ACCOUNTS of chemical research

pubs.acs.org/accounts Article

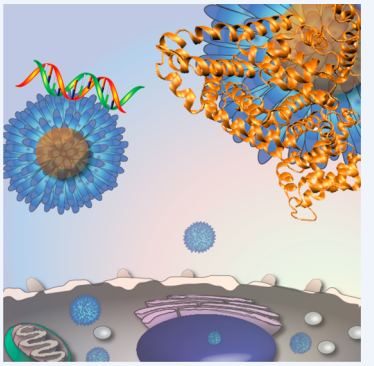
The Why and How of Ultrasmall Nanoparticles

Matthias Epple,* Vincent M. Rotello,* and Kenneth Dawson*

Cite This: *Acc. Chem. Res.* 2023, 56, 3369−3378 Read Online

ACCESS | Metrics & More | Article Recommendations

CONSPECTUS: In this Account, we describe our research into ultrasmall nanoparticles, including their unique properties, and outline some of the new opportunities they offer. We will summarize our perspective on the current state of the field and highlight what we see as key questions that remain to be solved. First, there are several nanostructure size-scale regimes, with qualitatively distinct functional biological attributes. Broadly generalized, larger particles (e.g., larger than 300 nm) tend to be more efficiently swept away by the first line of the immune system (for example macrophages). In the "middle-sized" regime (20−300 nm), nanoparticle surfaces and shapes can be recognized by energy-dependent cellular reorganizations, then organized locally in a spatial and temporally coherent way. That energy is gated and made available by specific cellular recognition processes. The relationship between particle surface design, endogenously derived nonspecific biomolecular corona, and architectural features recognized by the cell is complex and only purposefully and very precisely designed nanoparticle architectures are able to navigate to specific targets. At sufficiently small sizes (<10 nm including the ligand shell, associated with a core diameter of a few nm at most) we enter the "quasi-molecular regime" in which the endogenous biomolecular environment exchanges so rapidly with the ultrasmall particle surface that larger scale cellular and immune recognition events are often greatly simplified. As an example, ultrasmall particles can penetrate cellular and biological barriers within tissue architectures via passive diffusion, in much the same way as small molecule drugs do. An intriguing question arises: what happens at the interface of cellular recognition and ultrasmall quasi-molecular size regimes? Succinctly put, ultrasmall conjugates can evade defense mechanisms driven by larger scale cellular nanoscale recognition, enabling them to flexibly exploit molecular interaction motifs to interact with specific targets. Numerous advances in control of architecture that take advantage of these phenomena have taken place or are underway. For instance, syntheses can now be sufficiently controlled that it is possible to make nanoparticles of a few hundreds of atoms or metalloid clusters of several tens of atoms that can be characterized by single crystal X-ray structure analysis. While the synthesis of atomically precise clusters in organic solvents presents challenges, water-based syntheses of ultrasmall nanoparticles can be upscaled and lead to well-defined particle populations. The surface of ultrasmall nanoparticles can be covalently modified with a wide variety of ligands to control the interactions of these particles with biosystems, as well as drugs and fluorophores. And, in contrast to larger particles, many advanced molecular analytical and separation tools can be applied to understand their structure. For example, NMR spectroscopy allows us to obtain a detailed image of the particle surface and the attached ligands. These are considerable advantages that allow further elaboration of the level of architectural control and characterization of the ultrasmall structures required to access novel functional regimes and outcomes. The ultrasmall nanoparticle regime has a unique status and provides a potentially very interesting direction for development.



