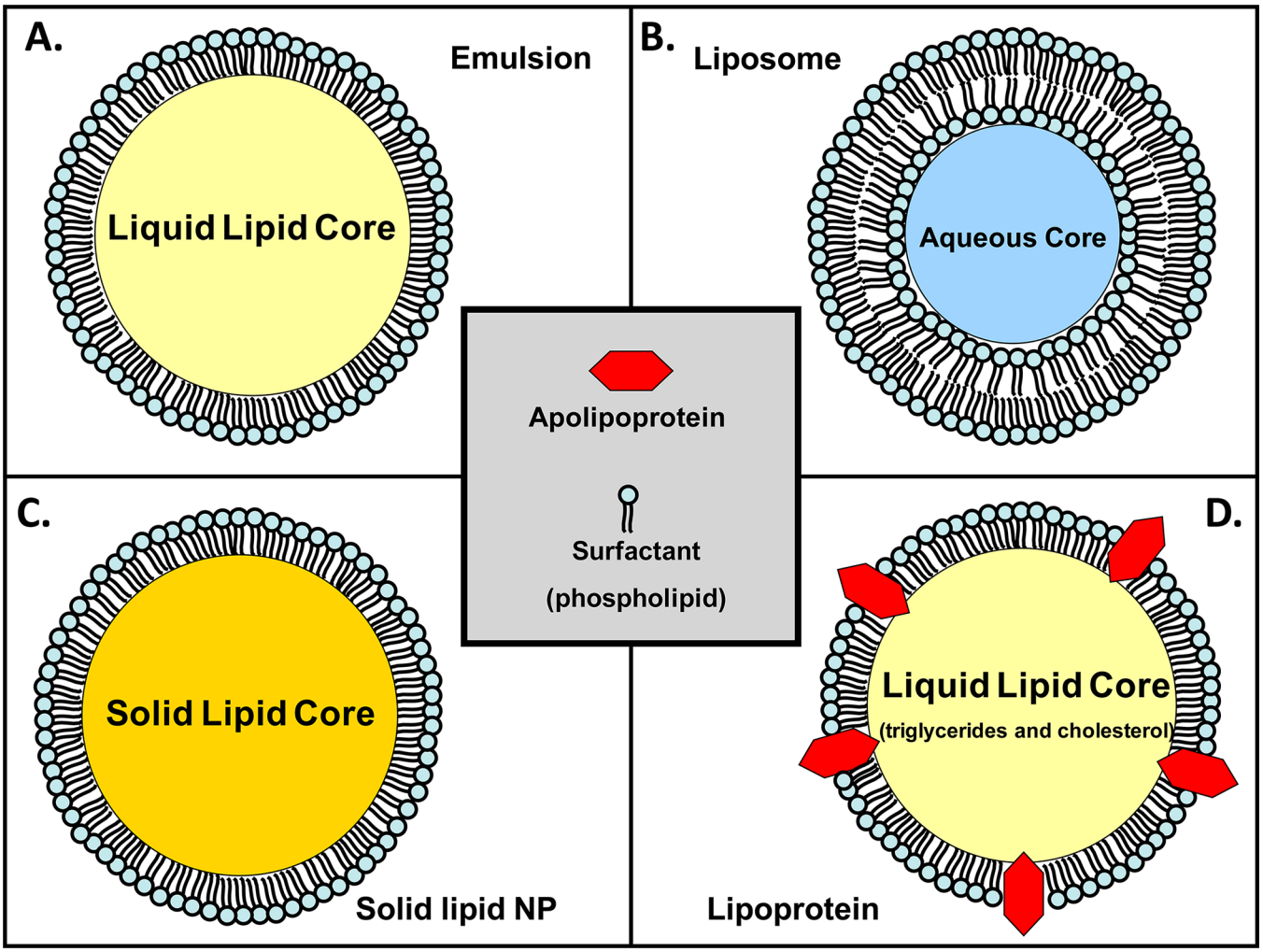

Figure 78. Schematic representation of common lipid-based NP structures: (A) emulsion/swollen micelle, (B) liposome, (C) solid lipid NP, and (D) lipoprotein.

water-in-oil-in-water.[1719,1720] Such emulsions are typically formed through spontaneous emulsification techniques where both the components and emulsification procedure, including how the various components (e.g., oil, water, and surfactant) are added to the system, are altered to vary the NP that is formed.[1710] The dispersed O-phase of emulsions are typically composed of vegetable oils (fatty acyls) or middle chain triacylglycerols. Surfactants may include nonionic block copolymers, lecithin (charged), and alcohols.[1699,1709,1721]

Conjugation of bioactives to lipid-based emulsions is almost exclusively achieved via encapsulation concurrent with emulsion synthesis (spontaneous emulsification). For aqueous soluble species, that is, peptides and proteins, the aqueous phase is doped with the bioactive, which is then incorporated into the emulsion through the spontaneous assembly of the water-in-oil emulsions. Numerous bioactives have been encapsulated in lipid-based NP emulsions for various applications, see Table 14. Notable examples include insulin,[1722,1723] immunoglobulin G,[1724] α-lactalbumin,[1725,1726] and fusion proteins.[1727] In the case where the bioactive is hydrophobic, O/W emulsions are used where the hydrophobic agent is solvated in the inner lipid phase surrounded by water. Spontaneously formed encapsulates of poorly soluble drugs within O/W emulsions are typically referred to as self-emulsifying drug-delivery systems.[1699,1709,1728] Emulsion-based therapeutic conjugates (i.e., encapsulates) have included nonsteroidal anti-inflammatory drugs such as dexamethazone palmitate,[1729] aceclofenac,[1730] and celecoxib,[1731] along with chemotherapeutics such as irinotecan,[84] PAX,[1732] and tamoxifen.[1733] Nanoemulsions have also been used as delivery vehicles for genes and siRNA.[1734]

4.3.4.1.2. Liposomes. Hailed as a "magic bullet" for pharmaceutical delivery applications following their creation in the 1960s, liposomes are the predominant form of lipid NP used for clinical applications.[1686,1687,1735] They consist of spherical assemblies approximately 50−200 nm in diameter with one or more phospholipid bilayers (see Figure 78). Phospholipids are a lipid subclass that includes glycerophospholipids and sphingolipids and are characterized by a hydrophilic phosphate-conjugated "head", a glycerol (glycerophospholipids) or sphingoid (sphingolipids) backbone, and a hydrophobic fatty acyl "tail" (see Figure 79). Liposomes composed of nonionic surfactant and phospholipid moieties are known as niosomes.[1736,1737] The amphiphilic nature of phospholipid molecules enables the spontaneous formation of bilayer structures when introduced to aqueous media (Figure 78). Liposomal-like NPs may also be produced with a unique class of lipids called bolaamphiphiles, which contain two hydrophilic head groups linked by a hydrophobic chain. Due to the presence of two hydrophilic head groups these lipids spontaneously form

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Table 14. Representative Bioactive Agents Conjugated to Lipid Nanoparticles

bioactive	nanoparticle type	conjugation (encapsulation/covalent)	reference
	Drugs		
dexamethazone palmitate, aceclofenac, celecoxib, irinotecan, paclitaxel, tamoxifen, α-tocopherol	emulsion	encapsulation	84, 1729−1733
doxorubicin, glutathione, amphotericin-B, nisin, pediocin AcH	liposome	encapsulation	1738−1744
camptothecin, tamoxifen, paclitaxel, diazepam, paramomycin, enrofloxacin	SLN	encapsulation	1745−1751
adriamycin, doxorubicin, paclitaxel, adriamycin iododeoxyuridine, vincristine,	lipoprotein	encapsulation	1752−1756
3′-azido-3′-deoxythymidine, 2′,3′-didehydro-3′-deoxythymidine	lipoprotein	covalent	1757
	Proteins/Peptides		
insulin, immunoglobulin G, α-lactalbumin	emulsion	encapsulation	1722, 1724−1726
HPV16 E7, interleukin-1, glutathione, superoxide dismutase	liposome	encapsulation	1707, 1743, 1758−1760
BSA, catalase	SLN	encapsulation	1761, 1762
	Nucleic Acids		
green fluorescent protein plasmid DNA, siRNA	emulsion	encapsulation	1734, 1763
siRNA	liposome	encapsulation	1764
p53 gene, plasmid DNA (pCMV-Luc)	SLN	encapsulation	1765, 1766
siRNA	SLN/lipoprotein	covalent	1765
	Imaging Agents		
fluorine	emulsion	encapsulation	1767
^{67}Ga, ^{99m}Tc, Gd−DTPA	liposome	encapsulation	1748, 1768−1771
^{125}I	lipoprotein	covalent	1772
^{125}I	lipoprotein	encapsulation	1773
	Multiple Bioconjugated Species		
^{19}F + QDs	emulsion	encapsulation	1774
mAb H18-7 antibody + doxorubicin	liposome	encapsulation	1775
QDs + biotin	liposome	QD-encapsulation/covalent, biotin-covalent	1776
QDs + anti-HER2 + doxorubicin	liposome	QDs + anti-HER2-covalent, DOX-encapsulation	1777
P 53 gene + anti-transferrin receptor antibody	liposome	P53 gene-encapsulation, anti-transferrin receptor antibody-covalent	1778, 1779
fluorescein−siRNA + anti-transferrin receptor antibody	liposome	fluorescein−siRNA-encapsulation, anti-transferrin receptor antibody-covalent	1780
docetaxel + galactose	SLN	docetaxel-encapsulation, galactose-covalent	1781
insulin + R8 CPP	SLN	encapsulation	1782
plasmid DNA (pCMV-Luc) + TAT peptide	SLN	encapsulation	1783

monolayers, as opposed to bilayers, when introduced to aqueous media. Use of bolaamphiphiles has been limited by the inability to efficiently synthesize the molecules or extract them from naturally occurring sources such as archaebacteria.[1704]

Phospholipids such as phosphatidylcholine (PC) or phosphatidylethanolamine (PE) are typically the main constituents of liposomes but other lipid classes, including sterol lipids (steroids; e.g., cholesterol), have also been utilized.[1704,1705,1736,1784] The size and structure of a liposome is governed by the type and concentration of lipids used, along with the processing conditions employed. Again, there are several processing techniques that have been used to prepare liposomes. The original method developed by Bangham involves hydrating a thin lipid film in an organic solvent, removing the organic solvent under vacuum, and replacing it with an aqueous solvent, which results in the spontaneous formation of liposomes.[1784−1786] Other processing techniques include freeze−thaw extrusion,[1787] dehydration−rehydration,[1788] double emulsification,[1789] reverse phase evaporation,[1790] and ether vaporization/injection.[1705,1791,1792] Further processing techniques, including controlled sonication, have been developed to enhance or control uniformity.[1699,1793−1797] The amphiphilicity of liposomal NPs allows for both hydrophobic and hydrophilic species to be loaded in the vesicle, even concurrently if desired. Liposomes have been used as biodelivery vehicles for numerous types of bioactives, including chemotherapeutics,[1704,1740] antifungals,[1704,1741] antigens,[1707,1758] peptides,[1707,1742,1798] enzymes,[1799,1800] cytokines,[1759,1800,1801] nucleic acids,[1802−1805] and various imaging agents (see Table 14).[1806−1808] Immunoliposomes, or antibody-decorated liposomes, are employed for the targeted delivery of therapeutics.[1775,1809]

4.3.4.1.3. Solid Lipid Nanoparticles. SLNs are characterized by an internal lipid core, which is solid at body temperature, and an emulsification or surfactant coating (Figure 78). The structure of a SLN closely resembles that of an emulsion or micelle except that the liquid lipid core is replaced by a solid core. Nanostructured lipid carriers (NLCs) and lipid drug conjugates are two of the more common forms of SLNs. NLCs were developed to overcome certain limitations of SLNs, such as limited drug loading and premature drug expulsion, which are common issues found when utilizing liquid cores. Assembly strategies include the utilization of "spatially different lipids", along with the mixing of liquid and solid lipids to disrupt crystallization, which both enhances loading capacity and prevents the rapid expulsion of the bioactive. In instances when the bioactive is more soluble in liquid lipids, increasing the liquid character of the NLC may also increase drug loading capacity. The three subtypes of NLCs include the "imperfect type", the "multiple type", and the "amorphous type". The combination of

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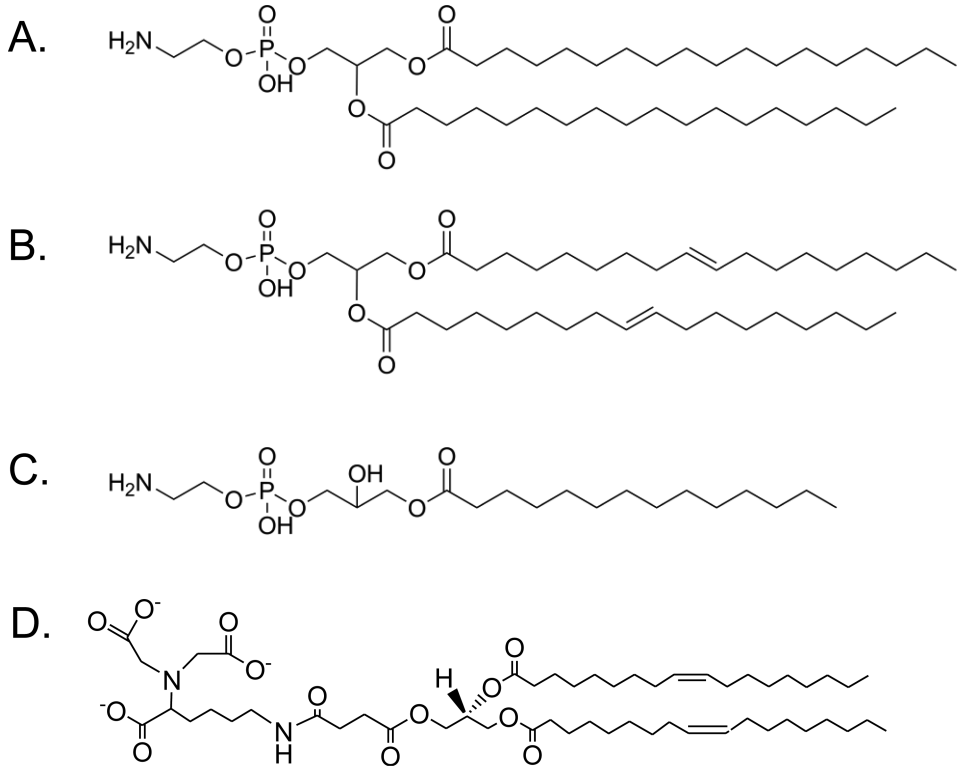

Figure 79. Representative lipid anchors used for bioconjugation. Modification or coupling chemistry is typically completed on the amino group at the distal end of the phospholipid or lipid molecules: (A) 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE), (B) 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), (C) 1-myristoyl-*sn*-glycero-3-phosphoethanolamine (MPE), and (D) 1,2-dioleoyl-*sn*-glycero-3-[(*N*-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (DOGS-NTA).

spatially different liquid (e.g., oils) and solid lipids results in the formation of an imperfect crystal matrix, referred to as the imperfect type. The multiple type NLC is characterized by segregated compartments or phases of liquid lipid within the solid lipid phase and results from a phase separation driven by the insolubility of excess liquid lipid molecules within the solid lipid phase. When incongruent solid lipid constituents are mixed together to form a solid yet amorphous inner phase, this is referred to as the amorphous type.[1810,1811]

Other advantages of SLNs include high drug stability, limited biotoxicity, facile production, and the ability to scale up synthesis to a commercial scale. Various types of lipids are used in the production of SLNs including glycerolipids (e.g., triglycerides and partial glycerides) such as tristearine,[1812] fatty acyls (e.g., fatty acids and waxes) such as stearic acid,[1813] and steroids such as cholesterol.[1765] Similarly, numerous surfactants have been utilized in the production of SLNs, including glycerophospholipids (e.g., lecithin[1814] and PC[1815]), short chain block copolymers (e.g., Poloxamer 188[1816]), and acidic molecules (e.g., butyric acid[1817,1818]). SLNs are particularly well suited for the encapsulation of lipophilic or hydrophobic bioactives. The encapsulation of hydrophilic bioactives into SLNs is commonly achieved by chemical modification with lipids such as fatty acyls, which yields modified lipid–drug conjugates.[1811,1819]

Two common SLN synthetic techniques include high shear homogenization and ultrasound, although various other emulsification techniques are also used. High shear homogenization uses high-pressure homogenizers to force a liquid through a narrow aperture. As the liquid is forced through, the strong shear forces generated are able to fragment the particles to sizes in the nanoscale range. High shear homogenization may be carried out at high temperatures (i.e., hot homogenization)[1816,1820,1821] or low temperatures (i.e., cold homogenization).[1816] Hot homogenization is typically used to load lipophilic drugs and bioactives into SLNs. Briefly, the lipids are heated above their melting point to create a liquid to which the bioactive is added. The bioactive-loaded melt is then dispersed in high-temperature aqueous media containing a surfactant. Finally, the system is put through the homogenization process and the generated particles are allowed to cool and crystallize, resulting in the formation of SLNs. Cold homogenization is typically utilized to load hydrophilic or temperature-sensitive bioactives, such as proteins, into SLNs. Here, the bioactive is dispersed in the lipid melt, which is then rapidly cooled. The system is then milled to create submicrometer particles.[1816,1822] Ultrasonication, which uses ultrasound at high temperatures to cavitate lipid emulsions, is another dispersion technique utilized to prepare SLNs.[1823]

Various microemulsion techniques have also been developed for the synthesis of bioactive-loaded SLNs (Table 14). In a typical protocol, the lipid is melted, and the bioactive is dispersed in the molten phase. An aqueous phase consisting of water, surfactant, and cosurfacant is heated to the temperature of the lipid molten phase and combined with the lipid molten phase to produce microemulsions. The microemulsions are then dispersed in cold water and mechanically agitated, resulting in the precipitation of the lipid phase in fine particles.[1822,1824] In solvent emulsification–evaporation, the lipid is dissolved into an organic solvent, which is, in turn, emulsified in a surfactant-containing aqueous phase. The solution is then slowly stirred to evaporate the solvent resulting in the precipitation of the lipid in the form of SLNs.[1825] To help this process, water is replaced by water-miscible polar organic solvents to create the equivalent

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of an O/W emulsion. The hydrophobic organic solvent is then evaporated, and the resulting SLN dispersion is introduced to an aqueous phase.[1826] Solvent emulsification techniques are typically utilized to encapsulate hydrophilic bioactives since this method avoids the need for high temperatures or pressures.[1698,1822] Other techniques for synthesizing SLNs include the utilization of supercritical fluids,[1827] spray drying,[1828] and membrane contactors.[1829]

4.3.4.1.4. Lipoproteins. Lipoproteins are spherical vesicles consisting of a lipid core (e.g., cholesterol and triglycerides), surrounded by a phospholipid monolayer that is embedded with various proteins known as apolipoproteins (Figure 78). The structural lipid characteristics of a lipoprotein are very similar to that of an emulsion or swollen micelle. Lipoproteins are used to transport hydrophobic molecules, such as cholesterols and other lipids, throughout the body. Lipoproteins are classified based upon their density, or rather the ratio of lipid to protein, and the main types include HDLs, LDLs, intermediate-density lipoproteins (IDLs), very-low-density lipoproteins (VLDL), and chylomicrometers. Lipoproteins typically range from 5 to 500 nm (HDL, 5−15 nm; LDL, 18−28 nm; IDL, 25−50 nm; VLDL, 30−80 nm; chylomicrometers, 100−500 nm).[1830,1831] The inherent biocompatibility of lipoproteins make them extremely attractive for delivery systems, yet application has been limited due to synthetic issues that stem from the inability to efficiently isolate lipoproteins and apolipoproteins from human serum in large quantities. As a consequence, several researchers have turned to developing synthetic or recombinant lipoproteins.[1832,1833] Despite these challenges, there are numerous examples of lipoprotein delivery systems that were developed for carrying chemotherapeutics (see Table 14).[1713,1752]

In cumulatively examining this brief synthetic overview, it is readily apparent that encapsulation is the most widely employed technique for passive or noncovalent conjugation of bioactives to lipid NPs and typically involves solvating the bioactive in either the aqueous (for hydrophilic species) or lipid (for hydrophobic species) phase during synthesis.[1699,1800,1834] We also note the presence of many variables, such as pH, which can help control or optimize the loading of various bioactives.[1835] Some other noncovalent bioconjugation techniques include heat treatments, which have been shown to enhance the interaction between antibodies and the liposomal surface,[1836] the utilization of haptens,[1837] and, of course, avidin−biotin conjugation.[1838]

4.3.4.2. Covalent Bioconjugation. A variety of techniques and chemistries have been utilized to actively couple bioactive species, whether therapeutic or diagnostic, to lipid NPs. There are three general strategies for covalent bioconjugation to lipid NPs. The first method uses the covalent attachment of a bioactive to a lipid "anchor" that is incorporated into the NP during or after synthesis. The typical site is an amine group located in the lipid headgroup. See for example, Figure 79, which shows the structure of several such moieties along with 1,2-dioleoyl-*sn*-glycero-3-[(*N*-(5-amino-1-carboxypentyl)-iminodiaceticacid) succinyl] (DOGS-NTA), a ligand that would allow any His_n-modified biomolecule to coordinate to the surface of the modified lipid NP, steric issues not withstanding.[1839] Several headgroup-modified lipids are available prefunctionalized with biotin and other bioconjugable groups. The second method is the direct covalent attachment of a bioactive to the surface of the preformed NP. Finally, the third method is the covalent attachment of a bioactive to a spacer moiety that is incorporated into the NP by a distal anchor moiety (i.e., indirect conjugation) and is essentially a derivative of the first method. Short chain heterobifunctional hydrophilic polymers, such as those based on PEG[1692] or glycolipids,[1840] are commonly used for the indirect conjugation of bioactives to lipid NPs. PEGylation of lipid NPs offers numerous advantages, including increased biocompatibility and enhanced circulation times.[1692,1841] Furthermore, the PEG moiety can be easily incorporated into the NP through lipid anchors such as 1,2-distearoyl-*sn*-glycero-phosphoethanolamine (DSPE),[1842,1843] ceramides,[1844] diglycerides,[1845] cholesterol,[1846,1847] or phosphatidic acid.[1848] Various forms of PEG-based heterobifunctional cross-linkers have also been utilized for lipid NP bioconjugation, where the reactive functions have included maleimides, amines, acrylates, epoxides, disulfides, thiols, aldehydes, azides, isocyanates, succinimidyl esters, and hydrazides.[1849] Overall, the entire spectrum of bioconjugation chemistries has been applied to functionalizing lipid NPs, and we only provide a limited overview here.

Lipid NPs displaying an accessible thiol group can be directly modified with other thiolated or maleimide-activated species. If the bioactive or lipid NP does not contain an accessible thiol group, one can be added via a heterobifunctional cross-linker or derived by reducing existing disulfide bonds within the structure. Traut's reagent (2-iminothiolane),[1850,1851] SATA,[1851−1855] SAMSA, SATP,[1856,1857] SPDP,[1858,1859] and SMPT are some of the more common cross-linkers used for lipid NP bioconjugation at thiol groups. It is important to understand all aspects of the chemistry and intended application of the conjugate when choosing among these approaches. For example, a limitation of using SAMSA is that the reaction yields a pendant carboxylic moiety that may affect the conformation of the attached bioactive if its quaternary or tertiary structure is affected by electrostatic interactions, such as may be the case for enzymes or ribozymes. In particular, *N*-succinimidyl-(4-(*p*-maleimidophenyl)) butyrate (SMPB) is commonly utilized to functionalize lipids with a maleimide group for subsequent conjugation with a thiol-containing bioactive.[1852,1860−1862] The NHS group reacts with a free amine group on the headgroup of the phospholipid resulting in a stable amide bond (Figure 79). The reverse of this reaction, where a maleimide functionalized bioactive is linked to a lipid at a thiol in its headgroup, can also be utilized.[1863]

Mulder and colleagues utilized thiol−maleimide chemistry to create paramagnetic, PEGylated immunoliposomes for theranostic applications. Liposomes were prepared using Gd−DTPA−BSA, PEG−DSPE, DSPC, and cholesterol via the film rehydration method. The H18/7 monoclonal antibody was thiolated using SATA and coupled to the preformed liposomes by a maleimide-functionalized PEG−DSPE lipid anchor (Mal-PEG−DSPE).[1864] In another example, Park et al. utilized thiol−maleimide chemistry to create DOX-loaded immunoliposomes decorated with PEG and anti-HER2 antibodies to target cancer cells.[1865] The researchers employed lipid film hydration−extrusion to synthesize liposomes from soy phosphatidylcholine (soy PC) and cholesterol. PEG-modified DSPE (PEG−DSPE) was incorporated during the synthesis and DOX was passively coupled with the liposomes through encapsulation. A recombinant scFV antibody fragment directed against the p185 (HER2) receptor tyrosine kinase (anti-HER2 mAb) was modified via the addition of a cysteine residue near the end of the carboxy terminus. This available thiol group was used to couple the anti-HER2 mAb directly to the

liposomal surface using a maleimido-functionalized lipid anchor (*N*-[4-(*p*-maleimidophenyl)butyryl]phosphatidylethanolamine) or indirectly by coupling the antibody to a maleimido-modified PEG−DSPE.[1865−1867] Recently, Yang and co-workers employed similar chemistry to couple an anti-HER2 antibody to liposomes for applications in cancer therapy.[1868] PEGylated liposomes were synthesized using a film hydration method and consisted of soy PC, cholesterol, methoxy-modified PEG−DSPE (mPEG−DSPE), maleimide-functionalized PEG−DSPE (mal-PEG−DSPE), and rhodamine-labeled PE. PAX was also encapsulated in the liposome during the synthesis process. After liposomal synthesis and drug entrapment, herceptin (anti-HER2 antibody) was thiolated using Traut's reagent and conjugated to mal-PEG−DSPE anchored into the liposome.[1868] Pastorino et al. created DOX-loaded liposomes decorated with Asp-Gly-Arg (NGR) sequences to target angiogenic endothelial cells. The liposomes were comprised of hydrogenated soy PC, cholesterol, PEG−DSPE, and mal-PEG−DSPE. Liposomes were synthesized using film hydration, and DOX was encapsulated during this process using a standard ammonium sulfate gradient. To enhance the accessibility of the NGR peptide, a cysteine residue was added to the amino terminus of the peptide, which was, in turn, coupled to the liposome via the mal-PEG−DSPE anchor.[1869] In some preparations, the authors also incorporated a radioactive lipid tracer, cholesteryl hexadecylether (^{3}H−CHE), to track the liposomes *in vivo*.

Carbodiimide chemistry is another coupling technique commonly utilized for the bioconjugation of lipid NPs. Again, the bioactive and the NP each display one of the amine and carboxyl groups needed to form the amide linkage. Weissig and colleagues utilized EDC to covalently couple α-ChT to an *N*-glutarylphosphatidylethanolamine lipid anchor, which was in turn incorporated into an egg PC comprised liposome.[1870] Recently, Edwards et al. developed dye-containing, SA-coupled liposomes using EDC chemistry for the purpose of detecting biotinylated nucleic acid targets. The liposomes, synthesized utilizing sonication and ether evaporation, were composed of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3[phosphor-*rac*-(1-glycerol)] (DPPG), and a carboxy-modified lipid derivative *N*-glutaryl 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (*N*-glutaryl-DPPE). The fluorescent dye, sulforhodamine B, was also encapsulated within the liposomes during synthesis. Coupling of SA to the liposome was achieved via *N*-glutaryl-DPPE using EDC.[1871]

Another lipid bioconjugation approach involves the attachment of bioactives to lipid NPs using hydrazide nucleophiles. In this approach, carbohydrate groups are oxidized to aldehyde groups, which in turn react with a hydrazide-functionalized anchor lipid or spacer group to yield a hydrazone bond. Oxidation is typically carried out using $NaIO_4$[1872] or galactose oxidase.[1873,1874] Similar to the other methods, each of the lipid anchors or the bioactives may potentially be modified with a reactive moiety; however, the bioactive (e.g., protein) is more commonly oxidized to produce the aldehyde groups, and the lipid anchor is modified with a hydrazide group.[1874−1876] Koning and co-workers employed this strategy in the development of multifunctional immunoliposomes for the treatment of colon cancer.[1872] The main components of the lipid carrier included egg PC, cholesterol, hydrazide-modified PEG−DSPE (Hz-PEG−DSPE), and methoxy-PEG−DSPE (mPEG−DSPE). The researchers created a lipophilic dipalmitoyl derivative of 5-fluorodeoxyuridine (FUdR-dP) by covalently coupling the chemotherapeutic 5-fluorodeoxyuridine prodrug to palmitoylchloride. This lipophilic derivative was then incorporated during synthesis to anchor the prodrug to the NP. In some instances, radioactive FUdR-dp was used to track the vesicles. After incorporation of the prodrug, the monoclonal CC52 antibody was coupled to the Hz-PEG−DSPE lipid anchors via the hydrazide moiety at the distal end of the PEG group and oxidized carbohydrate groups on the Fc region of the antibody.[1872] Potential limitations of this method include the necessity of oxidation, which may denature or inactivate some bioactives, along with low efficiency coupling.[1877]

Recently, researchers have utilized the acid-labile hydrazone bond to generate pH-sensitive linkages between lipid NPs and bioactives for "smart" drug delivery platforms that preferentially release drug in the acidic pH.[1878−1880] Biswas and colleagues developed a hydrazine-functionalized PEG−PE for reversible coupling of various bioconjugates.[1879] Monoclonal antinucleosome antibody 2C5 and antimyosin antibody 2G4, along with glycoproteins concanavalin A and avidin, were chosen as model ligands to validate the bioconjugation scheme. Briefly, liposomes were synthesized from egg PC and cholesterol using the film rehydration method. In some cases, rhodamine-labeled PE was also used for tracking. DSPE was thiolated using Traut's reagent and then reacted with MPBH to generate a reactive hydrazide group (DSPE−PEG-$CONHNH_2$). Antibodies and glycoproteins were incubated in $NaIO_4$ to produce reactive aldehyde groups and subsequently incubated with the hydrazide-modified DSPE anchor. Once the bioactives were coupled to DSPE−PEG-$CONHNH_2$, the polymer−lipid bioactive complex was embedded into preformed liposomes by lipid exchange.[1879]

Alternative bioconjugation strategies include standard cross-linking between amine and carboxyl groups, cross-linking between amines, glycolipid oxidation to produce aldehydes for subsequent linking, and click chemistries (see section 4.3.4.3). Homobifunctional cross-linking agents such as GA[1881] and suberimidate[1882] have been utilized to cross-couple amine groups associated with bioactives and lipid vesicles. It should be noted that there is a vast body of literature on the creation of carbohydrate-conjugated lipids (glycolipids), although the details of these techniques are beyond the scope of this review.[1883] The use of homobifunctional cross-linkers in glycolipid NP chemistry is rare due to the inability to control the homopolymerization reaction between the bioactive and lipid. Another potential issue arises with oxidation of the hydroxyl groups on glycolipids using $NaIO_4$, which can result in the generation of aldehyde groups that are capable of reacting with amine groups on the bioactive forming undesirable cross-linked species.

4.3.4.3. Click Chemistries. Researchers have also recently begun to utilize CuAAC and other "click" chemistries for the bioconjugation of lipid NPs due to the regiospecificity, chemoselectivity, and tolerance for a wide range of functional groups.[1884] Ma et al. explored azide reactivity in the development of a membrane-mimetic glycan array. A triphenylphosphine-functionalized DSPE−PEG was created by reacting DSPE−PEG-NH_2 with a 3-diphenylphosphino-4-methoxycarbonylbenzoic acid NHS-active ester. DSPC, cholesterol, and DSPE−PEG−triphenylphosphine were utilized to compose liposomal vesicles approximately 120 nm in size. The group then biotinylated or glycosylated the liposomes via the available phosphine on the DSPE−PEG−triphenylphospine, which reacted with an azide-functionalized biotin in a Staudinger ligation, see Figure 80.[1885]

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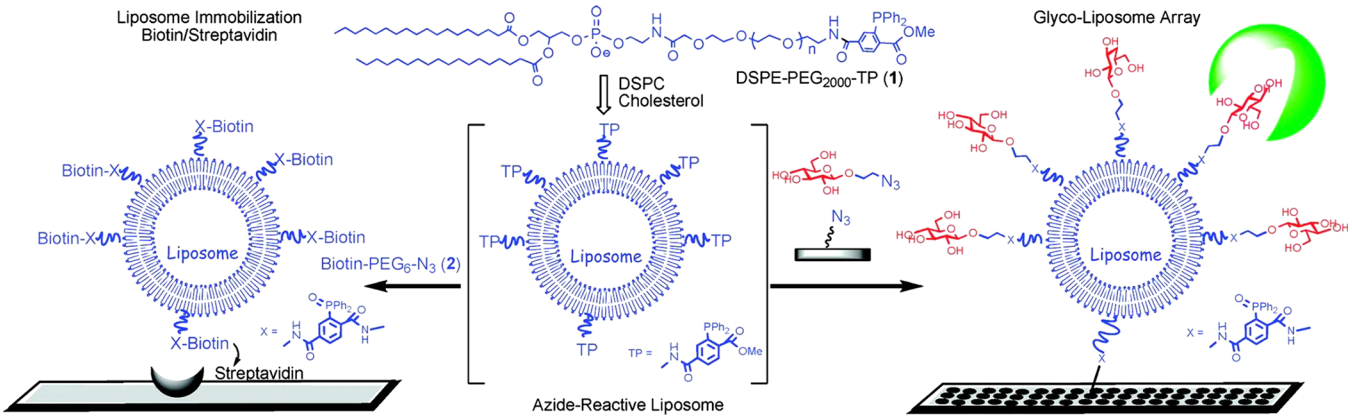

Figure 80. Schematic of chemically selective and biocompatible liposomal surface functionalization using biotin chemistry or immobilization and further glyco-functionalization via Staudinger ligation. Glyco groups are shown in red with recognition shown in green. Reprinted with permission from ref 1885. Copyright 2011 American Chemical Society.

Hassane and co-workers developed a novel azide−alkyne-based bioconjugation scheme to couple bioactives to the surface of preformed liposomes. An azide-functionalized derivative of mannose (azide−PEG−mannose) was coupled to preformed liposomes composed of DPPC, DPPG, and cholesterol via the alkyne-functionalized lipid anchor (2-(2-(2-(2,3-bis(hexadecyloxy)propoxy)ethoxy)-ethoxy)ethoxyethanolamine). The ligand conjugation occurs in a single under mild conditions, which makes it an ideal coupling technique for unstable bioactives, for example, enzymes.[1886] Similarly, Jolck et al. utilized CuAAC for the PEGylation of lipopeptides. An alkyne functionality was introduced to the peptide and served as a site-specific coupling site. The alkyne-modified peptide was then reacted with an α-methoxy-ω-azido-functionalized PEG group to generate the PEGylated lipoprotein. The multifunctional lipoprotein could be easily inserted into a lipid NP during synthesis or via exchange/insertion techniques.[1887]

Van Lengerich and co-workers developed a novel method to couple DNA conjugated vesicles to DNA-modified supported lipid bilayers using CuAAC chemistry.[1888] 1,2-*O*-Dioctadecyl-*rac*-glycerol was reacted with 2-cyanoethyl-*N*,*N*-diisopropyl-chlorophosphoramidite to produce a phosphoramidite-functionalized lipid anchor. DNA sequences were then coupled to the lipid anchor using phosphoramidite chemistry. In different instances, azide and alkyne groups were also coupled to the distal terminus of the DNA strands using azidobutyrate−NHS ester or propargyl-2-PEG-1-NHS ester, respectively. The group also synthesized an azide-functionalized 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (azido-POPE), which was incorporated into liposomal vesicles along with egg PC using film rehydration. Diffusing liposomal vesicles were then tethered to supported lipid bilayers via the CuAAC reaction and templated by DNA hybridization.[1888] Cavalli and colleagues exploited FRET to validate the effectiveness and efficiency of the CuAAC reaction for later coupling of bioactives to liposomal surfaces.[1889] Vesicles were synthesized to contain both an alkyne and lissamine−rhodamine-functionalized 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (alkyne-DOPE, LR-DOPE). An azide-functionalized *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amine dye was then coupled to preformed liposomes via the alkyne-modified DOPE. The observance of FRET between the dye clicked to the liposomal surface and the constituent LR-DOPE was used to validate the coupling technique and measure the reaction time.[1889]

4.3.4.4. Lipid-Based Nanoparticle Conjugates. Researchers have recently started to combine lipid NPs with other types of NPs in the development of synergistic, multifunctional platforms for applications that range from drug delivery to electrochemistry. QDs, for example, have attracted substantial interest due to their advantages over conventional imaging agents. Their utility for *in vivo* imaging applications has, however, been hampered by some concerns about biocompatibility and toxicity. Furthermore, as-synthesized QDs are typically not soluble in aqueous media.[30,552] By conjugating or entrapping QDs within lipid NPs, these potential limitations can be mitigated. The native QD may be easily solvated when confined within the hydrophobic matrix of a lipid NP without requiring any surface modification. Furthermore, the lipid NP provides a biocompatible coating for the QD that appears to limit systemic toxic exposure, decrease aggregation, and enhance the circulation time of the QD. Several groups have developed QD−lipid NP platforms for a multitude of applications.[1776,1777,1890−1892] Weng et al. developed DOX-loaded, QD-conjugated, anti-HER2 scFv immunoliposomes for cancer theranostic applications. Carboxylated CdSe/ZnS QDs were covalently coupled to a PEG−DSPE anchor using EDC chemistry. The QD-functionalized PEG−DSPE was then incorporated into the liposomes during synthesis. This resulted in specific targeting of the QDs in HER2-overexpressing SK-BR-3 and MCF-7/HER2 cells along with enhanced circulation times in athymic mice.[1777] Sigot and colleagues developed biotinylated, EGF decorated liposomes in which QDs were both conjugated to the surface and entrapped within the core. ITK-carboxyl biotinylated QDs with an emission maximum at 655 nm were entrapped within the liposomes during synthesis (film rehydration), while QDs with an emission maximum at 525 nm were conjugated to the surface of the preformed biotinylated liposomes by a SA linker. The group demonstrated that the particles were highly efficient at targeting, and breaching, EGFR-expressing tumor cells.[1776]

Several research groups have exploited the advantages of both lipid NPs and AuNPs in the development of novel multifunctional platforms.[1893−1895] Pornpattananangkul et al. coupled carboxyl-modified AuNPs to the surface of preformed liposomes via electrostatic interactions with the cationic headgroup of the phospholipids. The release of the AuNPs

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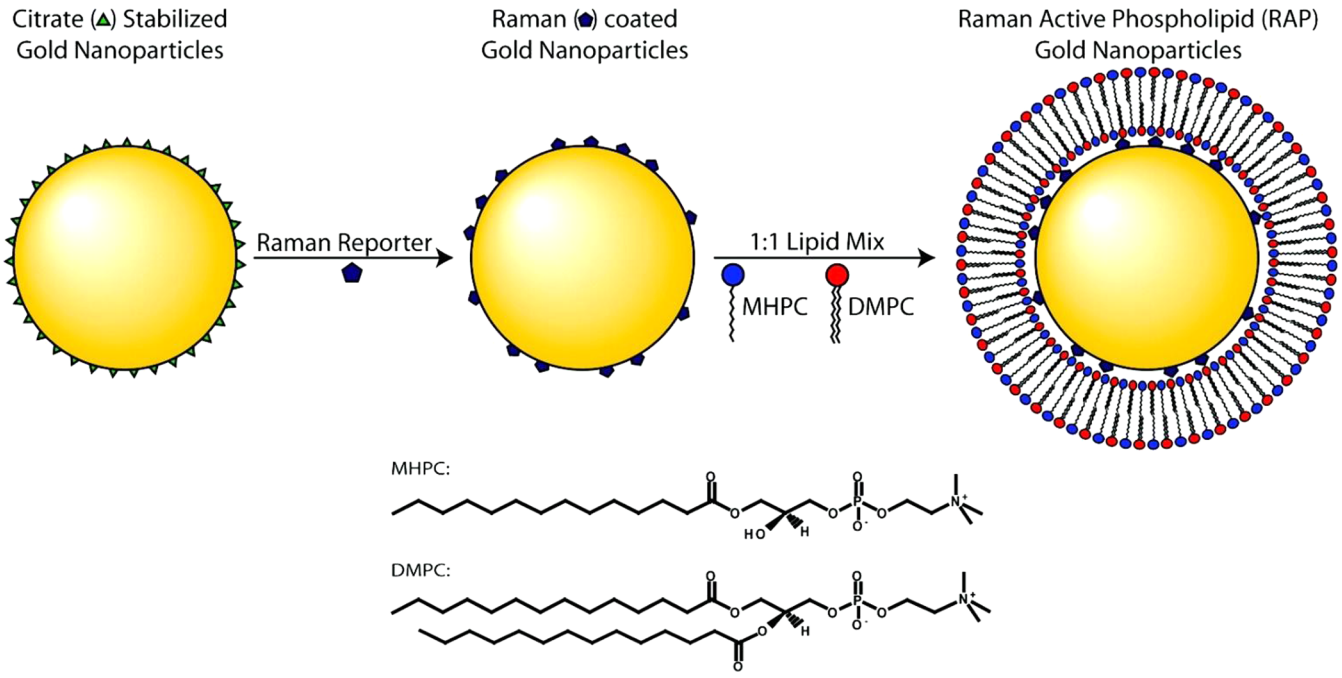

Figure 81. Schematic showing the synthesis of the Raman-active phospholipid AuNPs. The Raman-active species is the dye crystal violet. Reprinted with permission from ref 1897. Copyright 2010 American Chemical Society.