KEY REFERENCES

- van der Meer, S. B.; Hadrovic, I.; Meiners, A.; Loza, K.; Heggen, M.; Knauer, S. K.; Bayer, P.; Schrader, T.; Beuck, C.; Epple, M. New tools to probe the protein surface: ultrasmall gold nanoparticles carry amino acid binders. *J. Phys. Chem. B* **2021**, *125*, 115−127.[1] *A molecular tweezer was attached as supramolecular binder to ultrasmall gold nanoparticles. The selective interaction with cationic amino acids on the surface of the proteins hPin1 and Survivin was shown by NMR spectroscopy, isothermal titration calorimetry, and fluorescence polarization.*
- Moyano, D. F.; Saha, K.; Prakash, G.; Yan, B.; Kong, H.; Yazdani, M.; Rotello, V. M. Fabrication of corona-free nanoparticles with tunable hydrophobicity, *ACS Nano* **2014**, *8*, 6748−6755.[2] *Zwitterionic headgroups were developed that prevented corona formation with 2 nm core gold nanoparticles while enabling systematic control of surface hydrophobicity.*
- Boselli, L.; Polo, E.; Castagnola, V.; Dawson, K. A. Regimes of biomolecular ultrasmall nanoparticle interactions. *Angew. Chem. Int. Ed.* **2017**, *56*, 4215−4218.[3] *A*

Received: August 3, 2023
Revised: November 1, 2023
Accepted: November 6, 2023
Published: November 15, 2023

simple approach was presented to study the biomolecular interactions at the ultrasmall bionanointerface, allowing systematic linkage between microscopic molecular principles to predictions of in vivo biodistribution.

INTRODUCTION

Ultrasmall nanoparticles have a size of 1−3 nm and usually a spherical or globular shape.[4] They are smaller than many colloidal objects usually considered as an aggregate or a supramolecular assembly, e.g., micelles, liposomes, extracellular vesicles, or polyplexes of cationic and anionic polymers. Even dispersed polymers, plasmid DNA, antibodies, and proteins are usually somewhat larger. Here, we restrict our discussion to ultrasmall nanoparticles with a defined and purposefully made solid inorganic core, although many of the issues we discuss are relevant across all particles in this size regime. Chemically, particles with a metal core or a saltlike (ionic) core (like II−VI semiconductor quantum dots (QDs),[5] ultrasmall iron oxide nanoparticles (uSPIONs),[6] and ultrasmall nanodiamonds (USNDs) with a diameter below 3 nm fall into this category.[7] Generally, such nanoparticles are stabilized by an outer shell of ligands against agglomeration and Ostwald ripening, therefore we consider here dispersed objects with a solid core of 1−3 nm diameter and a hydrodynamic diameter of 4−10 nm due to the stabilizing shell.

The advantage of these ultrasmall particles in nanoscale diagnostics and therapeutics is clear, although the addition of biological function to ultrasmall particles by grafting molecular moieties is a somewhat more subtle issue. Certainly, the limited degree and short lifetime of the nonspecific corona in biological media potentially allows us to evade nanoscale cellular recognition machinery.[8] And, if we can simultaneously decouple ultrasmall cellular recognition from diffusive access and selective binding to otherwise inaccessible targets, we can make very flexible therapeutic and diagnostic constructs. Particle conjugates could then diffuse and selectively bind to targets within cells and behind biological barriers, according to more established molecular pharmacokinetic principles of absorption, distribution, metabolism, and excretion (ADME). Here we will summarize progress from our laboratories and highlight what needs to be done with a deliberate focus on ultrasmall gold nanoparticles.

How to Prepare Ultrasmall Nanoparticles

The synthesis of nanoparticles, ultrasmall and larger, has reached a considerable degree of maturity, and many excellent reviews on synthesis, purification, analysis, and applications are available.[9−14] Nanoparticles can be inorganic, organic, polymeric, and biological in nature, depending on the requirements of their corresponding application. If we consider biological applications, nanoparticles find applications in nanomedicine, e.g., in oncology or immunology including vaccination, where they act as carriers for drugs, often in combination with a targeting moiety on their surface. Notably, the RNA vaccines against COVID-19 consist of lipid nanoparticles with incorporated RNA.[15] Typical nanoparticles used in biomedicine have a size between 5 and 100 nm.[14,16,17] Ultrasmall nanoparticles, however, have been explored to a much lesser degree.[4]

Ultrasmall nanoparticles contain only a few hundred atoms or ions. We can distinguish ultrasmall inorganic nanoparticles of three different types: metallic, saltlike (ionic), and nanodiamonds. Metallic nanoparticles are mostly prepared bottom-up by chemical reduction of the corresponding metal salts in the presence of suitable capping agents.[9,13,18] Ionic nanoparticles like quantum dots (typical size 2 to 6 nm) are prepared in the same way by mixing the salt components, i.e., cations and anions.[5] Nanodiamonds are usually prepared top-down by detonation syntheses and less frequently by bottom up-synthesis, but a reproducible synthesis in the ultrasmall size range (3 nm and below) is not yet available.[7] While ultrasmall nanoparticles have some degree of diversity in terms of particle size distribution and particle shape, atomically precise nanoclusters have a well-defined stoichiometry and crystal structure and are prepared in the same way as ultrasmall nanoparticles but often in organic solvents.[19,20] Metallic nanoparticles consist of noble metals that do not oxidize or dissolve in contact with water or air. Consequently, the most prominent metals for this purpose are silver, gold, and the platinum group metals. Due to their high specific surface area, less noble metals like copper are easily oxidized if present as nanoparticle in contact with water.[21] Because of established syntheses and its high chemical stability, gold is by far the most commonly used metal used for ultrasmall metallic nanoparticles, with platinum and silver coming next. This also holds for atomically precise clusters where gold[22] and silver[23] enjoy a special prominence. As capping agents, organic ligands with the soft Lewis base donor atoms sulfur and phosphorus are generally preferred because they form a strong bond to the soft Lewis-acidic noble metals, in contrast to weakly binding oxygen-terminated ligands like citrate.[24] Besides monatomic clusters, bimetallic clusters[11] and ultrasmall nanoalloys[25,26] have also been prepared. In the following, we will concentrate on metallic nanoparticles, in particular gold nanoparticles, although many conclusions will also apply to particles of the same size but of a different chemical nature. We will also refrain from discussing atom-sharp clusters as there are many excellent reviews on this topic.[11,20,23,24,27,28]

Ultrasmall nanoparticles are always spherical or globular. There is currently no synthesis available that leads to uniform and well-defined anisotropic shapes. The underlying reason is probably that a nonspherical shape is thermodynamically unfavorable given the already very high specific surface area of ultrasmall nanoparticles. In contrast, anisotropic atom-sharp clusters are known, but these are better defined in terms of stoichiometry and crystal structure than ultrasmall nanoparticles.[12] Single-crystalline and twinned nanoparticle shapes have been theoretically derived and experimentally confirmed for gold nanoclusters, including a discussion on kinetics and thermodynamics.[29] If we consider a spherical gold nanoparticle of 2 nm diameter, it contains about 250 gold atoms. Thus, 1 g contains 1.23×10^{19} nanoparticles with a total specific surface area of 155 $m^2\ g^{-1}$. This is clearly a very high number that is comparable to zeolites. In contrast, 1 g of plasmonic gold nanoparticles (15 nm) has a specific surface area of "only" 21 $m^2\ g^{-1}$. This high specific surface area makes ultrasmall nanoparticles in a ligand-free, i.e. "naked" state, particularly interesting in heterogeneous catalysis,[12,20] including electrochemistry, but also underscores their value as high-capacity drug carriers.

Notably, ultrasmall nanoparticles are more difficult to isolate, purify, and characterize than conventional nanoparticles.[7,10,11,24,28] Applicable methods are nanofiltration (spin filtration), ultracentrifugation, precipitation, and dialysis. To our experience, precipitation (if possible) and nanofiltration are the easiest and most general methods. In ultracentrifugation, the separation of precipitate and supernatant is often difficult. Dialysis takes long and involves the handling of large solvent

https://doi.org/10.1021/acs.accounts.3c00459
Acc. Chem. Res. 2023, 56, 3369−3378

volumes. Nanofiltration does not require a sedimentation of the nanoparticles because the whole dispersion is passed through a filter. This is important if the nanoparticle core consists of a metal of lower density like silver or of carbon in the case of nanodiamonds. However, in terms of a defined surface functionalization in several reaction steps, the possibility for isolation of the nanoparticles via centrifugation is advantageous as it avoids the often-tedious separation of organic molecules via HPLC and similar techniques.