was triggered when the pH of the solution dropped below the pK_a of the carboxyl groups.[1896] Tam and colleagues entrapped AuNPs within liposomal vesicles to create Raman-active structures with enhanced biocompatibility.[1897] The structures were formed by mixing 1,2-dimyristoyl-*sn*-glycero-3-PC, 1-myristoyl-2-hydroxy-*sn*-glycero-3-PC, and crystal violet-coated 60 nm AuNPs, resulting in the encapsulation of the AuNPs within a liposomal shell, see Figure 81.[1897] Paasonen and colleagues modified the surface of AuNPs with either hydrophilic or hydrophobic coatings in order to load the NPs into the aqueous core or lipid bilayer of liposomes, respectively. The researchers were able to trigger payload release using UV light. It was theorized that the AuNPs were able to disrupt the phase and permeability of the lipid bilayer through the absorption of the UV energy and subsequent local heating.[1898]

IONPs are another type of NP material commonly incorporated into lipid NPs. For example, Hsu and co-workers created SLNs embedded with superparamagnetic IONPs (γ-Fe_2O_3), prepared using high-pressure homogenization, for hyperthermic applications. When introduced to a RF field, the particles increased the temperature of the surrounding solution from 37 to 50 °C in 20 min.[1899] Senpan et al. developed multifunctional emulsions embedded with Fe_2O_3 and Fe_3O_4 IONPs for theranostic applications. The colloidal IONP emulsions were synthesized using oleic acid-coated IO suspended in almond oil and encapsulated in a surfactant monolayer consisting of L-α-phopshatidylcholine, cholesterol, and DPPE.[1900] Cormode and co-workers developed a novel multifunctional HDL mimic where the hydrobophobic core entrapped either IONPs, AuNPs, or QDs for MRI, CT, and fluorescence imaging, respectively. The HDL vesicles were composed of 1-myristoyl-2-hydroxy-*sn*-glycero-3-PC, Gd–DMPE–DTPA, DSPE–PEG, rhodamine-conjugated DMPE, and apolipoprotein A, see Figure 82.[1892]

4.3.5. Other Nanoparticle Materials. *4.3.5.1. Liquid Crystal Nanoparticles.* Liquid crystals (LCs) are unique materials that manifest properties of both a conventional liquid and a solid crystal. The many different types of liquid crystalline materials and their unique properties are extensive and beyond the scope of the current discussion.[1901,1902] However, liquid crystal materials have only recently been incorporated in NPs or nanocolloids and their utility in a bioconjugate structure is just starting to be explored. Spillmann and co-workers designed an approach that allowed the self-assembly of polymerizable liquid crystals to control the molecular aggregation of a fluorescent molecule in a nanocolloid.[1903] A perylene derivative (PERC11) was synthesized to display terminal vinyl groups on either end of the molecule and incorporated into colloids using a two-phase miniemulsion followed by thermal polymerization. This yielded NPs with sizes ranging from 50 to 300 nm in diameter, and these were then size-selected using centrifugation to yield a sample displaying far lower polydispersity (<10%). More importantly, by controlling the concentration and aggregation of the internal perylene dye, the authors could also control the resulting emission colors, see Figure 83. To provide aqueous solubility and bioconjugation, a carboxylated surfactant was incorporated during synthesis, and standard EDC chemistry was applied to covalently attach NeutrAvidin to the colloidal surface. This allowed for binding to a biotinylated antibody and subsequent demonstration in a model sandwich immunoassay targeting the biothreat agent ricin. Interestingly, the authors also utilized biotinylated phycoerythrin to bind to unoccupied NeutrAvidin sites on the particle and obtained a 4-fold increase in the overall fluorescent signal. Although not exploited here, the polarization properties of LCs suggest that these NPs may be useful as labels in polarized light microscopy and other bioassay formats that also depend on polarization.

4.3.5.2. Opal Nanoparticles. Natural and synthetic opals are three-dimensional periodic colloidal crystals where a significant portion of the volume is air (≥20%). This resulting periodicity of high and low dielectric constants causes certain wavelengths of light to diffract in a manner that is described as a "pseudo-band gap" rather than the complete band gap associated with a

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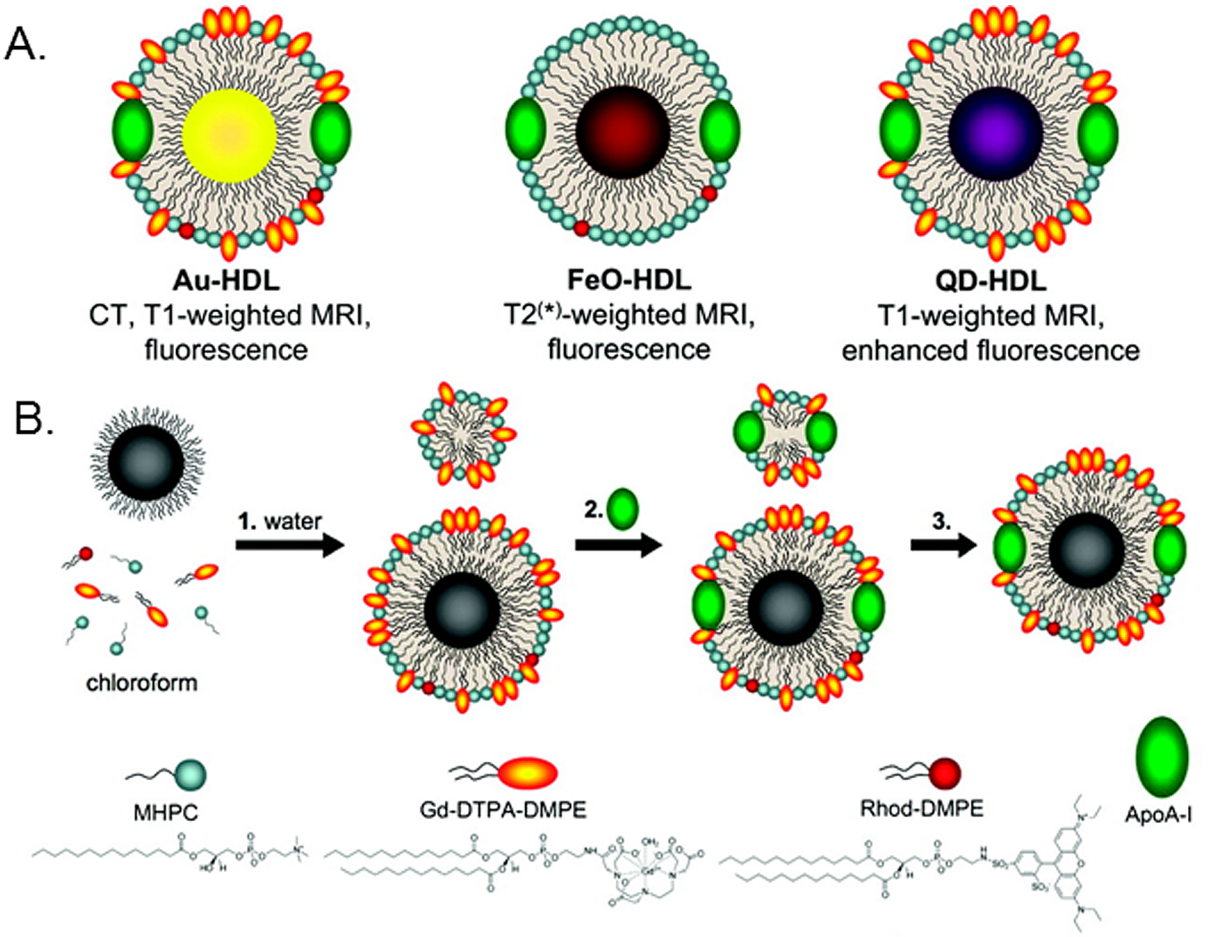

Figure 82. Nanocrystalline core HDL. (A) Schematic depiction of the different agents synthesized. (B) Summary of the synthesis procedure of the agents, where (1) the phospholipids and nanocrystals in chloroform are added to water, (2) apoA-I is added, and (3) the "empty" particles are removed. Reprinted with permission from ref 1892. Copyright 2008 American Chemical Society.

photonic crystal.[1904,1905] Inverse opals are prepared by filling the voids in a synthetic opal template with another material, followed by removing the original template to yield a macroporous close-packed arrangement of air spheres that also have photonic crystal properties.[1904,1905] Natural opal is a form of amorphous silica, whereas synthetic opal is often synthesized from polystyrene or PMMA. Inverse opal can be derived from SiO_2, TiO_2, CeO_2, and polymers such as polyacrylamide. The aforementioned photonic properties have suggested some utility for colloidal and inverse opals in biological applications, and preliminary bioconjugation reports have started to appear.

Lange et al. synthesized colloidal photonic crystals from monodisperse core−shell particles where the shell included glycidylmethacrylate as the monomer.[1906] The particles were then crystallized into artificial opals, which dramatically improved their mechanical stability, and reacted with a commercial NTA precursor and triethylamine to yield NTA-functionalized NPs. The NTA groups were used to immobilize histidine-appended silicatein, a sponge-derived enzyme that catalyzes biosilica formation from monomeric silicon compounds. The silicatein−opal construct was then utilized as a nanoreactor that could synthesize and immobilize AuNPs from auric acid precursors directly onto the surface of the core−shell colloid. Although the opal photonic properties were not directly exploited in this construct, this example does suggest the possibility of a hybrid material with access to the unique properties of both AuNPs and opals. In contrast to this synthetically intensive approach, Swinerd et al. created bioderived inverse opals directly from regenerated silk fibroin.[1907] Rather than focus on optical properties, they found the silk inverse opals to be incredibly elastic and able to withstand and recover from large compressive loads by a reversal of pore deformation, along with demonstrating what they term "super-hydrophobicity". Here, the results suggest that these materials have strong potential for biocompatible elastic scaffolds, drug release, and self-cleaning applications. The ability to "dope" these silk-derived opal colloids with other biologicals such as peptides or drugs would certainly help achieve some of the potential noted. Lastly, the Asher group has been quite prolific at developing nanoscale opal sensors, although they are more geared toward array formats.[1908−1910]

4.3.5.3. Hybrid Nanoparticle Materials. There are a wide array of composite NPs that are synthesized or constituted from two or more disparate materials, and these are difficult to discretely classify in a materials-directed scheme. Rather than metal alloys or multilayer core−shell−shell structures, these composites bring together different NP materials in sometimes unique combinations. The diversity of these materials, the different chemistries applied to their bioconjugation (albeit mostly analogous to those already described herein), and their final applications are again far beyond the scope of this review. Several prototypical examples of these NPs are, however, briefly

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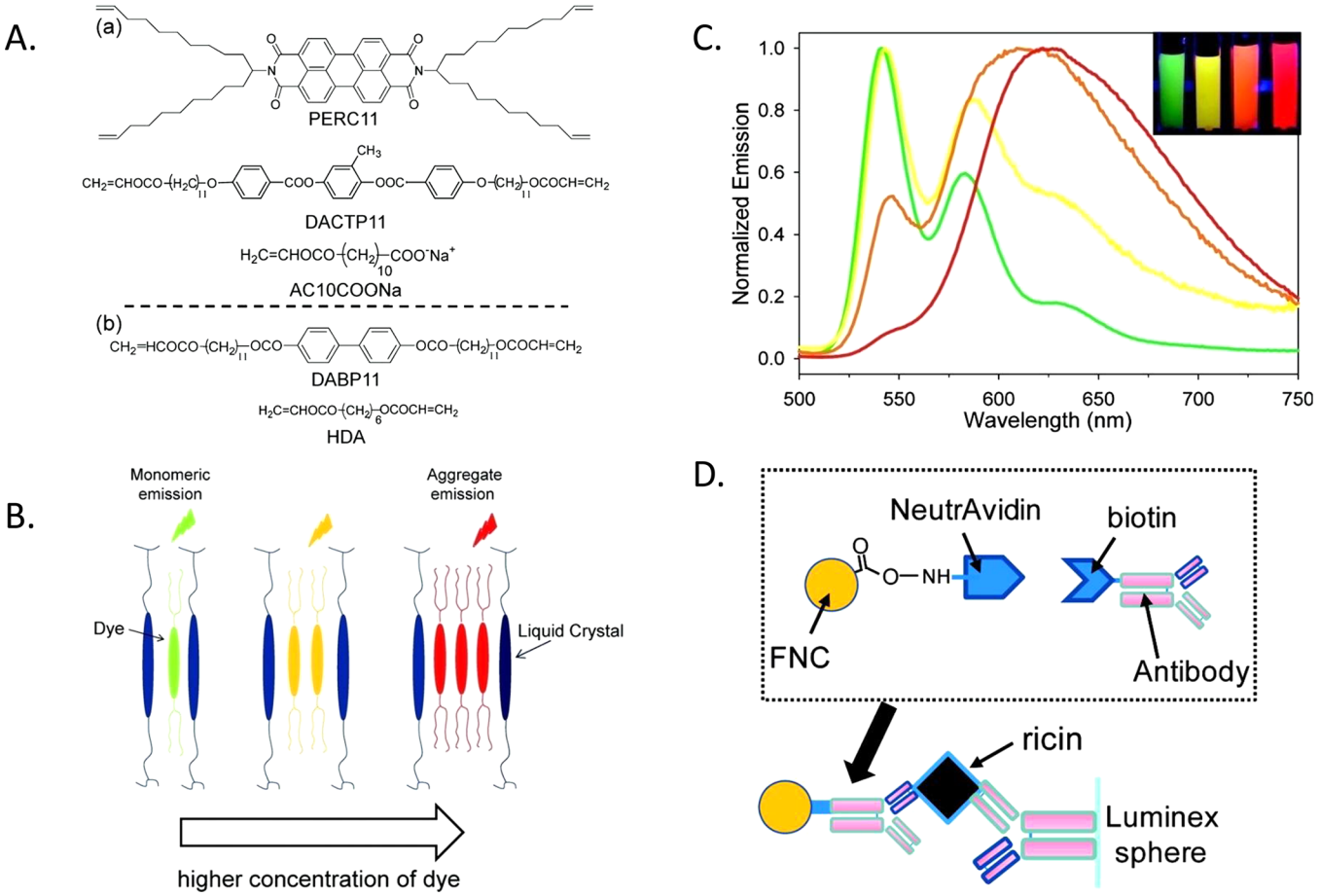

Figure 83. Liquid crystalline NPs. (A) Molecular structures of the components of liquid crystal NPs. (a) Perylene tetracarboxylate diimide derivative PERC11, liquid crystalline diacrylate cross-linking agent DACTP11, and polymerizable carboxylate surfactant AC10COONa. (b) Alternative cross-linking agents DABP11 and HDA. (B) Schematic of dye and cross-linking agent interaction. Increasing the dye to cross-linker ratio leads to aggregate formation and a red shift in the emission spectrum. (C) Increasing the ratio of PERC11 to cross-linker in the nanocolloids controllably red shifts the emission spectra. Emission spectra of populations containing 0.6 (green), 1.5 (yellow), 2.5 (orange), and 4.8 (red) mol % of PERC11. (D) Schematic representation of fluorescent nanocrystal (FNC)−NeutrAvidin (FNC−NA) coupling to biotinylated anti-ricin antibody to complete a sandwich immunoassay. Reprinted with permission from ref 1903. Copyright 2009 American Chemical Society.

reviewed here for illustrative purposes. The reader will note that many of these NMs are designed to have some non-standard properties that would not be available within an assembly derived from a "single" material. These include, for example, extreme surface roughness, a large cargo carrying capacity, multimodal spectroscopic properties, or multiple surface-displayed moieties at differing valences.

Lee's group used a bioinspired approach to synthesize "nanocorals" for cellular targeting and possible sensing applications.[1911] The nanocorals were prepared starting from close-packed PS nanosphere arrays that underwent oxygen plasma etching to induce shrinking and deep surface trenches. The arrays were then covered with a specific thickness of Au and released by sonication. Anti-HER2 antibodies were adsorbed to the PS template, and the materials demonstrated specific binding to a BT474 breast cancer cell line. The antibody adsorption to the PS component is extremely user-friendly since it only requires incubation and washing steps. The authors further suggest that the added Au surface, with its intrinsic roughness, could also provide for surface-enhanced Raman spectroscopic imaging. Kong et al. also used PS as a templating material to create magnetically vectored nanocapsules that were able to penetrate tumors and release drug on demand.[1912] IONPs (10 nm) were incorporated into PS spheres, which were then encapsulated in a silica shell. The PS was either dissolved or burnt away, and the anticancer drug camptothecin was coencapsulated within the particle. The magnetic properties were then exploited in two ways: the first was to actively direct the capsules toward a colony of tumor cells; the second was to drive the release of the drug through the minimally permeable silica shell. Although the functional capability of this NM is complex, the bioconjugation or drug encapsulation portion is quite simple. In contrast, Chen and colleagues used a very different approach to create similar magnetic nanocapsules.[1913] As shown in Figure 84, ellipsoid Fe_2O_3 NPs were first coated by both solid and mesoporous silica using standard sol−gel chemistry. The NPs were then etched in an ammonium solution to prepare what they term "rattle" or "yolk−shell" type materials. Following a H_2 reduction step, the Fe was converted to magnetic Fe_3O_4, and the chemotherapeutic DOX was coencapsulated by electrostatic interactions with the silica surface and pores. The resulting construct was shown to provide both MRI contrast and toxicity toward cancer cells *in vitro* and in a mouse tumor graft. Shi's group also utilized PS NPs as a scaffold and similarly encapsulated Fe_2O_3 nanocrystals for hyperthermic treatment.[1914] In this case, amine-functionalized

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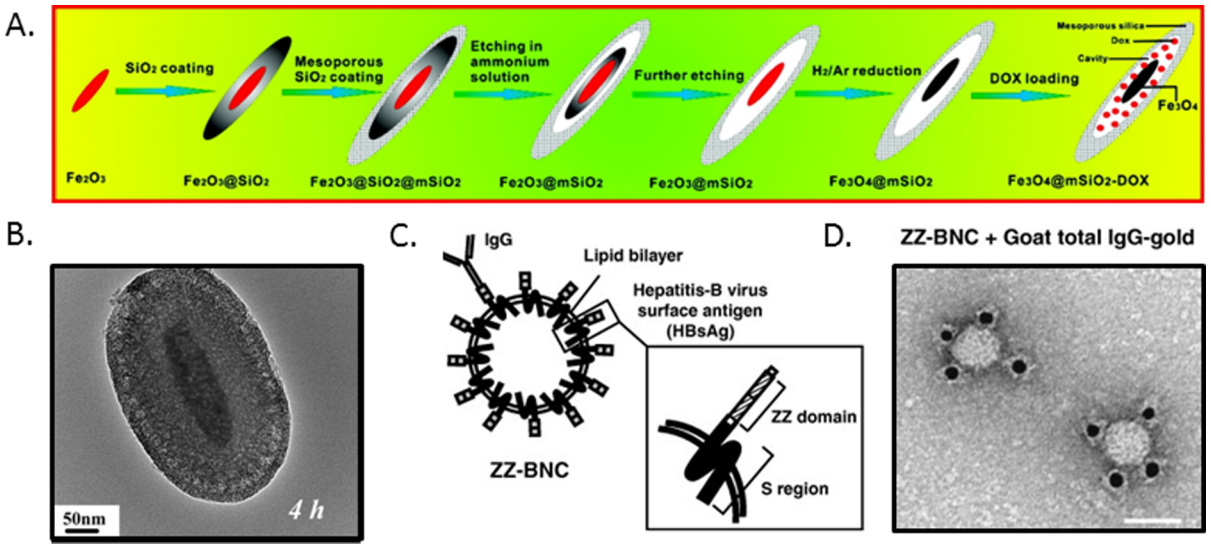

Figure 84. Mesoporous NPs. (A) Schematic for the preparation of hollow core/shell structured mesoporous drug-loaded nanocapsules. (B) TEM images of magnetic mesoporous composites obtained by etching in an ammonium solution for 4 h. Reproduced from ref 1913. Copyright 2010 American Chemical Society. (C) Schematic structure of a ZZ-bionanocapsule (BNC). (D) TEM images of ZZ-BNC conjugated with 10-nm AuNP-labeled goat total IgG used for visualization purposes. Scale bar = 40 nm. Reprinted from ref 1916, Copyright 2010, with permission from Elsevier.

QDs were conjugated to PEO on the surface of the PS by EDC−NHS chemistry to provide imaging capability. PAX as then loaded onto the surface using a layer of biodegradable PLGA. EDC−NHS chemistry was also used to add ethylenediamine to the surface, which provided amine groups for attaching anti-PMSA antibodies for targeting with the same chemistry. The resulting nanocarrier system thus encompassed five different materials, each providing a different desired utility.