How to Analyze Ultrasmall Nanoparticles

Characterization of ultrasmall nanoparticles is challenging.[4,14,28,30] Common methods for characterization of nanoparticles can sometimes be impossible to apply. Scanning electron microscopy (SEM), dynamic light scattering (DLS), or nanoparticle tracking analysis (NTA) often fail because the particles are too small. For size analysis of dispersed particles, analytical ultracentrifugation (AUC) and differential centrifugal sedimentation (also known as analytical disc centrifugation) are both possible. However, as the density difference between a particle together with its hydrated ligand shell and the solvent is the dominant parameter, it is difficult to analyze particles that consist of a small metallic core in combination with a large ligand shell. For instance, a gold nanoparticle with a 2 nm core and a 2 nm thick shell around (with a density close to the solvent) has an effective density of about 1.64 g cm^{-3}, i.e., much less than the original density of gold (19.3 g cm^{-3}). Small angle scattering (usually of X-rays; SAXS) is a powerful method to study individual and partially agglomerated nanoparticles on the ultrasmall length scale.[31] The high density of noble metals helps due to the high contrast between nanoparticles and solvents. SAXS also gives information on the solid core, i.e., it ignores any organic coating. For dried particles, transmission electron microscopy is the method of choice to study particle shape, size, and size distribution, generally performed in vacuum and showing only the inorganic core. Mass spectrometry (e.g., ICP-MS) on single particles can also give valuable insight.[32]

The arrangement of atoms inside a nanoparticle can be provided by the crystal structure. Contrary to what many people believe, many nanoparticles are not small single cut-outs of a bulk metal crystal, i.e., out of a cubic or hexagonal close packing.[29] This variation is also evident from a number of single-crystal X-ray structure analyses of metalloid clusters that clearly indicate that the structure is based on covalent bonds between the metal atoms and not a close-packing of spheres.[11,24,28] Even if the nanoparticles are close-packed like the bulk metals, they are often crystallographically twinned, i.e., they consist of several single crystals.[27] Notably, a pentagonal bipyramid (Marks' decahedron) occurs often; according to theoretical predictions, it is already imprinted at the nucleation stage, followed by crystal growth.[29] In general, the diverse nature of ultrasmall nanoparticles renders them much less defined in terms of atom arrangement than atom-sharp metalloid clusters.

The internal structure of a nanoparticle can be addressed by diffraction methods, usually X-ray diffraction and electron diffraction. The small size of the particles makes recording and interpretation of diffraction patterns challenging because the diffraction peaks are very broad. Nevertheless, structure determination by Rietveld refinement is possible,[33,34] and the structural elucidation via pair-distribution functions (PDF) has also given deep insight into the arrangement of atoms.[35] X-ray photoelectron spectroscopy (XPS) yields the oxidation state of metals and ligands (e.g., the binding sulfur atoms).[33,34] Solid-state NMR spectroscopy is usually not applicable to the inorganic part (the core) of nanoparticles as many metals are not susceptible to NMR.[36] X-ray absorption spectroscopy (EXAFS) gives element-specific pair distribution function information on solid particles, mainly the solid core.[37] It is remarkable that apparently purely metallic ultrasmall nanoparticles were shown by both XRD and XPS to contain oxidized metals and even metal oxides.[33]