In contrast to the previous examples, Paulo et al. used silica NPs as a building block for creating antifungal materials.[1915] The NPs were modified with APTES and 3-(trihydroxysilyl)-propylmethylphosphonate. Dextran aldehyde was oxidized to allow immobilization of amphotericin B via imine bond formation between the polymer and amine group of the drug. The remaining aldehydes were used to form imine bonds with the amine groups on the surface of the NP. $NaBH_3CN$ was then used to reduce the imine bonds to more stable secondary amines. These functionalized NPs demonstrated fungicidal properties against several strains of yeast, were more potent than colloidal Ag, and could be reused up to five times without losing activity. Iijima and colleagues used a biological route to develop yeast-derived hollow bionanocapsules with a diameter of 30 nm.[1916] The bionanocapsule is essentially a liposome that displays HBV surface antigen L proteins fused in tandem to a sequence of the IgG Fc-interaction region from protein A (the ZZ domain), see Figure 84C. For visualization purposes, Figure 84D highlights an example where the bionanocapsule was functionalized with AuNP-labeled goat IgG. This allows the composite bionanocapsule to bind antibodies and be applied in ELISAs. More interestingly, the capsules could also carry therapeutics, which suggests drug delivery applications.

Perhaps one of the more elegant hybrid materials was recently developed by the Brinker group.[1917] They reported the assembly of NP-supported lipid bilayers (protocells) that synergistically combined properties of both liposomes and nanoporous silica particles, see Figure 85. A variety of standard bioconjugation chemistries, chemical cross-linkers, lipid assembly, and encapsulation techniques were utilized to prepare the final conjugates. The authors modified the construct with a peptide that targets human hepatocellular carcinoma, which resulted in a 10 000-fold greater affinity for these cells over normal hepatocytes, endothelial cells, or immune cells. The protocells were also loaded with diverse combinations of therapeutics (e.g., drugs, siRNA, and toxins) and contrast agents (e.g., QDs) and further modified with other fusogenic peptides to promote endosomal escape and nuclear accumulation of selected cargos. Indeed, the enormous cargo capacity of these materials enabled a single protocell loaded with a drug cocktail to kill a drug-resistant human hepatocellular carcinoma cell, representing a million-fold improvement over comparable liposomes.

Tseng's group combined a supramolecular synthetic approach with a digital microreactor to program structural and functional diversity into a library of complex multifunctional NPs that were formed completely by self-assembly.[1918] The molecular building blocks included cyclodextrin-grafted branched PEI, adamantanamine (Ad)−polyamidoamine dendrimer (Ad−PAMAM-3), Ad−PEG, RGD−PEG−Ad, and TAT−PEG−Ad, as well as plasmid eGFP- and firefly luciferase-encoding DNA. These were all intended to function in concert to both create and vary the desired NPs, see Figure 86. The synergistic cyclodextrin−PEI and Ad−PAMAM were used to construct the cationic hydrogel networks that can encapsulate anionic DNA forming the cores of the NPs. Therefore, the DNA loading capacity was dependent on the net positive charges embedded in the hydrogel networks. Second, Ad−PEG serves as a capping and solvation reagent that constrains continuous growth of the DNA-encapsulated hydrogel networks while also conferring aqueous solubility, structural stability, and some surface passivation. The two functional RGD−PEG−Ad and TAT−PEG−Ad ligands can be incorporated onto the surfaces of the NPs via dynamic exchange to enable targeted delivery to cell populations expressing appropriate integrin receptors and facilitate cellular uptake, respectively. The authors demonstrated that systematically altering the mixing ratios of the five molecular building blocks and DNA in the microreactor could imbue the NPs with programmable and distinct structural or functional properties, such as size or surface chemistry, that reflected the underlying combinatorial library. Optimized NPs

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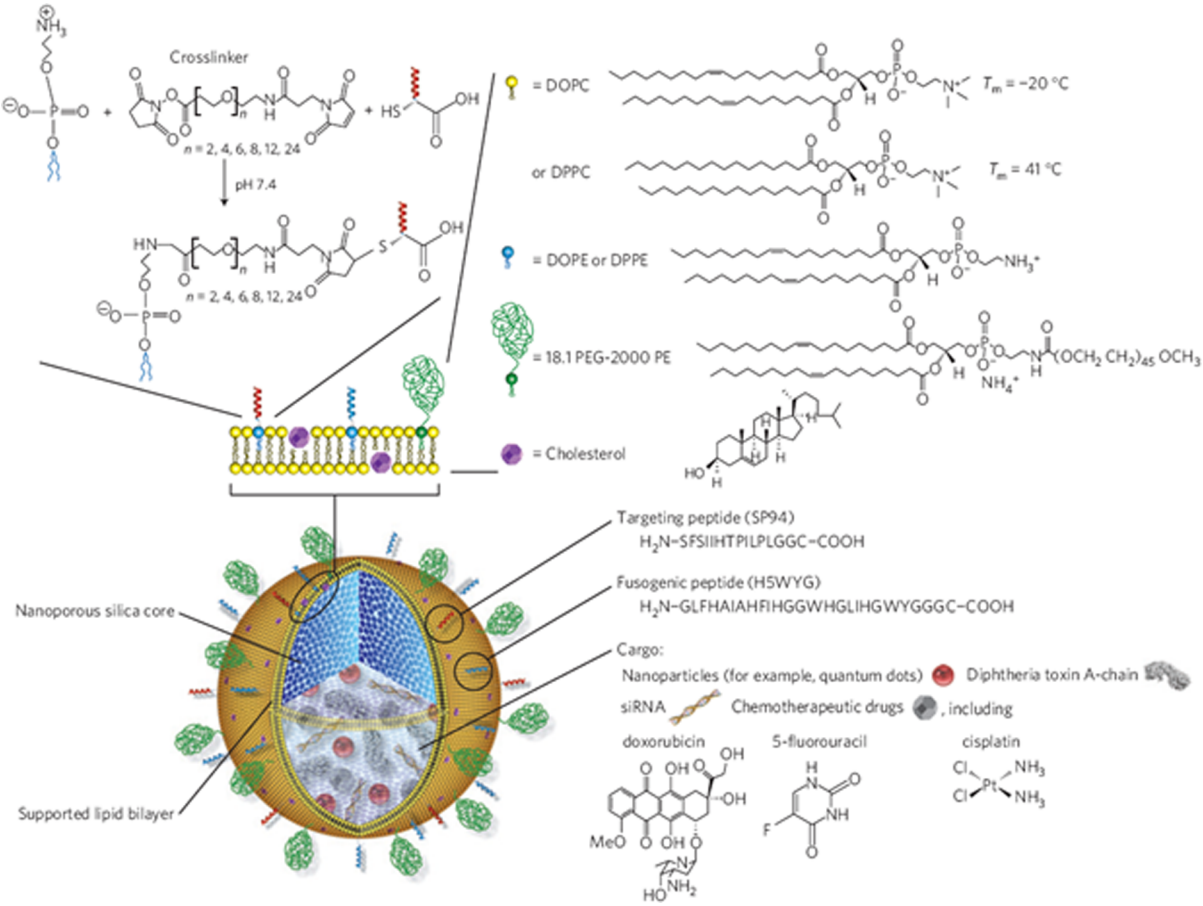

Figure 85. Schematic illustration of the nanoporous particle-supported lipid bilayer (SLB), depicting the disparate types of therapeutic and diagnostic agents that can be loaded within the nanoporous silica core, as well as the ligands that can be displayed on the surface of the SLB. Targeting and fusogenic peptides are chemically conjugated to PE (DOPE or DPPE), present in the SLB at 1−5 wt %, by a heterobifunctional cross-linker with a PEG spacer arm (nD24). The SLB, composed of either fluid (DOPC) or nonfluid (DPPC) zwitterionic PC lipids with 30 wt % cholesterol, is further modified with 5 wt % PEG-2000 PE to enhance colloidal stability and decrease nonspecific interactions. Reprinted by permission from Macmillan Publishers Ltd., *Nature Materials*, ref 1917, Copyright 2011.

were then delivered to a number of different tumor cell lines where they demonstrated significantly higher transfection efficiencies than controls utilizing Lipofectamine or PEI. The fact that this diversity can be achieved with noncovalent chemistry and a mix of such diverse and modular building blocks confirms that the power of combinatorial chemistry can be implemented to provide potent NP constructs.

4.3.5.4. Top-Down Fabricated Nanoparticle Materials. In contrast to many of the "bottom-up" fabrication processes that are the basis for most of the synthetic NMs discussed here, a review of this field would not be complete without mentioning the strong potential offered by "top-down" NP synthesis. As the name implies, bottom-up approaches involve molecular or atomic scale synthetic chemistry, that is, growing a nanocrystal, while the converse top-down methodology allows the processing of a given NM on the desired size scale.[1919] Bottom-up NMs are most-often spherical in shape with significant levels of polydispersity not being uncommon during synthesis. For more complex control over shape, far more complex multistep synthetic chemistries are typically needed.[1920] The primary benefits of top-down chemistry include synthesis of complex shape-specific materials that have reproducible sizes and minimal size distributions.[1919,1921]

Top-down engineering of complex yet biocompatible NMs is typified by the seminal work of the DeSimone group.[1919,1921,1922] Their particle replication in nonwetting templates (PRINT) approach has proven quite versatile and adept at providing useful NMs derived from poly(ethylene glycol diacrylate), triacrylate resin, poly(lactic acid), poly(pyrrole), PLGA, and even proteins such as albumin.[1922,1923] Figure 86C,D provides an overview of the PRINT process and some micrographs of representative materials synthesized using this methodology, respectively. PRINT-derived materials appear to be particularly useful for drug delivery given their exceptionally high loading capacity.[1922] Similarly, the work of the Mitragotri laboratory is also prominent in this field, and their work is exemplified by generating NPs of various shapes for understanding phagocytosis and creating red blood cell mimicking NMs.[1924,1925]

5. DEVELOPING BIOCONJUGATION CHEMISTRIES

These strategies encompass more complex second or third generation attempts at NP bioconjugation and are thus a more focused development in bionanotechnology. That is, they exploit multiple established or cutting edge materials, chemistries, and biological components to achieve biomolecular attachment to NPs. They are classed into two functional categories: bioconjugation of the NP by direct chemical modification and enzyme-catalyzed ligation.

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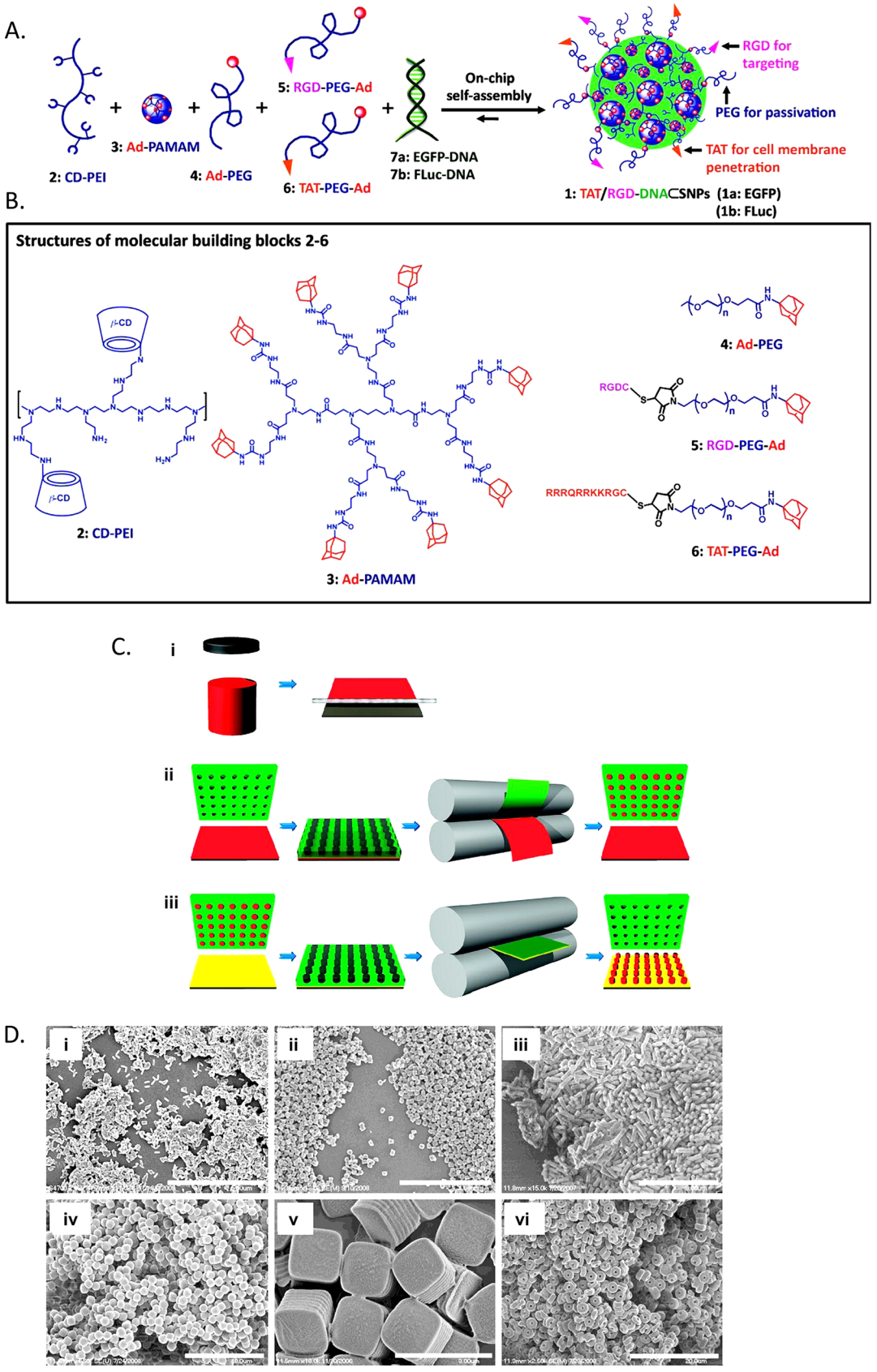

Figure 86. DNA encapsulated supramolecular NPs. (A) Graphical schematic of the self-assembly approach for producing a combinatorial library of DNA encapsulated supramolecular NPs in which a broad structural and functional diversity can be programmed by systematically altering the mixing ratios of the five functional molecular building blocks (B), cyclodextrin-grafted branched polyethylenimine, CD-PEI (**2**), adamantanamine–polyamidoamine dendrimer, Ad–PAMAM (**3**), Ad–PEG (**4**), RGD–PEG–Ad (**5**), and TAT–PEG–Ad (**6**), as well as DNA plasmid encoding eGFP (**1a**) and firefly luciferase, FLuc (**1b**). Reprinted with permission from ref 1918. Copyright 2010 American Chemical Society. (C) The PRINT

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Figure 86. continued

Process: (i) Delivery sheet casting. PLGA and docetaxel are dissolved in DMF and DMSO (4:1 solvent ratio) to create a true solution (red). A mayer rod is then used to draw a film from this solution on a PET substrate. The solvent is removed under heat generating a solid state solution film referred to as the delivery sheet, because it will deliver the composition to the mold. (ii) Particle fabrication. A perfluoropolyether elastomeric mold (green) is brought into contact with a PLGA (red) film, passed through a heated nip (gray) and split. The cavities of the mold are filled. (iii) Particle harvesting. A filled mold is brought into contact with a high energy film or excipient layer (yellow) and passed through the heated nip without splitting. After cooling, the mold is removed to reveal an array of particles on the high-energy film or excipient layer. (D) Representative PLGA nano- and microparticles fabricated by the PRINT process: (i) 80 nm × 320 nm cylinders; (ii) 200 nm × 200 nm cylinders; (iii) 200 nm × 600 nm cylinders; (iv) 1 μm sphere approximates; (v) 2 μm cubes with ridges; (vi) 3 μm particles with center fenestrations. Scale bars: (i) 5 μm, (ii) 4 μm, (iii) 3 μm, (iv) 10 μm, (v) 3 μm, and (vi) 20 μm. Reprinted with permission from ref 1922. Copyright 2011 American Chemical Society.

5.1. Chemical Bioconjugation

Direct chemical modifications include "click" chemistry, FlAsH/CrAsH approaches, and native or chemoselective protein- or peptide-based ligation chemistries. Some basic aspects of these methods were discussed in sections 2.8−2.10 and throughout where appropriate (see also ref 14 for a recent review). Several other recently described chemistries that fall under similar criteria and that have only seen limited NP application are also mentioned here.

5.1.1. Tetrazine Chemistry. Tetrazines are six-membered aromatic ring compounds that contain four nitrogen atoms within the ring and encompass many different isomers and derivatives. They are considered somewhat unstable and have previously found application in energetic chemistry. Weissleder's group pioneered the use of these compounds in NP modification and bioconjugation based upon an irreversible inverse Diels−Alder reaction with strained dienophiles. In one of the first iterations, the authors synthesized the tetrazine derivative 3-(4-benzyl-amino)-1,2,4,5-tetrazine (BAT), which demonstrated good aqueous stability, and used it in conjunction with the strained olefin norbornene for QD modification, see Figure 87A.[1926] To facilitate this, the QDs were first surface-modified with a polymeric imidazole ligand that had pendant amines functionalized with an NHS ester derivative of norbornene. An NHS ester activated BAT was prepared for cognate labeling of EGF protein and allowed subsequent QD−EGF conjugation. The high reaction rate of this conjugation chemistry allowed the authors to demonstrate labeling of extracellular EGFRs with preformed QD−EGF conjugates and also demonstrate an *in situ* conjugation to tetrazine−EGF prebound onto live cells, see schematics in Figure 87B. Further studies utilized *trans*-cyclooctene-modified antibodies for conjugation to tetrazine-modified magnetofluorescent NPs or QDs, see Figure 87C.[1927] These conjugates demonstrated intracellular conjugation in semipermeabilized cells, helping to identify protein biomarkers and phosphoprotein signal mediators within both the cytosol and nucleus. The authors also noted a site-specific amplification of NM binding within the cellular context. Mehl recently reported on a stable tetrazine-containing amino acid that could be site-specifically encoded at any location in a protein using an amber codon approach.[1928] Utility was highlighted by ligation to conformationally strained *trans*-cyclooctenes both *in vitro* and *in vivo*. This latter approach may allow for the production of proteins with site-specific tetrazine moieties preintroduced before purification or coupling to appropriately modified NPs *in vivo*. The recent description of [4 + 1] cycloaddition where isonitriles were coupled to tetrazines in aqueous media is also very promising.[1929]

5.1.2. Hydrosilylation and Epoxy−Alkene Linkages. Alkenes and alkynes are known for their capacity to react with unoxidized H-passivated Si surfaces.[1190,1930] These reactions can be catalyzed by UV light or heat.[1931,1932] Hydrosilylation has found extensive use in functionalizing Si surfaces or NPs and, to a lesser extent, H-terminated Ge (i.e., hydrogermylation) and diamond materials.[1933] In the context of Si, Ge, or diamond NPs, small bifunctional molecules with a terminal alkene or alkyne have the potential to be used similarly to the bifunctional thiolates widely used to derivatize AuNPs and II−VI QDs. For example, Erogbogbo et al. modified luminescent Si NPs with undecylenic acid via hydrosilylation and further conjugated the NPs with lysine, FA, anti-mesothelin, or apo-Tf using standard EDC/NHS chemistry.[1181] Alternatively, bifunctional epoxy−alkenes can provide reactivity without activation, as recently reported by Jeanquartier and colleagues.[1934] The authors utilized 1,2-epoxy-9-decene to initially functionalize the surface of a Si wafer and linked the epoxy function to an esterase enzyme derived from the pathogenic bacteria *Burkholderia gladioli*. Characterization with XPS and FTIR confirmed the linkage, and activity was verified with a colorimetric substrate. Although not demonstrated for NPs *per se*, the strong potential arises from the potential ability to functionalize Si, Ge, and diamond NPs in a straightforward two-step process. The liability is the poor chemoselectivity of the epoxy group, which is well-known to react with nucleophiles such as -SH, -NH_2, or -OH, and potentially even -COOH, in a ring-opening process.[80] This can result in the nonspecific attachment of a protein, which would yield a heterogeneous orientation around a NP.