Counterbalancing the challenges, ultrasmall nanoparticles offer several advantages in terms of analysis. First, NMR spectroscopy can be used to characterize the ligand shell of dispersed nanoparticles.[10,38,39] NMR peaks are usually broadened due to the vicinity of the metal core, slowed ligand motion, and relaxation. Although this method is restricted to the nuclei of the ligand (^{1}H, ^{13}C), the extent of line-broadening is much smaller than in experiments where the metal core (e.g., ^{195}Pt) of solid nanoparticles is probed by solid-state NMR spectroscopy.[40] The NMR spectra of the attached ligands can be quantitatively analyzed to study the ligand shell, including the number of ligand molecules attached to the surface of each nanoparticle. Their number is remarkably high, with a molecular footprint of about 0.1 nm^2 for small molecules like glutathione[41] or cysteine.[42] This means that a nanoparticle of 2 nm diameter can easily carry 100 ligand molecules. This is not surprising as the majority of the atoms of a 2 nm nanoparticle are on the surface, and the radius of curvature is very small.[4] NMR spectroscopy can also be used to probe the surface structure of metallic nanoparticles that have different crystallographic and chemical environments for ligands (Figure 1).[42] Diffusion-ordered spectroscopy (DOSY) is another valuable NMR tool to determine the hydrodynamic diameter of dispersed nanoparticles.[38,42]

Plasmonic metal nanoparticles with a typical diameter of 5–25 nm show a distinct UV/vis absorption.[43] They efficiently quench the fluorescence of attached ligands. In contrast to larger nanoparticles, ultrasmall metallic nanoparticles show little or no surface plasmon resonance, i.e., they do not have a prominent

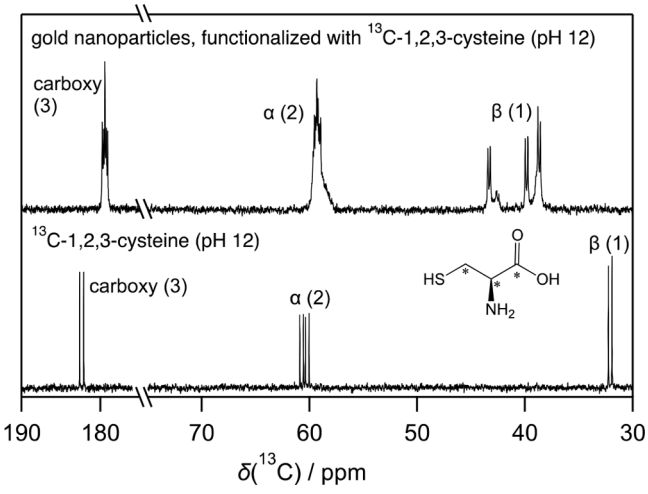


Figure 1. ^{13}C NMR spectra of ultrasmall gold nanoparticles (1.8 nm), functionalized with ^{13}C-1,2,3-labeled cysteine (top) and of dissolved ^{13}C-1,2,3-labeled cysteine (bottom). Note the peak shift and the peak broadening of cysteine after binding to the nanoparticles. The split of the β-carbon peaks indicates at least three different magnetic environments for cysteine on the gold surface. Adapted from ref 42. Copyright 2019 American Chemical Society.

https://doi.org/10.1021/acs.accounts.3c00459
Acc. Chem. Res. 2023, 56, 3369–3378

UV−vis absorption on their own.[43] Therefore, ultrasmall nanoparticles with a diameter below 1.5 to 2 nm do not quench fluorescence as strongly as larger particles.[44] Therefore, they can be easily labeled with fluorophores, e.g., to trace them in cell culture or tissues, something that is not possible with the larger-labeled plasmonic nanoparticles. In addition, the number of ligands on the nanoparticle surface can also be determined by UV/vis spectroscopy if the ligand contains a UV/vis-active chromophore, important to determine the dose of an attached drug molecule. The characteristic UV absorption of attached nucleic acids also permits their quantification on the particle surface.[45]