5.1.3. Hydrazide Reactive Peptide Tags. In pursuit of new site-specific protein labeling reactions, Weiss's group recently described a phage selection process that isolated 20-mer peptide sequences with nucleophilic characteristics toward certain types of hydrazide derivatives.[1935] Using reaction-based selection criteria, the authors isolated reactive carbonyl-containing peptides in a phage display library by screening against a Boc-(*tert*-butyloxycarbonyl)-hydrazide modified Tentagel resin. Several peptide sequences were derived, and the authors surmised that these interacted with hydrazides in various ways, including (i) oxidation of the peptide followed by hydrazone bond formation, (ii) high-affinity noncovalent interactions, (iii) hydrazide oxidation followed by peptide nucleophilic attack, and (iv) peptide nucleophilic attachment on the hydrazide carbonyl functional group, which displaced the hydrazine. The hydrazide reactive peptides were then fused to lysozyme and demonstrated specific covalent labeling to biotin or rhodamine B hydrazide derivatives in crude bacterial lysates, confirming the ability to accomplish site-specific modifications. These results, along with the fact that a hydrazide group on the micrometer particles in a Tentagel resin could interact with the phage by some or all of the aforementioned mechanisms, suggest that scaling down to NP size may derive the same results. This could provide a rather simple chemical strategy for site-specifically attaching

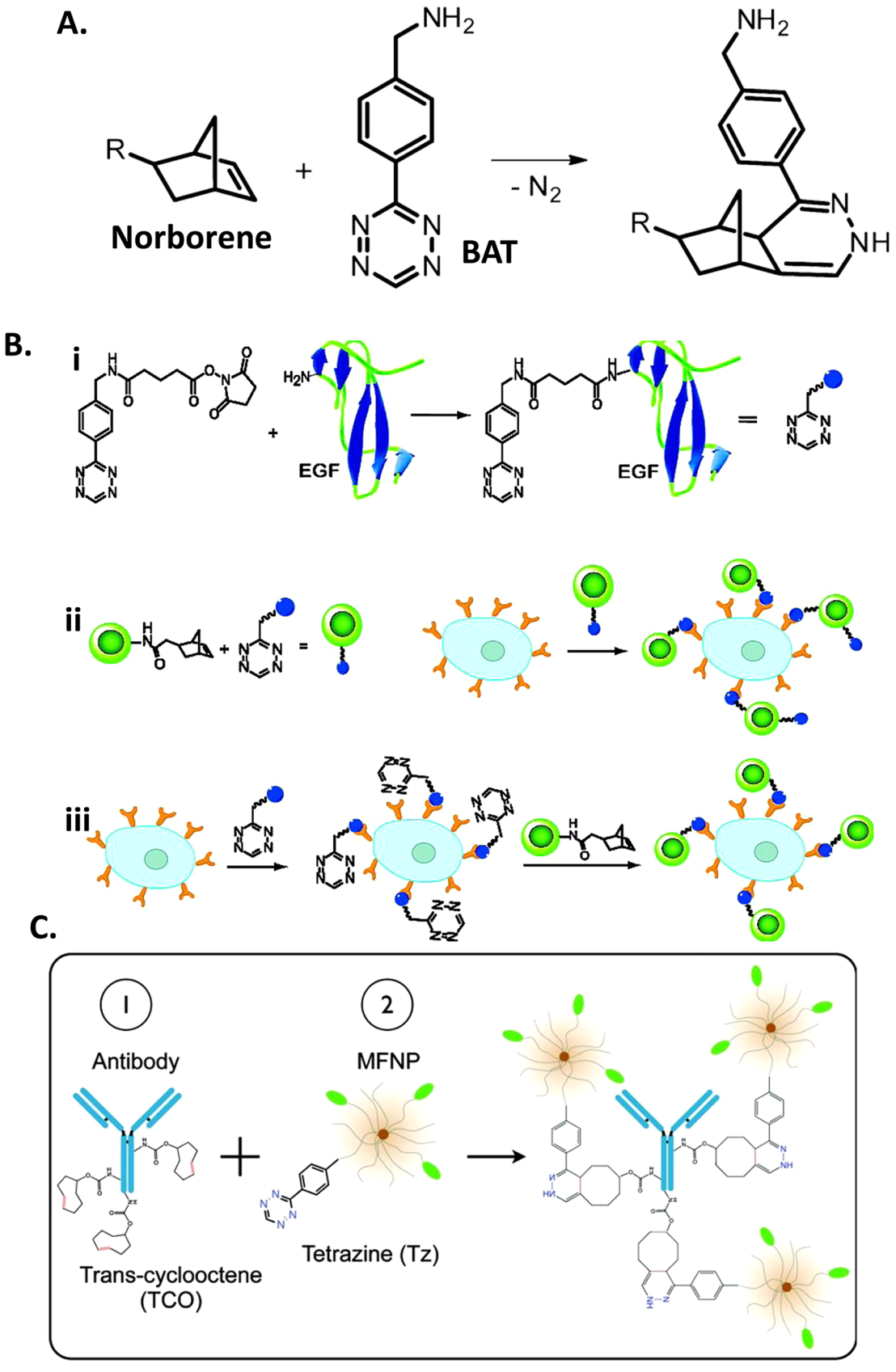

Figure 87. BAT−norbornene chemistry. (A) Schematic of the chemistry between BAT and norbornene. (B) (i) Conjugation of NHS-activated BAT to EGF; (ii) labeling of cells with preformed QD−EGF constructs; (iii) *in situ* conjugation of norbornene-functionalized QDs to BAT−EGF joined to EGFRs on live cells. (C) Targeting scheme using a TCO-modified antibody followed by Tz NP to amplify NP binding. Panels adapted from refs 1926 and 1927. Copyright 2010, 2011 American Chemical Society.

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appropriately modified fusion proteins (or peptides) directly to hydrazide-activated NPs.

5.2. Enzyme-Catalyzed Bioconjugation

Borrowing directly from cellular biochemistry, more than a dozen enzyme-catalyzed systems capable of either post-translational modification of defined substrates or self-labeling have been described. These were developed primarily for selective labeling of substrate or fusion proteins with organic fluorophores or affinity handles, such as biotin, and were primarily targeted toward cellular labeling applications. These systems, however, can function either *in vitro* or *in vivo*, with the latter usually requiring a cell-permeable substrate. It is only recently that their potential for NP bioconjugation has begun to be investigated, and some examples have already been highlighted above.

5.2.1. Post-Translational Modification. In these systems, protein substrates and other biomolecular moieties undergo site-specific post-translational modification (specific attachment of a fluorescent or functional entity) by the enzyme as long as the ligand to be attached expresses an appropriate acceptor tag, which is usually an amino acid sequence or modified chemical functional group.

5.2.1.1. Biotin Ligase. The desire to provide site-specific access to protein biotinylation chemistry has driven the development of biotin ligase enzymatic systems as an alternative to more heterogeneous chemical labeling (see Biotin–Avidin Chemistry, section 2.5.2). The prototype for this labeling approach has been *E. coli* biotin ligase (BirA), which transfers endogenous biotin in an adenosine triphosphate-dependent manner to a specific lysine side chain found in a 15-residue acceptor peptide.[190] Modification of various substrate proteins with the acceptor peptide sequence is common and has allowed recombinant production of sensing proteins in biotinylated form[1936] or cellular membrane labeling with a variety of biophysical probes.[1937,1938] The Ting Lab has taken the lead in adapting this approach for fluorescent labeling with semiconductor QDs.[1939,1940] They demonstrated that extracellular receptors in HeLa cells and neurons could be modified with acceptor peptide sequences and specifically biotinylated by BirA present in the growth media. This allowed for the rapid (2 min) and specific *in vivo* labeling of the membranes with SA-conjugated QDs,[1932] which was reproduced by another group using CHO cells.[1941] A follow-up study included BirA in combination with yeast biotin ligase and an evolved yeast acceptor peptide to achieve orthogonal two-color QD labeling of cell surface proteins, where the different acceptor peptide sequences defined the labeling specificity.[1940] A slightly different orthogonal approach encompassing BirA and polyhistidine–NTA interactions also allowed two-color QD tracking of single interferon receptor subunits on live cells.[1942] Ting also utilized BirA to selectively label adeno-associated virus particles.[1943] This capsid was engineered to display an available acceptor peptide sequence that was modified with a ketone isostere of biotin by BirA. Subsequent conjugation to hydrazide- and hydroxylamine-functionalized cyclic CPPs and fluorophores facilitated viral cellular uptake and fluorescent tracking.

BirA utility for NP biomodification in different formats appears to be expanding. This enzyme has also been applied to labeling hollow protein NPs composed of the hepatitis B virus surface antigen. Using SA as a linker, these NPs could be made to display various biotinylated ligands such as antibodies and synthetic peptides.[1944] The use of multivalent SA acted to effectively multiply the number of ligands displayed, increasing them from ~1 per modification site to potentially 3. In a fascinating adaptation, biotinylated magnetic NPs were constructed by displaying acceptor peptide on the surface of bacterial magnetic particles (BacMPs) biosynthesized by a *Magnetospirillum magneticum* strain referred to as AMB-1, see Figure 88.[1945] It was postulated that BacMPs displaying

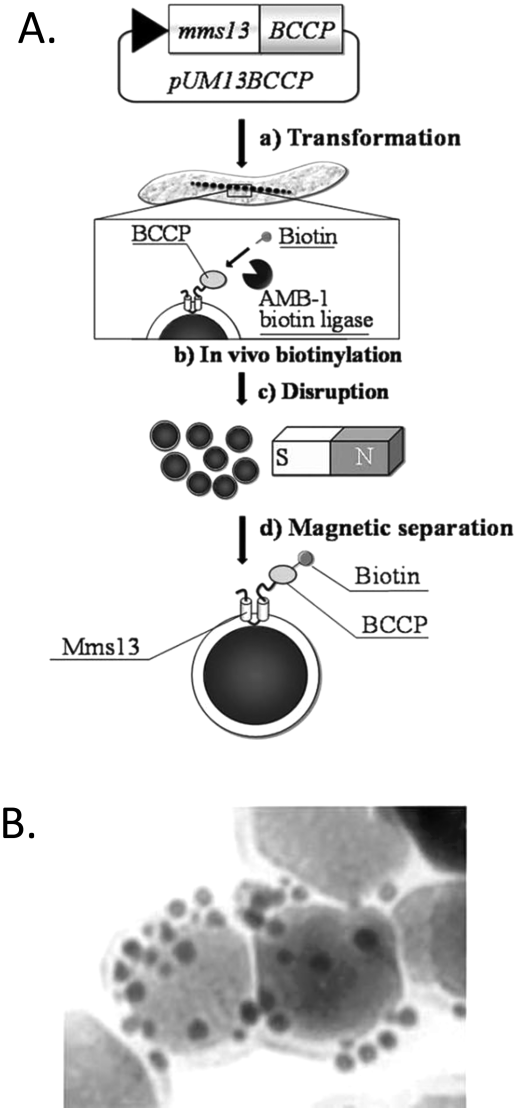

Figure 88. *In vivo* biotinylation. (A) Schematic diagram of the preparation and *in vivo* biotinylation of BCCP (biotin carboxyl carrier protein)–BacMPs. Plasmid pUM13BCCP containing an Mms13–BCCP fusion gene was used to transform wild-type bacteria AMB-1 (step a), and BCCP–BacMPs were biotinylated by endogenous AMB-1 biotin ligase (step b). The AMB-1 transformant harboring pUM13BCCP was then broken open to release BCCP–BacMPs (step c), and BCCP–BacMPs were magnetically separated and purified by stringent washing (step d). (B) TEM images of gold nanoparticles bound to BCCP–BacMPs via SA–biotin interaction. Figure reproduced with permission from ref 1945. Copyright 2008 American Society for Microbiology.

portions of recombinant biotin carboxyl carrier protein were biotinylated *in vivo* within this cell line by endogenous biotin ligase. Furthermore, AuNP–BacMP composites could be constructed by *ex vivo* interactions between purified biotin–BacMPs and AuNP–SA. The authors suggested this process as

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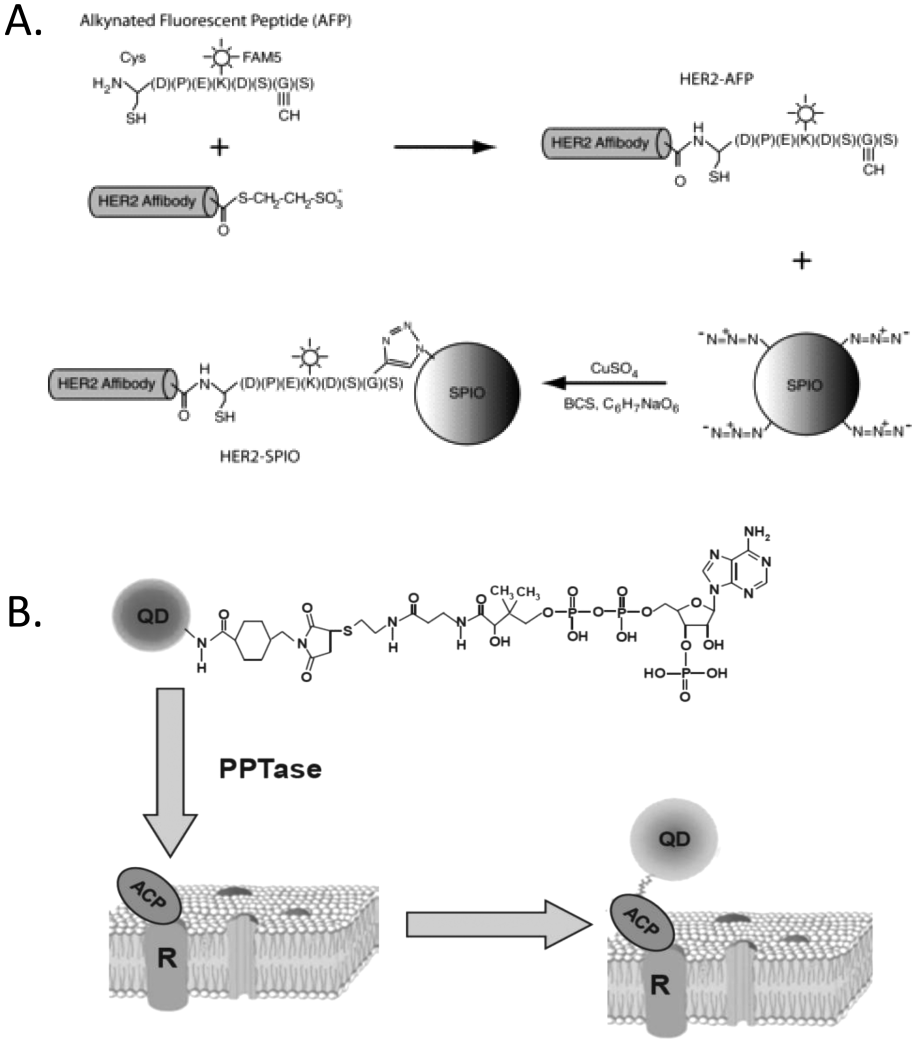

Figure 89. Schematic of EPL−click and ACP conjugation. (A) Expressed protein ligation between a HER2 Affibody containing a C-terminal thioester and an alkynated fluorescent peptide (AFP) containing an N-terminal cysteine results in the chemoselective attachment of a "clickable" alkyne group onto the affibody (HER2-AFP). Subsequent CuAAC between azide-modified SPIO-NPs and HER2−AFP results in the site-specific attachment of the HER2 Affibody onto the SPIO-NPs. Reproduced with permission from ref 1952. Copyright 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (B) Schematic of acyl carrier protein labeling of cell surface receptors. By application of the enzyme PPTase, a single QD functionalized to CoA is transferred to the ACP protein fused to the receptor. Reproduced from ref 1957. Copyright 2010 BioTechniques. Used by permission.

a potential simple and low-cost method for producing biotin- or SA-labeled magnetic NPs.

5.2.1.2. Intein-Mediated Protein Ligation. Inteins are polypeptide sequences found within proteins that are capable of chemically excising themselves and rejoining the parent protein with a peptide bond in a reaction catalyzed by an active thioester intermediate.[1946−1948] This reaction chemistry is also referred to as intein-mediated protein splicing or expressed protein ligation. To date, more than 200 intein sequences ranging in size from 100 to ∼800 amino acids have been identified in diverse protein families, and their wide utility has led to targeted applications including protein synthesis, surface immobilization, and protein conjugation with numerous fluorescent and affinity probes.[1947,1949,1950] The stepwise mechanics of the chemistry and the diversity of intein-mediated processes are rather complex, and the interested reader is referred to refs 1946−1948 and 1950 and references therein. In one of the few examples of application to NP conjugation, Rao's group elegantly utilized a modified intein chemistry to label QDs and create protease sensors.[1951] *Renilla* luciferase−protease−substrate−intein fusions were genetically engineered and exposed to carboxylated QDs surface-modified with adipic dihydrazine via carbodiimide/EDC chemistry. The nucleophilic hydrazide attacks the thioester intermediate in the intein fusion protein forming a stable adduct, followed by intein cleavage and religation to yield the final functional QD−protease−substrate−*Renilla* luciferase conjugate. Addition of the luciferase substrate coelenterazine resulted in efficient BRET between the luciferase and the proximal QDs. Further addition of a specific protease cleaved the fusion protein's peptidyl attachment to the QD, altering BRET and allowing monitoring of proteolytic activity.[1951]

In another example, Tsourkas's group applied an intein-mediated conjugation to improve the tumor targeting of designer NP systems by attaching a HER2 Affibody (i.e., antibody mimic) to a SPIO-NP. See Figure 89A for a schematic of this strategy, which also incorporated a second CuAAC step.[1952] A HER2 Affibody containing a C-terminal thioester was ligated

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with an alkynylated fluorescent peptide (AFP) containing an N-terminal cysteine and resulted in the chemoselective and site-specific attachment of a target alkyne group onto the Affibody (HER2−AFP). Subsequent CuAAC between SPIO-NPs displaying surface azide-modifications and HER2−AFP resulted in the site-specific attachment of the HER2 Affibody onto the SPIO-NPs. The final HER2−SPIO-NPs were found to be receptor specific in both cellular studies and murine tumor models, and demonstrated improved contrast enhancement versus constructs generated using more conventional EDC bioconjugation chemistry. Moreover, the authors highlighted the versatility of this chemistry by extending it to other liposomal and dendrimeric NP systems.[1952] Reulen and colleagues also demonstrated a two-step process where recombinant single-domain antibodies were generated with a C-terminal thioether for attachment to micellar particles via NCL.[1953] Use of intein-based approaches for protein and NP modifications are facilitated by the availability of the IMPACT vector system.[1954] The IMPACT (intein mediated purification with an affinity chitin-binding tag, www.NEB.com) Kit incorporates engineered protein splicing elements and inteins and allows purification of recombinant proteins via a single column in reactive form.

5.2.1.3. Carrier Proteins. Peptidyl and acyl carrier proteins (ACP) can be specifically modified with a variety of cargoes and chemical groups by phosphopantetheinyl (PPT) transferase, which catalyzes the transfer of the PPT unit from coenzyme A (CoA) to a conserved serine in the carrier protein.[1938,1955,1956] Because both the carrier protein and the transferase tolerate a wide range of substitutions at the CoA terminal end, this system has been used to label ACP-fusion proteins with a variety of fluorophores and affinity handles, including biotin and digoxigenin.[1955] By a strategy conceptually similar to that demonstrated with biotin ligase above, a PPT was utilized to specifically label ACP fusion proteins displayed on yeast cells with SA-conjugated QDs.[1955] Harm's group also utilized PPT to label transmembrane receptors with QDs for single-molecule tracking experiments, see Figure 89B.[1957] Using commercial 655 nm emitting QDs surface-functionalized with an aminated amphiphilic polymer, they began by blocking most of the amines with a t-Boc group and then converting no more than one amine per QD to a thiol-reactive site by using SMCC. These sites were then linked to the terminal thiol of CoA for subsequent PPT-catalyzed labeling of the ACP protein, which was fused to either the parathyroid hormone receptor or the bone morphogenetic protein type II receptor, allowing for single-receptor tracking studies of both. The 1:1 labeling stoichiometry made interpretation of the results more facile because it excluded any cross-linked receptors. CoA-modified QDs have also been joined to a carrier protein modified MBP (which retained its sugar binding function in subsequent assays) or used to label the membrane of transformed CHO-TRVb cells expressed with a tagged Tf receptor that also displayed the acceptor sequence.[1941] The recent introduction of shorter peptidyl tags that still allow PPT labeling should facilitate the application of this modification strategy to more NP materials.[1958]

5.2.2. Enzymatic Self-Labeling. *5.2.2.1. HaloTag.* Recombinantly modified haloalkane dehalogenase (HaloTag, www.Promega.com) can be utilized to covalently bind synthetic HaloTag ligands, which consist of a chloroalkane linker attached to fluorescent dyes, affinity handles, or even solid surfaces.[1959] In the wild-type dehalogenase enzyme, the His272 residue functions as a base to catalyze hydrolysis and release of the substrate intermediate allowing enzyme regeneration. The HaloTag enzyme is mutated to express a Phe272, which is ineffective as a base and traps the reaction intermediate as a stable covalent adduct. This system has been demonstrated for both *in vitro* and *in vivo* labeling with an fluorophores.[1959] Potential scenarios for utilization in the current context include the NP surface being modified with an appropriate HaloTag ligand, which would facilitate the binding of a HaloTag fusion protein and provide oriented display of the fusion protein partner. Conversely, modifying the NP surface with HaloTag enzyme may allow binding to a preferred substrate that is chemically unavailable for direct NP surface modification. Anticipating the potential of this system, Rao demonstrated that carboxylated QDs could be modified with an aminated chloroalkane ligand to allow subsequent decoration of the QDs with a HaloTag−*Renilla* luciferase fusion protein.[1960] The attached luciferase was shown to engage in BRET with the QDs at a rate that tracked with increasing QD chloroalkane ligand surface functionalization. The HaloTag system has also been utilized to achieve *in vivo* membrane labeling of cells with SA-functionalized QDs[1961] and for functionalization of microbeads with plant-receptor-like kinases for receptor−ligand screening assays.[1962]

5.2.2.2. Other Enzymatic Modification Systems. Membrane-localized target proteins have been fused to the cutinase enzyme allowing their subsequent covalent binding to QDs prefunctionalized with a high-affinity cutinase substrate suicide inhibitor.[1941] This approach has also been demonstrated for labeling integrin lymphocyte function-associated antigen-1 on the surface of K562 erythroleukemic cells with red QDs.[1963] GST has also been shown to specifically bind AuNPs expressing a mixed surface of modified ethylene glycol and its substrate, the tripeptide GA.[440] GA-modified magnetic NPs have further been applied to purifying several GST fusion proteins.[1964] GA systems may have great potential because engineering GST fusion proteins for either labeling or purification over GA media is a common technique[1965] and may also allow for oriented protein attachment to GA-decorated NPs. The recent report of a series of highly fluorogenic substrates for GST suggests a strategy that allows engineering of other ligands for attachment to GST and incorporation within different NP labeling and modification schemes.[1966]

5.2.3. Potential Limitations. In cumulatively examining the enzymatic labeling systems described above, there are several common threads. The systems have been developed almost exclusively for fluorescent cellular labeling, and in the context of NPs, almost all have been initially demonstrated for this purpose with semiconductor QDs. This is not surprising because this represents a "proof-of-concept" adaptation, and QDs are arguably the most prevalent NP analog of fluorophores. Although some enzymatic labeling systems are available commercially, several issues need to be considered in their use. Successful implementation requires expertise in both molecular biology and chemical modification of NMs, techniques not commonly found in the same laboratory. Each system has different reaction rates and specificity, and not all substrates will be specific or compatible with all NP chemistries. The number of commercially available substrates for most systems is still very limited, and specific affinity- or dye-labeled substrates may require complex custom synthesis. Regardless, the ability to site-specifically label NPs or attach specific (fusion) proteins, in what will be in most cases an optimal orientation and, for all intents and purposes, a bioorthogonal manner, will continue to drive the development of these approaches.

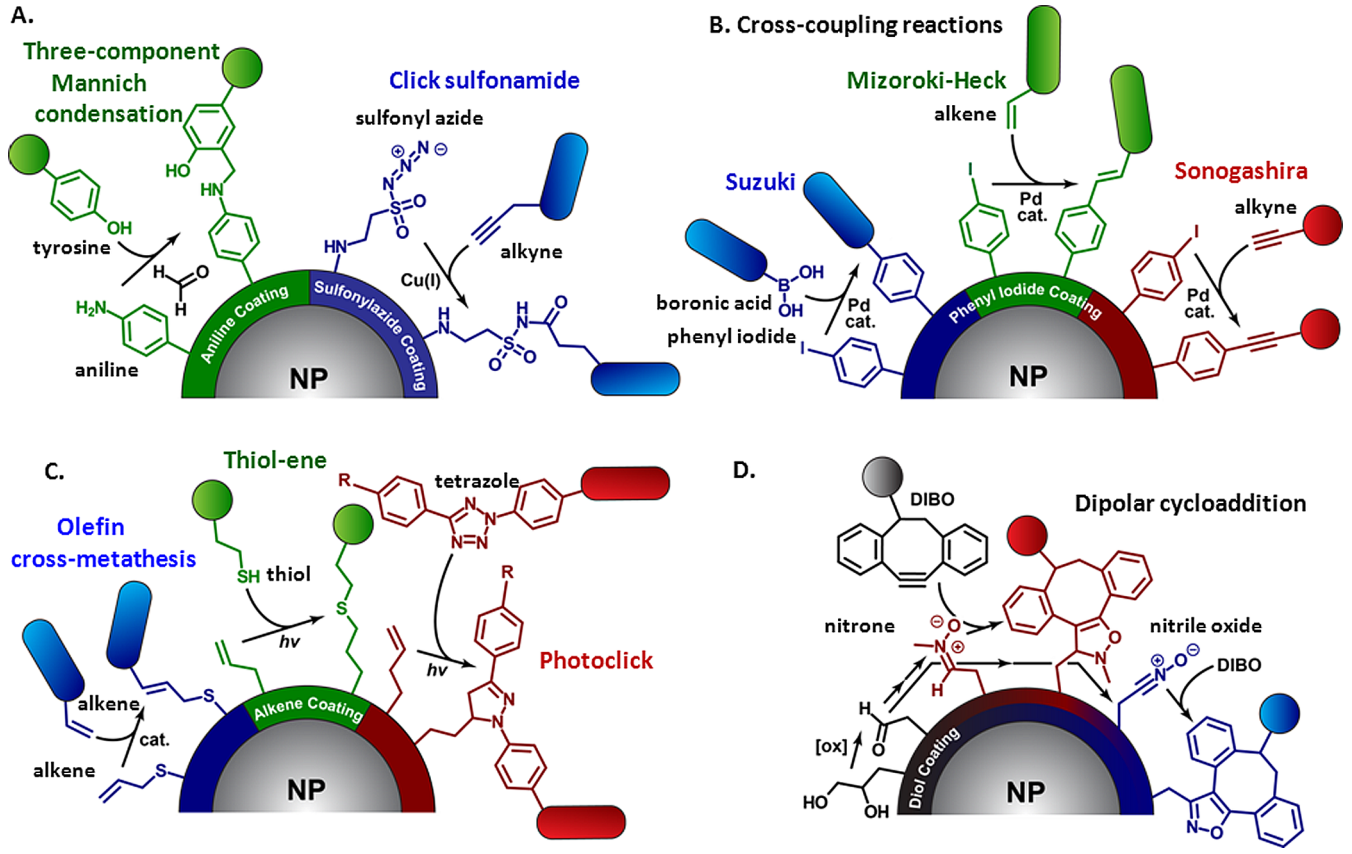

Figure 90. Promising chemistries for NP bioconjugation. (A) (green) Three-component Mannich condensation with a tyrosine residue and aniline-modified NPs; (blue) click sulfonamide reaction between sulfonylazide-modified NPs and an alkyne tag. (B) (blue) Suzuki, (green) Mizoroki–Heck, and (red) Sonogashira cross-coupling reactions between phenyl iodide modified NPs and boronic acid, alkene, and alkyne tags, respectively. (C) (blue) Olefin cross-metathesis, (green) thiol–ene, and (red) "photoclick" reactions between alkene-modified NPs and alkene, thiol, or tetrazole tags, respectively. (D) Multistep, one-pot modification of a vicinal diol coated NP to yield (red) a nitrone or (blue) a nitrile oxide for a 1,3-dipolar cycloaddition with a dibenzocyclooctyne derivative (DIBO).

5.3. Promising Chemistries for Nanoparticle Bioconjugation

The development of highly selective and efficient bioconjugate chemistries for labeling proteins without perturbing their structure or function is a major focus of research in chemical biology. Thus, novel protein labeling methods are likely to continue to be at the forefront of NP bioconjugation. Interestingly, NPs may actually offer greater opportunities for implementing new methods of bioconjugation. As noted by Lin et al., reactions for protein modification must occur in predominately aqueous solvents, at low or ambient temperatures, and avoid pH extremes.[1967] Proteins tend to unfold and lose their function away from these conditions. In contrast, many types of NPs are quite robust over a wide range of solvents, temperature, and pH; rather, the primary limitation tends to be the NP coating properties, which determine colloidal stability. Thus, compared with protein conjugates, the choice of solvent system may be less restricted in the preparation of oligonucleotidyl- and peptidyl-NP bioconjugates, which are not prone to irreversible denaturation. As mentioned several times, non-natural nucleotides (e.g., cyclooctyne derivatives[1968]), non-natural amino acid residues (e.g., fluorogenic mimics[1969]), and terminal modifications can also be readily incorporated during the chemical synthesis of oligonucleotides and peptides, potentially offering greater convenience than protein modifications. In this section, we briefly discuss some selected bioconjugate chemistries that have yet to be applied to NP materials but hold strong potential for future use.

5.3.1. Targeting Natural Protein Residues. Although tyrosine is more abundant in proteins than cysteine, it is still a largely underutilized and potentially useful site for modification, being more frequently buried within proteins than accessible at their surfaces.[1970] Its reactivity is also orthogonal to that of lysine and cysteine residues. The Francis group has selectively labeled protein tyrosine residues using a three-component Mannich condensation with formaldehyde and aniline derivatives of a fluorescent dye or peptide.[1970,1971] These reactions were done using 20–25 mM formaldehyde, 20–200 μM protein, and 2–25 mM aniline-modified dye or peptide. These concentrations are higher than would be ideal for NP bioconjugation; however, it is conceivable that aniline-modified NPs would permit the use of lower reagent concentrations due to the locally high concentration of functional groups at the NP surface, see Figure 90A. A second concern is that multivalent protein–NP conjugates could be more prone to protein–protein cross-linking by formaldehyde than isolated proteins in bulk solution, resulting in loss of activity (intra-NP) or agglomeration (inter-NP). Nonetheless it is a useful addition to the bioconjugation toolkit since it targets the more naturally prevalent tyrosine residues.

5.3.2. N-Terminal Transamidation. Another strategy developed by the Francis group is the aqueous N-terminal transamidation of proteins using pyridoxal 5′-phosphate to

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yield a terminal ketone suitable for oxime ligation.[168] This method was applied to fluorescently label antibodies.[168] In the context of NPs, this reaction is interesting because it can covalently tether a protein to a NP by its terminus, which would provide for optimal site-specific orientation and may be more applicable to a wider variety of NP materials than the self-assembly of proteins onto QDs or AuNPs using terminal polyhistidine tags. In principle, any alkoxyamine-modified NP should be a suitable substrate for this chemistry. The Che group recently reported a modification of this strategy using ketenes.[1972] Several proteins including insulin, lysozyme, RNaseA, and BCArg were selectively modified at room temperature with an alkyne-functionalized ketene, providing for subsequent site-specific N-terminal modification with a dansyl azide compound.

5.3.3. Aryl Halides and Cross Coupling. Aqueous Mizoroki–Heck, Sonogashira, and Suzuki cross-coupling reactions have emerged as viable methods of modifying proteins.[1973] These Nobel Prize winning reactions have preeminent positions in organic synthesis and couple an aryl halide with an alkene, terminal alkyne, or boronic acid, respectively, using a Pd catalyst, see Figure 90B. Considering proteins, *p*-iodophenylalanine[1974] and *p*-boronophenylalanine residues[1975] or cysteine residues selectively modified with an iodobenzyl group[1976] have enabled labeling via Suzuki cross-coupling. Mizoroki–Heck and Sonogashira couplings with proteins have also relied on *p*-iodophenylalanine incorporation,[1977−1979] as well as homopropargylglycine.[1980] Cross-coupling reactions are also highly attractive due to their chemoselectivity, bioorthogonality (reversible ester formation between boronic acids and saccharides notwithstanding), and stable ligation products. Their application to proteins, however, has largely been limited by the search for suitable catalysts under mild aqueous conditions.

Considering NMs, water-soluble, boronic acid functionalized polymer,[1981] IO,[741] Au,[1982] silver,[1983] and semiconductor[1984] NPs have been prepared, and would appear, *a priori*, to be suitable substrates for Suzuki cross-couplings. Terminal alkyne functionalized Au,[1985,1986] IO,[1987,1988] silica,[1989] capsid,[1512,1990] and other NP materials originally prepared for CuAAC chemistry may also be suitable for Sonogashira coupling, see Figure 90B. Cognate aryl halide, boronic acid, alkene, and alkyne modifications (as appropriate) can be incorporated into oligonucleotides and peptides during synthesis. However, indirect routes to these functionalities may be more widely accessible. For example, N-termini or lysine residues on proteins or peptides and amino-terminated linker modifications on oligonucleotides or NPs can be potentially modified using commercially available reagents (e.g., iodobenzoic acid, carboxyphenylboronic acid) and cross-linkers (e.g., carbodiimide). We also suggest that a more general scaffold for bioconjugation may be achievable using NPs solubilized with PEG-based copolymers or ligands that can accommodate iodophenyl groups at pendant or terminal sites. Such a configuration could hypothetically support serial, or even parallel, bioconjugation with three different species that display boronic acid, alkene, and terminal alkyne functions.

A potential challenge with Pd-catalyzed cross-couplings at NPs may be the compatibility of catalysts with certain NP materials. Fortunately, it should be possible to address such issues through careful selection of NP coatings. The widespread availability of efficient Pd catalysts that are soluble in water or water/organic solvent mixtures could ultimately launch NP bioconjugation using these chemistries. Interestingly, Mizoroki–Heck, Sonogashira, and Suzuki couplings catalyzed by Pd NPs have been reported.[1991] Given the ever growing array of composite NP materials (e.g., Pd/AuNPs[1992]), it will be interesting to see whether NPs will ever be able to catalyze their own bioconjugation via carbon–carbon bond formation.

5.3.4. Metathesis. Olefin metathesis is another Nobel Prize winning reaction that is sought after for use in protein modification. This chemoselective reaction joins two alkenes through a new carbon–carbon double bond formed in the presence of a metal catalyst.[1993] To date, the principle impediment to protein modification using olefin cross metathesis has been the poor aqueous compatibility of catalysts. However, the Davis group has recently reported that allyl sulfides, which can be introduced at cysteine residues,[1994] are privileged substrates for aqueous cross metathesis and suitable for protein labeling using a Ru-based Hoveyda–Grubbs second generation catalyst.[1995] The caveat was that the solvent system was 30% *t*-butanol in aqueous buffer; nonetheless, some degree of protein activity was retained following the reaction.[1995]

Olefin metathesis at NP reaction substrates should provide greater flexibility in both catalyst and solvent selection than proteins alone (Figure 90C). PEGylated NPs generally have colloidal stability in water and polar organic solvents, and could permit the preparation of peptidyl and oligonucleotidyl NP conjugates using more optimal solvents and catalysts, followed by phase transfer to aqueous buffers for subsequent use in biological applications. NP-coordinating PEG ligands should accommodate terminal alkenyl groups, while pendant chains of polymer coatings could be modified similarly. Importantly, alkenyl-modified NPs may have considerable versatility; they are potential substrates for Mizoroki–Heck reactions, olefin metathesis, and photochemical thiol–ene or tetrazole–alkene click reactions (*vide infra*).

5.3.5. Photochemical Reactions. Photochemical reagents and methods are well-known in protein labeling and other bioconjugate techniques.[80] Commercially available aryl azides and diazirines are commonly used for cross-linking, although they are somewhat nonselective. More recently, "photoclick" coupling strategies have been adopted for bioorthogonal photochemical protein modification. These have included the photoinitiated thiol–ene reaction[1996,1997] and tetrazole–alkene cycloaddition (Figure 90C).[1998]

The thiol–ene reaction comprises the addition of a thiol to an alkene through a free radical mechanism. The reaction can be initiated chemically or by using light between 365 and 405 nm and proceeds nearly quantitatively in aqueous solvent.[1996,1997] Compared with olefin metathesis, the thiol–ene reaction is advantageous in that it proceeds without a catalyst and is not currently limited to allyl sulfides. In an elegant example of its potential, the thiol–ene click photochemistry was adopted for the oriented surface-immobilization of farnesylated proteins directly from cell lysate.[1999]

The application of the thiol–ene reaction to preparing NP bioconjugates is surely just around the corner. Thiol-modified QDs have been prepared through functionalized silica coatings,[576,2000] and this strategy is applicable to many other materials. In addition, polymer NPs have been synthesized using thiol–ene polymerization,[2001] and magnetite NPs have been coated using modular trialkoxysilanes from a library prepared using thiol–ene photochemistry.[2002] Thiol-functionalized NPs should also be suitable for reaction with proteins that are enzymatically prenylated or (bio)chemically tagged with an alkene functionality. This approach may be preferable to the converse, because it has been speculated that sulfenyl radicals formed at cysteine residues in proteins could lead to

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undesirable side reactions.[235] Nonetheless, the preparation of many peptidyl and oligonucleotidyl conjugates should be amenable to NPs displaying alkene functional groups.

The tetrazole−alkene reaction, which is another example of a 1,3-dipolar cycloaddition, has been recently reviewed by Lim and Lin.[1998] The reaction proceeds through photocycloreversion of the tetrazole to generate a nitrile imine that concertedly reacts with an alkene to yield a pyrazoline cycloadduct. This process is largely bioorthogonal, with endogenous alkenes being a potential exception (albeit that the predominant biological *cis*-alkenes are a poorer cycloaddition substrate than exogenous *trans* alkenes[1998]). Moreover, the reaction is fast in aqueous solvents, with efficient photoactivation at 302 nm. It has been applied to the modification of several proteins, including: palmitylation of GFP that had been labeled with a tetrazole via intein-mediated ligation in a prior step,[2003] and labeling of a genetically encoded *O*-allyltyrosine-containing Z-domain protein in live cells.[2004] In the latter case, intrinsic pyrazoline fluorescence of the product was used to follow the reaction. The non-natural amino acids, *p*-(2-tetrazole)-phenylalanine and homoallylglycine, have also been used for photoclick labeling of myoglobin and β-galactosidase, respectively, with 254 and 302 nm UV illumination.[2005,2006]

Considering NP bioconjugation with the tetrazole−alkene photoclick chemistry, AgNPs and QDs coated with coordinating tetrazole ligands[2007,2008] may be suitable substrates for the reaction, although this is uncertain due to the potential perturbation of electronic structure by NP binding. A more robust strategy is expected to be functionalization of polymer coatings with pendant tetrazole groups. Overall, the primary advantage of the tetrazole−alkene and thiol−ene reactions over similar bioorthogonal chemistries (e.g., olefin metathesis, tetrazine−norbornene) is the ability to actuate the reaction using UV light. Although photodamage to biological samples (e.g., cells) is a concern, the work with protein modification suggests that the efficiency of these reactions is sufficient to minimize such damage. UV actuation thus avoids challenges associated with catalysts, such as the solubility of organometallic complexes or the cytotoxicity and luminescence quenching of Cu ions, and also permits spatial and temporal control over reactivity. Photoclick chemistry could potentially be used to tag proteins *in vivo* using optically or magnetically active NP materials (analogous to molecular contrast agents), to modify NPs *in situ* during an experiment, or as a surrogate "protecting group" for the dual conjugation of NP materials in a one-pot reaction.

5.3.6. Strain-Promoted and Cu-Catalyzed Click Reactions. Although the SPAAC reaction is arguably the most renowned reaction with strong potential for bioorthogonal labeling, it is certainly not the only strain-promoted 1,3-dipolar cycloaddition. Another such reaction, called the "strain-promoted alkyne nitrone cycloaddition" (SPANC), has recently been reviewed by Debets et al. in the context of bioorthogonal protein labeling (Figure 90D).[2009] In one format, chemokine interleukin-8 protein was modified with a nitrone at an N-terminal serine residue for an *in situ* reaction with a PEGylated cyclooctyne derivative using a one-pot, three-step oxidative protocol with an intermediate aldehyde.[2010] Given the commercial availability of cyclooctyne derivatives, the application of the SPANC reaction with NPs will likely be predicated on the modification of NP coatings with nitrones. Analogous to N-terminal protein modification, this may be most readily accomplished using mild oxidative methods since direct aldehyde functionalization of NPs is rare and bifunctional cross-linkers such as glutaraldehyde are very poorly controlled. An alternate route to bypass this issue may involve a prefunctionalization of the NP with a bifunctional NHS−aldehyde linker or similar analog. Additionally, the Bertozzi group's recent demonstration of genetically encoded aldehyde tags on proteins can help with the opposite configuration.[2011] NPs coated with vicinal diols (e.g., sugars) or 2-amino alcohols are perhaps more accessible and are suitable for use with periodate oxidation to form aldehydes.[80] For example, IONPs coated with dextran were oxidized to aldehyde-functionalized particles in this manner.[2012] The caveat is that not all NP materials will necessarily be amenable to oxidative methods.

Similar to the SPANC reaction, Sanders et al. have reported that cyclooctyne derivatives can also undergo 1,3-dipolar cycloaddition reactions with nitrile oxides and diazocarbonyl derivatives.[2013] These are referred to as the "strain-promoted alkyne nitrile oxide cycloaddition" (SPANOC) and "strain-promoted alkyne diazocarbonyl cycloaddition" (SPADC) reactions. The nitrile oxide can be installed in a one-pot reaction similarly to the nitrone described above: periodate oxidation of glycans or glycoproteins produces an aldehyde, which is reacted with hydroxyl amine to form an oxime that is subsequently oxidized to the corresponding nitrile oxide by [bis(acetoxy)iodo]benzene.[2013] The SPANOC reaction was noted to be more than an order of magnitude faster than the corresponding SPAAC, SPANC, and SPADC reactions,[2009] suggesting good potential for the bioconjugation of NP materials tolerant to oxidative conditions.

Another potentially useful bioorthogonal click reaction is that between sulfonylazides and terminal alkynes to form stable *N*-acylsulfonamides in the presence of a Cu(I) catalyst and aqueous solution.[2014,2015] As recently reviewed by Kim et al., this so-called "click sulfonamide" reaction is part of a growing catalog of Cu-catalyzed multicomponent reactions that utilize sulfonyl, phosphoryl, and select acyl azides.[2016] Within bioconjugation, the click sulfonamide reaction has been recently applied to the site-specific immobilization of peptides, carbohydrates, and proteins.[2017] The limitations of this chemistry are analogous to those of the CuAAC, but represent a carbonyl-free route to amide formation. The application of this chemistry to NPs may parallel that of the classic CuAAC with aryl or alkyl azides, utilizing alkynyl NP coatings.[1512,1985,1987−1990,2001] However, the modification of NPs or biomolecules with cognate sulfonyl azide groups is, at present, considerably less convenient. Sulfonylazides are typically prepared from the reaction of sodium azide with sulfonyl chlorides. Convenient bifunctional starting materials such as 2-aminoethanesulfonic acid or 2-mercaptoethanesulfonic acid require protection and deprotection steps to prepare the corresponding sulfonyl azides, which would then be suitable for biomolecule and NP modification using traditional methods. In contrast, a variety of alkyl azide modified NPs have been prepared for use with the CuAAC reaction,[454,1509,1986,2018,2019] suggesting that application of the click sulfonamide reaction as a bioconjugate technique may be limited in the near future.

5.3.7. Serial and Parallel Bioorthogonal Reactions. While bioorthogonal reactions have been highly successful for protein labeling and tracking, it is widely recognized that individual proteins play roles in complex signal cascades involving other proteins. Moreover, visualization of protein expression or location does not necessarily report on the state of the protein (e.g., folded/unfolded or active/inactive). As a consequence, attention has turned to the simultaneous labeling of multiple proteins or the dual labeling of an individual protein to enable FRET. As an example of the latter, Brustad et al. expressed a protein with single *p*-acetylphenylalanine (a ketone bearing

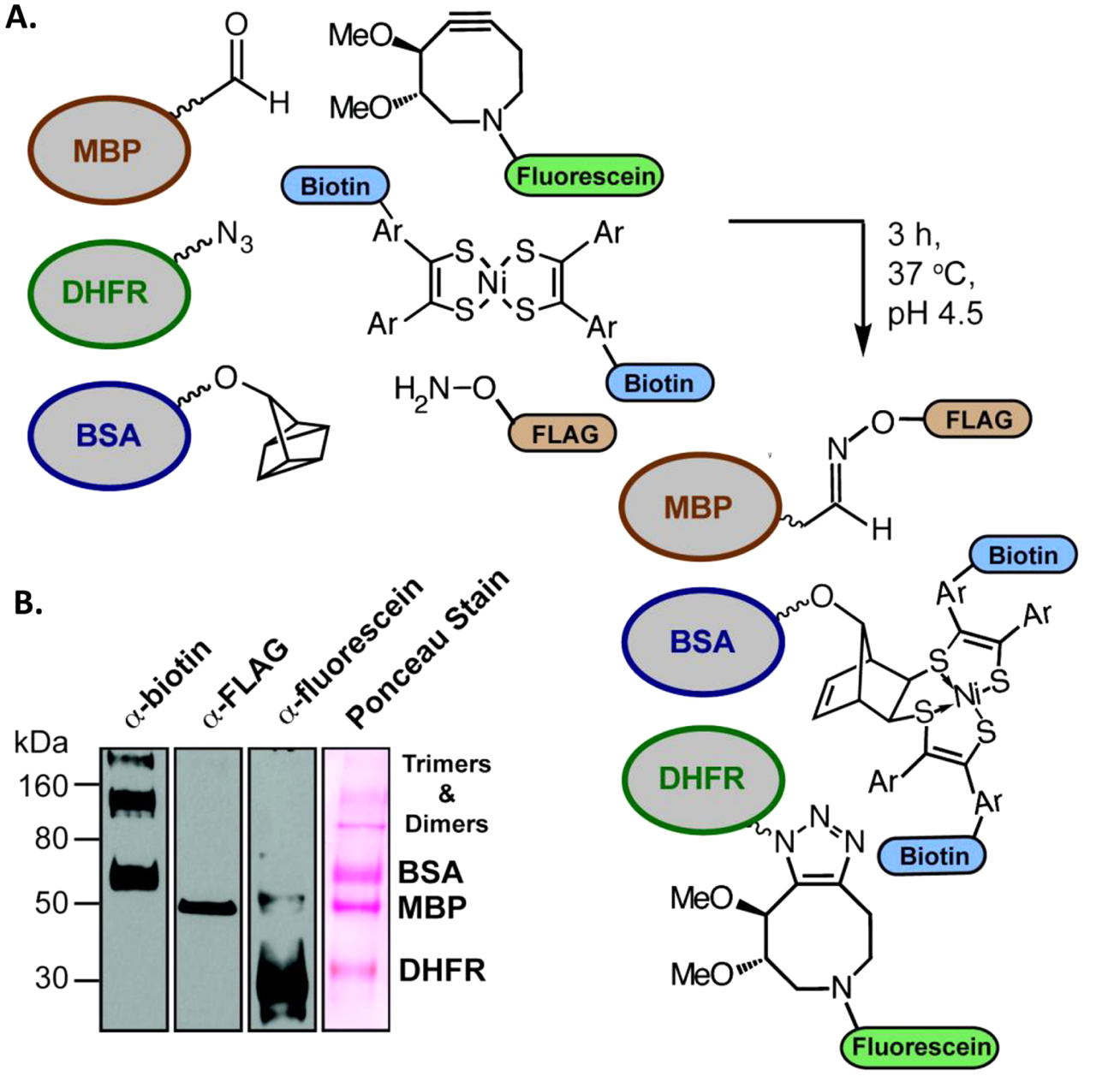

Figure 91. Simultaneous bioorthogonal chemistry. (A) A mixture of quadricyclane BSA (QCBSA), azidoDHFR (AzDHFR), and aldehyde-tagged maltose binding protein (CHO-MBP) was simultaneously reacted with the indicated groups. (B) The mixture was separated into three portions, and each portion was analyzed by Western blots probing with a different antibody: α-biotin–HRP (quadricyclane ligation), α-fluorescein–HRP (Cu-free click chemistry), or α-FLAG–HRP (oxime ligation). The Ponceau stain indicates all three proteins were present. Oligomer bands are observed for BSA and DHFR. Adapted from ref 2021. Copyright 2011 American Chemical Society.

non-natural amino acid) and cysteine residues, which then underwent a dual site-specific labeling using commercially available maleimide and alkoxyamine donor/acceptor fluorescent dyes.[2020] This was possible due to the orthogonality of the thiol Michael addition and oxime ligation chemistries. In another context, Sanders et al. prepared glycoside clusters labeled with a fluorescent dye or biotin using the sequential application of orthogonal metal-free click chemistries to a bifunctional azide–oxime linker.[2013] A dibenzocyclooctynol dye derivative was first reacted with the linker azide via the SPAAC reaction. This was followed by the addition of a dibenzocyclooctynol derivative of the glycoside cluster with a mild oxidizing agent, thereby converting the oxime to a nitrile oxide *in situ* for a SPANOC reaction with the linker. Most recently, Sletten and Bertozzi concurrently modified BSA, dihydrofolate reductase (DHFR), and MBP using parallel quadricyclane ligation, SPAAC, and oxime ligation reactions.[2021] The proteins were successfully tagged with quadricyclane, azide, and aldehyde groups, respectively, for reaction with biotinylated Ni(II) bis(dithiolene) complexes, dimethylazacyclooctyne fluorescein, and aminoxy-modified FLAG peptide, see Figure 91. Steinhagen also recently demonstrated a simultaneous one-step immobilization on a surface of a protein by combining expressed protein ligation and CuAAC.[2022] Postmodification characterization confirmed that the native protein structure and function were maintained, suggesting this approach could potentially be adapted for immobilization on NP surfaces.

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The examples above, particularly the demonstration by Sletten and Bertozzi, highlight the extended potential of bioorthogonal chemistries. In most applications for chemical biology, the interest in parallel, orthogonal reactivity is for multiplexed intracellular detection of different proteins. This concept is equally applicable to certain NPs because, for example, a rainbow of QDs could be concurrently derivatized with different biomolecular probes using orthogonal chemistries. However, another conceptual application of parallel orthogonal chemistries is the one-pot multifunctionalization of a single NP population, which capitalizes on the (bio)-chemically tailorable surface area available with many types of NP. In most bioapplications, NP conjugates are designed for one of three predominant roles: targeting, detection, or delivery. The concept of multifunctional NPs has attracted considerable interest (e.g., theranostics) but continues to be hindered by the challenge of reliably and controllably appending multiple functional (bio)molecular ligands to a NP. However, the emergence and validation of mutually orthogonal chemoselective/bioorthogonal reactions has made this a viable endeavor. The major challenge is to now develop NP coatings that have suitable chemical handles for parallel bioconjugate reactions. Alternatively, bioorthogonal reactions that utilize a common functional group (e.g., iodophenyl groups for Pd-catalyzed couplings; alkene groups for metathesis, thiol−ene, and photoclick reactions) may be suitable for serial conjugation at appropriately derivatized NPs. In either case, the result may be a one-pot or *in situ* preparation of NPs capable of cellular targeting, sensing, and therapeutic delivery as a single vector.

5.3.8. Enzymatic Bioconjugation: SNAP Tag and Other Modifications. The engineered human DNA repair enzyme alkylguanine-DNA alkyltransferase (SNAP-tag, www.neb.com) can also be used as a self-labeling tag. A variety of modified O^6-benzylguanine derivatives can function as substrates for this enzyme and are attached to the alkyltransferase by irreversible transfer of an alkyl group to a cysteine residue.[2023] Since its first description, this system has been dramatically improved through engineering faster, more-efficient enzymes and a wider range of fluorescent, affinity handle, and bifunctional substrates, yielding a multitude of *in vivo* cellular labeling demonstrations.[1938] Recent modifications of the SNAP-tag enzyme now also specifically target O^2-benzylcytosine derivatives, which can enable an orthogonal labeling approach using both enzymes.[2024] Beyond cellular labeling, this technology has already been applied to assembling proteins on surfaces in an oriented manner.[2025] Given the relative ease of modifying a variety of substrates with benzylguanine derivatives, it is only a matter of time until this system is utilized to label NPs in a manner akin to the HaloTag. Yao's group recently reported on a fluorogenic non-natural amino acid that can mimic phosphotyrosine and self-immobilize to a protein tyrosine phosphatase or another nearby protein during enzymatic activity.[1975] Although the mechanism of activity is quite complex, the ability of this amino acid analog to be linked, either by itself or as part of a peptide, to a protein substrate suggests potential utility in the context of NP bioconjugation. The fluorogenic amino acid analog is synthesized with a caged phosphate that allows UV excitation to control its availability and activity. Additional enzyme-based modification systems with NP-labeling potential include dihydrofolate reductase, which can covalently bind trimethoprim,[2026,2027] transglutaminase, which can attach cadaverine-modified probes to small glutamine (Q) expressing peptide substrates termed Q-tags,[2028] and lipoic acid ligase, which can functionalize acceptor peptides with various substituted substrates.[2029]

Bertozzi's group also recently described a genetic tag that allows proteins to undergo site-specific introduction of an aldehyde.[2030] This tag consists of a specific six-residue sequence (LCTPSR) found within a 13-mer conserved consensus sequence. When introduced into proteins that are coexpressed with a heterologous formylglycine-generating enzyme, the cysteine residue undergoes cotranslational oxidation and modification to formylglycine. It was shown that proteins bearing this unique aldehyde group could be chemoselectively modified by reaction with aminooxy- or hydrazide-functionalized reagents, including biotin derivatives. This approach was recently demonstrated for producing complex aminooxy glycan modified proteins and could potentially allow proteins to be produced with site-specific aldehyde tags for subsequent chemoligation to NPs displaying the cognate chemical groups.[2011]

Overall, the ability to re-engineer enzymes to selectively modify target proteins and other (NP) substrates in a unique, orthogonal manner will add to the growing repertoire of bioconjugation chemistries and benefit not only biolabeling in general but also NP modification.

6. CHARACTERIZATION OF NANOMATERIAL BIOCONJUGATES

While the ultimate test of successful NM bioconjugation is functionality in the desired application, proper characterization of NM bioconjugates is being recognized as an essential element not only in the design and production of these unique materials but also for the subsequent interpretation, reproducibility, and comparison during their intended use.[3,2031,2032] Royce Murray, the former editor of *Analytical Chemistry*, recently summed up this idea in an eloquent editorial entitled "Nanoparticles: An Emerged and Lasting Frontier" where he stated, "As important as these applications are or may become, researchers sometimes charge into NP use with inadequate attention to what the NPs actually are. When used as chemical substances, or carriers thereof, NPs should not be deployed in ignorance of their composition and, ultimately, structure."[2033] Clearly, the interpretation and reproducibility of results can be especially difficult unless the NM bioconjugate under study is well understood, and this may involve characterization at multiple stages during a study (i.e., as prepared, as stored, or when diluted into the matrix used). The importance of the latter point is exemplified by the protein corona that can result upon exposure to biological environments (see section 2.3).[90] Understanding impurities or left over reaction products in the system is also important, especially in the emerging field of nanotoxicology, where the presence of, for example, surfactants or endotoxins in the NM bioconjugate solution can result in false indications of toxicity.

There are a number of physicochemical and bioconjugation metrics that are of particular interest when characterizing NM bioconjugates because they influence subsequent behavior in an application. These include but are not limited to composition, purity, size, particle or conjugate mass, shape, aspect ratio, surface area, polydispersity in the aforementioned characteristics, surface properties, colloidal stability and solubility, ζ potential, hydrodynamic radius, conjugate valence and the distribution thereof, biomolecular orientation(s) within the NM bioconjugate, and the activity, affinity, or avidity of the final conjugate.[16] Reliable measurement of NM bioconjugate concentration can be challenging and is often approximated but is critically important in almost all

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applications. A variety of well-established and emerging analytical methods have been successfully applied to the characterization of NMs and their bioconjugates; a general overview of these methods is presented in Table 15.[16] A comprehensive discussion of these methods is far too broad to be presented here; however, a recent review provides an extensive list of the relevant techniques with discussion of representative examples that highlight the benefits and liabilities of each.[3]

Analytical techniques for NMs typically fall into one of five main categories: separation-based, scattering, microscopy, spectroscopic, and thermal, with a sixth and final category reserved for new and emerging technologies. No single analytical method provides complete and definitive characterization, and thus a combination of techniques is ultimately required to fully characterize the physicochemical and bioconjugation metrics important to the intended use. The choice of technique(s) used is also dependent on a number of logistic and pragmatic factors, including cost, availability to the researcher, the expertise required, resolution capabilities, ease of data interpretation and potential artifacts or ambiguities, the ability to evaluate more than one metric, and the need for either bulk or single particle analysis or both. Sample preparation is, of course, another key consideration for many of these techniques. This can include drying, suspension in ultrapure liquids or special solvents, dilution or concentration, labeling, and other specialized protocols. Such sample manipulations can, in turn, influence the NM bioconjugate properties. Therefore, when interpreting the results of such methods, researchers should be mindful of potential effects. Clearly, analytical chemistry and instrumental analysis will play a pivotal role in the characterization of increasingly complex NM bioconjugates. The techniques and instruments briefly described in Table 15, along with the corresponding preparative methods, are continually evolving to meet the high demand and challenges required for nanoscale characterization analysis. Given the growing importance of NMs and especially their bioconjugates in life sciences, and similar to the calls for a dedicated discipline focused on nanotoxicology (*vide infra*), it is not unrealistic to suggest the need for an analytical subdiscipline focused almost exclusively on NM and NP bioconjugate characterization.

7. NANOPARTICLE TOXICITY

With the rapid expansion of NP usage into the biomedical realm and in food products[2034] and cosmetics such as sunscreen,[968] the study of NP toxicity both to humans and to the environment is extremely important. There are legitimate concerns that NPs may be more toxic than their microsized counterparts due to their increased surface-to-volume ratio. There is also concern that due to their small size they may accumulate in the lung by inhalation or penetrate through the skin.[968] Additionally, given the strong focus on developing NP bioconjugates for a myriad of *in vivo* imaging and drug delivery applications, the issue of toxicity within a particular usage also needs to be thoroughly considered. Concomitant to this, a generalized skepticism about the potential *in vivo* effects of NPs, which cannot be anticipated from *a priori* knowledge of ionic or bulk analogs, has also been growing in the popular consensus. The issue of NP toxicity is exceedingly complex, and interestingly, this very statement may be one of the only points in the debate surrounding this subject that almost all can agree on. Here, we neither argue for or against any positions on this subject nor focus on the applications of any NP materials in particular. Rather, we seek to point out the complexity of the subject by highlighting the

Table 15. Common Characterization Techniques for NM Bioconjugates[a]

technique	examples	typical information obtained	general comments
separation	high-performance liquid chromatography (HPLC), nanofluidic size exclusion, field flow fractionation (FFF), electrophoresis, analytical ultracentrifugation, electrospray differential mobility analysis (ES-DMA)	confirms NM bioconjugation, ζ potential, hydrodynamic radius, size distribution, biomolecule-to-NM ratio, postproduction stability, aggregation or agglomeration state, purity	routinely used to purify NM bioconjugates, requires optimization for each NM system under investigation, cost ranges widely, may be nondestructive to sample
scattering	dynamic light scattering (DLS), fluorescence correlation spectroscopy (FCS), resonance light scattering correlation spectroscopy, ζ potential, Raman techniques, X-ray diffraction, small angle scattering techniques	NM structure, morphology, hydrodynamic size, aggregation or agglomeration state, biomolecular conformation upon NM bioconjugation, postproduction stability, surface charge, confirms NM bioconjugation	cost ranges widely, in some cases limited to specific materials (e.g., fluorescence), typically provides average bulk analysis of NM bioconjugate (not single molecule), sample preparation key to analysis
microscopy	scanning electron microscopy (SEM) and transmission electron microscopy (TEM), atomic force microscopy (AFM), near-field scanning optical microscopy (NSOM), optical light microscopy	NM size and shape (aspect ratio), individual NMs characterized, composition, in some cases can measure or image biomolecule with suitable staining although more typically used to characterize the core NM	apart from light microscopy single particle resolution obtained, sample preparation key to analysis, specific instrument conditions required, technical expertise required, significant number of particles representative of total sample must be analyzed.
spectroscopic	UV−visible absorbance, circular dichroism (CD), fluorescence spectroscopy, infrared spectroscopy, nuclear magnetic resonance, magnetic resonance imaging (MRI), mass spectrometry	concentration, confirms NM bioconjugation, biomolecular conformation in NM bioconjugate, average NM-to-bioconjugate ratio, postproduction stability, mass of NM bioconjugate	provides bulk analysis of NM bioconjugate (not single molecule), intrinsic spectroscopic properties required, sample preparation key to analysis, may require fairly concentrated samples
thermal	thermal gravitational analysis (TGA), differential scanning calorimetry (DSC), isothermal titration calorimetry, thermophoresis	determine the amount of biomolecule conjugated and the NM/biomolecule's thermal stability	sample preparation key to analysis, technique optimization required, can require highly concentrated samples
emerging	CytoViva with hyperspectral imaging, XiGo Acorn analysis, resonance frequency devices (quartz crystal microbalances and suspended cantilevers, single particle tracking (NanoSight and others), Coulter counter devices	surface area, activity of NM bioconjugates, NM size, concentration, confirms NM bioconjugation, aggregation or agglomeration state	sample preparation key to analysis, technique optimization required, newer techniques may require further optimization, in some cases require precalibration with known size or NM standards

[a] Adapted from ref 3.

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relevant parameters that need to be considered and suggest that the context of the final application should perhaps be the most important determinant of what constitutes toxicity.

The underlying complexity is a direct reflection of the vast number of variables involved in every format, all of which need to be strictly defined (see also Table 2). Without a well-defined NP or NP bioconjugate material, any toxicity results are essentially meaningless because it is exceedingly hard to compare results or ultimately identify the causal linkages between the materials, components, or structure and toxicity; in fact the latter are critical to mitigate the responsible issues.[3,2031,2032] Indeed, a detailed characterization of the material in question is now generally accepted as the appropriate starting point prior to the toxicity assessment.[3,2031,2032] Starting with just the NP itself, these variables include the constituent materials (are any potentially toxic), its structure (core vs core−shell), hard size, hydrodynamic size, shape, surface area, roughness and porosity, surface charge, hydrophobicity, solubility, aggregation state, stability, aspect ratio, ligand structure and chemistry (PEGylated vs other materials) if utilized, and photophysical or chemical properties (photoactive or catalytic). Due to the stochastic nature of most bioconjugation reactions, a further level of complexity arises if the NP is modified with biologicals. Now the metrics of interest can include type(s) of biomolecule attached, ratio, polydispersity, NP bioconjugate size, orientation of the biomolecule, stability of the conjugate, and activity, to name but a few.[3] These metrics become exponentially more complicated if the NP is functionalized with multiple biological or chemical entities.

Of equal consideration is the intended application of a NP bioconjugate. A probe or sensor intended for monitoring a biochemical process in a cellular system over several hours is far different from that intended for long-term use in animal models or humans. For cellular studies, the relevant issue is not to significantly perturb the system under investigation for the duration of the experiment such that the results can be considered to reflect a realistic environment. It should be pointed out that many such studies take place in transformed cell lines, which, for all intents and purposes, are cancer cells. Thus, the background for experimental utility is already radically altered. Further, almost all molecular dyes and fluorescent proteins are toxic to some extent (depending upon dosage); however, this is never debated nor is their use generally precluded in biological research, within transformed or primary cell cultures, unless it perturbs cellular function over the experimental time scale. Implementation of proper controls and relevant toxicity assays are the appropriate and accepted means to address these latter points. Animal systems ratchet up the complexity with added issues of dosage, delivery time, circulation time *in vivo*, clearance, fate, experimental time, format, and both short- and long-term response and effects. When considering NP bioconjugate utility in humans, the issue is actually much simpler and more clearly defined. Appropriate regulatory bodies (i.e., FDA) will examine the pertinent toxicological data, and similar to all other drugs and probes, a decision based on risk/benefit potential and efficacy will be made for a very specific and limited application. Indeed, this paradigm will be true for all NM utility in a similar pharmaceutical role. What is not clear at the moment is what exactly will be required in terms of full characterization and supporting information for a particular regulatory submission and whether this will be standardized or will need to be addressed on a case-by-case basis.[3]

In general, concerns about NP toxicity are being taken very seriously across the board and are being addressed through continuing studies of toxicity in various cellular and animal models.[395,572,2035−2041] There are numerous reviews on this subject summarizing current techniques for measuring both genotoxicity (DNA damage)[2042] and cytotoxicity (cell death).[2035] Currently, toxicity focuses on three major areas: cellular uptake and localization, *in vitro* toxicity (cellular proliferation, necrosis, and apoptosis), and *in vivo* toxicity in animal models. A summary of methods commonly used is reviewed in ref 2036. *In vitro* toxicity analysis includes cellular uptake and localization studies primarily through microscopy methods, cellular viability assays (proliferation, necrosis, and apoptosis), and mechanistic assays measuring DNA damage and oxidative stress. These are the preferred toxicity assays for NPs because they are low cost, easy, and efficient. However, these assays have numerous caveats, including the fact that the size, biocompatible coatings, and other formulations of NPs can cause varied results. Furthermore, results can vary depending on the type of assay used. These assays also may not predict *in vivo* toxicity. Many of the initially published assays used unrealistically high concentrations of NPs, which are not physiologically relevant. The assays may also miss certain aspects of toxicity. For example, a NP that causes cellular senescence would be considered toxic but would not affect some viability assays.[2035] Further, it is now well established that many NP materials directly interfere with standard "first line" toxicity assays such as the *in vitro* limulus amoebocyte lysate (LAL) assay.[2043]

In vivo toxicity assays, usually in mouse and rat models, are meant to give the most complete picture of NP toxicity in a living organism. The advantage in these studies is the ability to study long-term exposure effects, tissue localization, biodistribution, retention and excretion, and other aspects of pharmacokinetics. A timely review of the status of *in vivo* testing of NPs was published in 2007.[2044] Examples of animal measurements include weight change, fertility, longevity, changes in blood serum chemistry and cell population, tissue morphology, and biodistribution.[2036] While this is currently the gold standard for studying NP toxicity, multiple variables should be considered including dosing concentration, delivery route, and the targeted organs or cells of interest, again, with the intended application of that material in focus. Also, biodistribution quantification often requires radiologic or fluorescent tags, which may alter the clearance route.[2036] While rat and murine models do allow for a whole organism analysis, they are not necessarily always reflective of human toxicity. Moreover, a joint NCI, NIST, and FDA program has established the Nanotechnology Characterization Laboratory (NCL, http://ncl.cancer.gov/), which provides an "assay cascade" to characterize all biologically relevant NP (or NP bioconjugate) physical attributes along with pertinent *in vitro* biological properties and *in vivo* compatibility. Importantly, researchers may submit materials directly for analysis free of charge. Combining high-throughput assays with computational assessment also looks to be a very promising way to address this issue.[2031] Some promising preliminary data in this regard has already shown that quantitative structure−activity relationship models can predict the cytotoxicity for a subset of metal oxide NPs.[2045] To help address this important issue, many who work in this field have called for the establishment of a dedicated subdiscipline solely focused on "nanotoxicology".[44,2020,2032,2041,2046−2048]

For the interested reader's consideration, we provide a brief list of some notable review articles concerning the toxicity of several representative NP materials, see Table 16. The reader

Table 16. Selected Review Articles on Nanoparticle Toxicity

material	comments	references
NPs in general	reviews of issues pertinent to NP toxicity; some perspectives from leaders in the field	64, 75, 968, 2034, 2035, 2037, 2042, 2044, 2048−2053
NPs in general	reviews on available techniques for monitoring NP toxicity and environmental exposure	2054−2057
quantum dots	includes data on some bioconjugated QD materials	581, 2058−2061
gold NPs	review of Khlebtsov and Dykman is the most comprehensive to date	66, 395, 2038, 2039, 2062
silver NPs	AgNPs have been proposed for wide-ranging antimicrobial activities	413, 2062−2065
carbon NPs	includes references on both fullerenes and nanotubes	2040, 2053, 2066−2074
titania NPs	commonly found in many sunscreen formulations	2074−2077

will note almost immediately that there is no standardized material or even assay for any of the NPs in question, and the surveys compare across many different preparations, chemistries, cell lines, doses, bioconjugates, etc. Again, this is a direct reflection of the disparate nature of the variables and diversity of (bioconjugated) materials. It is obvious that as NP bioconjugates are developed, improved, and applied in the near future, the amount of information available on NP toxicity will expand dramatically and more standardized protocols may become available for particular materials or applications. Pragmatism is also being applied at the initial design stages to remove any potential toxic elements from the NPs; this is especially true, for example, with newer non-Cd-containing QDs.[565,566] Paradoxically NP toxicity may be a specifically desired characteristic in the context of certain constructs for PDT and targeted tumor treatments, and research in this regard may actually focus on increasing these effects in a very controlled and localized manner.

8. CONCLUSIONS AND PERSPECTIVE

Although intended to be somewhat comprehensive, the preceding, in reality, only provided a superficial overview of the diverse nanotechnologies that rely on bioconjugation chemistries. Moreover, it allows us to appreciate the research contributions originating in the biomedical sector, especially in regard to nanoparticle mediated drug delivery and theranostics, which are driving this field. The range of NPs and NMs being utilized are almost uncountable as are their potential applications. As more NP materials are developed and their nanoscale properties elucidated, there will clearly be a desire to incorporate them within bioconjugates as well. As we have seen, materials currently being utilized include noble metal NPs and clusters, semiconductor QDs, viral nanoplatforms, carbon allotropes, self-assembled DNA structures, metal oxide NPs, and supramolecular protein assemblies, to name but a paltry few. The utility envisioned for these NMs within the context of a bioconjugated structure spans from molecular electronics to drug delivery. Despite these two examples appearing almost orthogonal to each other in nature, the final application of all such conjugates, in almost all cases, will directly reflect the fidelity of the underlying chemistries used to assemble them. It is here that much remains to still be addressed in this nascent but increasingly important field.

Coming back full circle to the ideal criteria desired from such chemistries, as iterated in Figure 2, we see that almost all the examples described here fail to meet many, if not most, of these initial goals. This is not to say that the individual assemblies were not functional in their intended application but rather that they were probably capable of far more. The broad reliance exhibited so far on utilizing chemistries originally developed for protein labeling to assemble many of the NP bioconjugates reflects how much more needs to be accomplished not only in developing new chemistries but, more importantly, in making them available and accessible in almost any desired format. Standard or "classical" bioconjugation chemistries remain a powerful set of tools in the NP bioconjugation chemistry kit and will undoubtedly always have a role here. Moreover, there are many applicable lessons to be learned from the application of each type of newly developed bioconjugation chemistry or new permutations of established chemistries. However, the need for diverse nonheterogeneous and site- or regiospecific chemistries within this "toolbox" is critical. The heterogeneity commonly found with the NP materials themselves, and in the resulting bioconjugates, in terms of both orientation on the NP and the attachment ratio, remains an important related concern. However, improved chemistries can most certainly help address this. For example, should a given conjugate display a distribution of valencies, having them all site-specifically attached and functional is far superior to a large proportion being oriented in a nonfunctional manner. On average, this conjugate should manifest far higher avidity and activity than the latter. As briefly mentioned in the characterization overview (section 6), the ability to separate individually desired constructs or fully characterize all the products from a conjugation reaction can also be another mechanism to address heterogeneity. Toxicity is far more complex, and much clearly needs to be done here. It is also important to point out that beyond medical or *in vivo* application, this may not be a major concern for a particular construct, beyond proper disposal.

The number of bioorthogonal and click-type chemistries being applied in preliminary NP bioconjugation is very encouraging in this direction although it is also here that we can illustrate some of the important remaining issues. For such chemistries to work optimally, cognate bioorthogonal groups or partner pairs must be introduced onto both the NP and the biological in question. One part of this equation is more easily addressed, and that is the NP because the requisite functionalities can be more easily introduced chemically during synthesis or preparation. The other part of the equation, the biological, remains only partially addressed. Small biologicals of synthetic origin (i.e., peptides, DNA, lipids) can be prepared with the necessary handle site-specifically inserted as desired, but what about larger proteins, such as antibodies, that may either be harvested or recombinantly produced? In many cases, the necessary groups are now introduced by relying again on standard labeling chemistries, such as heterogeneous amine-targeted conjugation with an NHS ester activated derivative. Site-specific introduction of appropriately modified non-natural residues during protein expression can be one avenue to address this concern; however, this requires specialized techniques, dedicated laboratory infrastructure, and considerable knowledge. This latter point brings us to the necessary skill sets to successfully engineer a multifunctional NP bioconjugate. Given the complexity envisioned for such conjugates, within theranostics and NP-mediated drug delivery in particular, contributions will be required from diverse fields such as materials scientists, chemists, biophysicists, molecular biologists, and pharmacologists. However, a major divide still remains present to some

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degree among these diverse fields. Fortunately, this divide is being steadily eroded by the establishment of multidisciplinary academic departments and research institutes along with targeted funding mechanisms.

As for the NP bioconjugates themselves, although considerable progress has been made over the past decade, it is clear that this field is still in its infancy. Looking at the elegant design and capabilities of "state of the art" constructs such as many of the functionalized VNPs (section 4.2.5), Kim's ferritin-based InCell SMART-I (section 4.2.4, Figure 65),[1472] or Brinker's protocellular NP-supported bilayer construct (section 4.3.5.3, Figure 85),[1917] it is exciting to imagine what the next generation of materials will look like and be capable of. Currently, far more is still desired from even the most advanced of these constructs. For example, within drug delivery, one major goal is that of a controlled and triggered delivery or release (which may be externally actuated) under the right conditions and in a specific or localized *in vivo* environment. Some preliminary photonic mechanisms to accomplish this have already been prototyped.[2078] We can envision that the ideal chemistries to accomplish this for NP bioconjugation would resemble a "plug and play" toolbox where the "recipe" for assembling a given NP bioconjugate, with all the desired properties (biological ratio, orientation, attachment affinity, etc.) would always be readily available perhaps even in a preassembled kit form. Regardless of whether this final goal is ever achieved, a countless number of increasingly complex, versatile, and useful NP bioconjugate constructs will be designed, synthesized, and applied in the process.

ASSOCIATED CONTENT

Supporting Information

A table listing some suppliers of selected NPs, NP bioconjugates, and related materials. This material is available free of charge via the Internet at http://pubs.acs.org.

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Russ Algar completed a Ph.D. in analytical chemistry at the University of Toronto in 2010, under the supervision of Ulrich Krull. He then moved to the Center for Bio/Molecular Science and Engineering at the U.S. Naval Research Laboratory as a postdoctoral researcher (through the College of Science at George Mason University) working with Igor Medintz. In the summer of 2012, he moved to the University of British Columbia as an assistant professor in the Department of Chemistry. His current research interests are in exploiting optically active nanoparticles and their interfacial properties for the development of multiplexed or multifunctional biosensors and diagnostics, quantitative probes for intra- or extracellular measurements, and theranostics.

Lorenzo Berti received his B.Sc. (Laurea) in Chemical Engineering from the University of Bologna, Italy, in 1995 and a Ph.D. in Organic Chemistry from the same institution in 2000. Afterwards he was a postdoctoral researcher at the University of California, Berkeley, under the supervision of Prof. Richard Mathies where he worked on the synthesis of universal fluorescent probes for DNA sequencing and genotyping. In 2003, he joined the National Institute for Nanostructures and Biosystems at Surfaces of the Italian National Research Council (CNR) as a Research Scientist, where he became interested in nanoparticles and nanotechnology while developing new chemistries to explore the interface between

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Eunkeu Oh received an M.S. in physics from POSTECH (Pohang University of Science and Technology) and worked for Samsung for over 11 years starting in 1996. In 2006, she obtained a Ph.D. in biological science from KAIST (Korean Advanced Institute of Science and Technology). She subsequently joined the Naval Research Laboratory through Johns Hopkins University to focus on the development of nanoparticle-based optical materials and their biological application. She received the Jang Young Sil prize from the Korean government and the best annual patent award from Bioneer. She also received a postdoctoral fellowship from the Korean Research Foundation.

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LIST OF ABBREVIATIONS

AAC	azide−alkyne cycloaddition
ACP	acyl carrier protein
AFP	alkynated fluorescent peptide
AgNP	silver nanoparticle
ALG	alginate
APTES	(3-aminopropyl)triethoxysilane
APTMS	(3-aminopropyl)trimethoxysilane
AuNP	gold nanoparticle
BacMPs	bacterial magnetic particles
BCP	block copolymer
BirA	*E. coli* biotin ligase
BMV	brome mosaic virus
Boc	*tert*-butyloxycarbonyl
β-PE	β-phycoerythrin
BRET	bioluminescent resonance energy transfer
BS^3	bis[sulfosuccinimidyl] suberate
BSA	bovine serum albumin
CaNPs	Ca-based NPs
CCMV	cowpea chlorotic mottle virus
C-dots	carbon nanodots
CEA	carcinoembryonic antigen
CHI	chitosan
CHO	Chinese hamster ovary cells
ChT	chymotrypsin
CNBr	cyanogen bromide
CNT	carbon nanotube
CPP	cell-penetrating peptide
CPMV	cowpea mosaic virus
CaP NP	calcium phosphate NPs
CT	computed tomography
CTAB	cetyltrimethylammonium bromide
CrAsH	FlAsH probe that contains a second carboxy group
CuAAC	copper(I)-catalyzed azide−alkyne cycloaddition
CuSNPs	cupric sulfide NPs
DCC	*N*,*N*′-dicyclohexylcarbodiimide
DHFR	dihydrofolate reductase
DHLA	dihydrolipoic acid
DMPE	1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine
DOPE	1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine
DOTA	1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
DOX	doxorubicin
DPPC	1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine
DPPE	1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine
DPPG	1,2-dipalmitoyl-*sn*-glycero-3[phosphor-*rac*-(1-glycerol)]
DSPC	1,2-disteroyl-*sn*-glycero-3-phosphocholine
DSPE	1,2-distearoyl-*sn*-glcyero-phosphoethanolamine
DTPA	diethylene triamine pentaacetic acid
DTSSP	3,3′-dithiobis[sulfosuccinimidylpropionate]
EDC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
EGF/EGFR	epidermal growth factor receptor
eGFP	enhanced green fluorescent protein
ELP	elastin-like polypeptide
ELISA	enzyme-linked immunosorbent assay
EPR	enhanced permeation and retention
FA	folate/folic acid
4FB	4-formylbenzamide
FDA	United States Food and Drug Administration
FHV	flock house virus
FITC	fluorescein isothiocyanate
FlAsH	fluorescein arsenical helix binder
FRET	Förster (fluorescence) resonance energy transfer
GA	glutathione
GFP	green fluorescent protein
GMBS	*N*-(γ-maleimidobutyryloxy) succinimide
GMR	giant magnetoresistive
GPTMS	glycidoxypropyltrimethoxysilane
GOx	glucose oxidase
GST	glutathione-*S*-transferase
HAA	hyaluronate, hyaluronic acid
HBV	hepatitis B virus
hCG	human chorionic gonadotropin
HCPT	10-hydroxycamptothecin
HDL	high-density lipoprotein
HeLa	cervical cancer cell line derived from tumor tissue obtained from Henrietta Lacks
HER2	human epidermal growth factor receptor 2
His_n	polyhistidine
HRP	horseradish peroxidase
HSA	human serum albumin
Hsp	heat shock protein
HyNic/HYNIC	6-hydrazinonicotinic acid
ICG	indocyanine green
IgG	immunoglobulin G

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IO	iron oxide
IONP	iron oxide nanoparticle
IONP@Au	iron oxide core/gold shell NPs
IR	infrared
LbL	layer-by-layer
LDL	low-density lipoprotein
LSPR	localized surface plasmon resonance
Luc	luciferase
MAA	methacrylic acid
MBP	maltose binding protein
MEIO	magnetism-engineered iron oxide
MPBH	4-[4-*N*-maleimidophenyl]butyric acid hydrazide
MRI	magnetic resonance imaging
Mts-Atf-biotin	methanethiosulfonate−azidotetrafluoro−biotin
MWNT	multiwalled nanotube
NCL	native chemical ligation
ND	nanodiamond
NGR	asparagine-glycine-arginine
NHS	*N*-hydroxysulfosuccinimide/*N*-hydroxysuccinimide
NM	nanomaterial
NP	nanoparticle
NPIA	*p*-nitrophenyliodoacetate
NTA	nitrilotriacetic acid
NV	nitrogen vacancy
O/W	oil-in-water
PAA	poly(acrylic acid)
PAX	paclitaxel
*p*aF	*p*-aminophenylalanine
PAMAM	poly(amido amine)
PC	phosphatidylcholine/phosphocholine
PE	phosphatidylethanolamine
PEG	poly(ethylene glycol)
PEI	polyethyleneimine
PEO	poly(ethylene oxide)
PDGF	platelet-derived growth factor
PDT	photodynamic therapy
PdNP	palladium nanoparticle
pI	isoelectric point
PL	photoluminescence
PLA	poly(lactic acid)
PLGA	poly(lactic acid-*co*-glycolic acid)
PMAA	poly(methacrylic acid)
PNA	peptide nucleic acid
PPT	phosphopantetheinyl transferase
PS	polystyrene
PS-I	photosystem I
PSA	prostate-specific antigen
PtNP	platinum nanoparticle
PVC	poly(vinyl chloride)
PVP	poly(vinyl pyrrolidone)
QD	quantum dot
QY	quantum yield
RAFT	reversible addition−fragmentation chain transfer polymerization
RGD	Arg-Gly-Asp, integrin binding tripeptide
ROS	reactive oxygen species
SA	streptavidin
SAM	self-assembled monolayer
SAMSA	*S*-acetylmercaptosuccinic anhydride
SATA	succinimidyl-*S*-acetylthioacetate
scFV	single-chain variable fragment
SEB	staphylococcal enterotoxin B
SERS	surface-enhanced Raman spectroscopy/scattering
SEM	scanning electron microscope
SiO_2 NP	silicon dioxide nanoparticle
Si-NP	silicon nanoparticle
siRNA	small-interfering RNA
SLNs	solid lipid nanoparticles
SMCC	succinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate
SMPT	succinimidyloxycarbonyl-α-methyl-α-(2-pyridyldithio)toluene
SPADC	strain-promoted alkyne diazocarbonyl cycloaddition
SPANC	strain-promoted alkyne nitrone cycloaddition
SPANOC	strain-promoted alkyne nitrile oxide cycloaddition
SPDP	*N*-hydroxysuccinimidyl 3-(2-pyridyldithio)-propionate
SPECT	single-photon emission computed tomography
SPIO	superparamagnetic iron oxide
ss	single stranded
sulfo-LC-SPDP	sulfosuccinimidyl 6-[3′-[2-pyridyldithio]-propionamido]hexanoate
sulfo-SMCC	sulfo-succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carbonate
S/V ratio	surface area-to-volume ratio
SWCNT	single-wall carbon nanotube
TAT	transactivator of transcription of the human immunodeficiency virus (HIV)
TEM	transmission electron microscope
TEOS	tetraethyl orthosilicate
Tf	transferrin
TiO_2 NPs	titanium dioxide NPs
TMV	tobacco mosaic virus
TNT	trinitrotoluene
TPP	tripolyphosphate
UV	ultraviolet
VLDL	very-low-density lipoprotein
VLP	virus-like particle
VNP	virus nanoparticle

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