Nanoparticles for Targeting and Interaction with Biomolecules: Ligand Attachment

There are many reasons for attaching molecules to ultrasmall core particles, ranging from an increased local concentration of drugs on the particle surface, application in magnetic extraction, or the more specific task of targeting cells and tissues. For some of these applications adsorptive attachment may be sufficient, while others require attachment via a covalent bond. For applications in cell/tissue targeting, the obvious prerequisite is that the grafted moiety has a sufficiently strong affinity and specificity to the target and does not cross-interact off-target. Also, where we wish to decouple cellular recognition and defense mechanisms from specific molecular interactions directed at the target, it is necessary to consider direct off-target molecular interactions caused by the attachment chemistry as well as the nonspecific biomolecular corona. Several physiochemical methods have been developed to follow the transition across regimes where the particle corona is relatively fixed (and recognized by cells) to one where it fluctuates rapidly (and thereby, not recognized).[3,46] A rational ultrasmall particle design would require a time scale for fluctuations of the kinetically evolving protein corona to be faster than typical receptor recognition times for the particle-corona complex.[47] The application of this approach is now potentially feasible in a high-throughput mode in which many variants of the ultrasmall particle core and grafting design can be evaluated in complex milieu, and it is therefore useful to summarize the current status of approaches to grafting and how these could be further evolved to rational engineered ultrasmall particles. Figure 2 illustrates the protein corona formation and dynamics on ultrasmall nanoparticles.

Along these lines, ligands can be attached to nanoparticles by covalent bonds based on the strong sulfur-metal bond that is characteristic for all noble metals and can be considered as covalent[48] although ligand exchange may occur with dispersed nanoparticles.[49] Of course, this creates a better defined and more stable attachment of a ligand than a purely adsorptive bond. The covalent conjugation can be done during the synthesis, i.e., during the reduction of the metal ions to the metal. However, not all (bio)organic ligands survive the harsh conditions during reduction, i.e., the presence of oxidizing and potentially coordinating ions like Ag^{+} or Au^{3+} and of strongly reducing agents like $NaBH_4$. Nevertheless, peptides can be easily attached via cysteine to serve as capping agent and targeting molecule at the same time.[50,51]

For more sensitive ligands and for a better control over stoichiometry, it is preferable to attach the ligand after the particle has been prepared and stabilized. Ligand-exchange reactions[49] and versatile conjugation methods, e.g. click chemistry, can be used for decorating particles. The copper-catalyzed azide-alkyne cycloaddition (CuAAC) is especially suitable due to its versatility. A robust method to convert the amino groups of primarily attached glutathione molecules into azide groups has been developed that permits the subsequent attachment of alkyne-carrying ligands to the nanoparticle surface (Figure 3).[41] NMR spectroscopy (1D and 2D) enables kind and

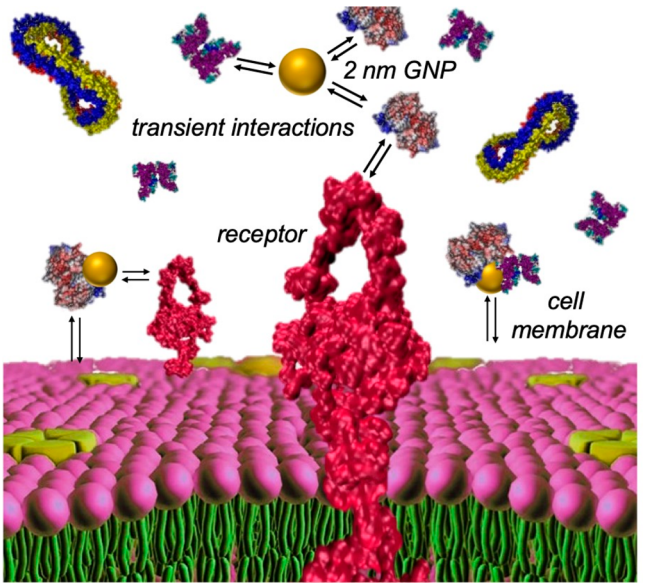


Figure 2. When particles (gold sphere) become ultrasmall, their interactions with ambient biomolecules become sufficiently short-lived and limited in variety (gold sphere with proteins in the environment) so that the particles can interact directly with cells and cellular machinery. Since they do not exist as long-lived complexes between particles and biomolecules from the environment, they are not recognized in a biological sense by the various receptors (red) and other components that trigger complex biological defenses of living organisms. In principle, this can allow ultrasmall particles to cross membranes, biological barriers, and evade immune responses, in much the same ways as some small molecule drugs with appropriate physicochemical properties. Adapted from ref 47. Copyright 2020 American Chemical Society.

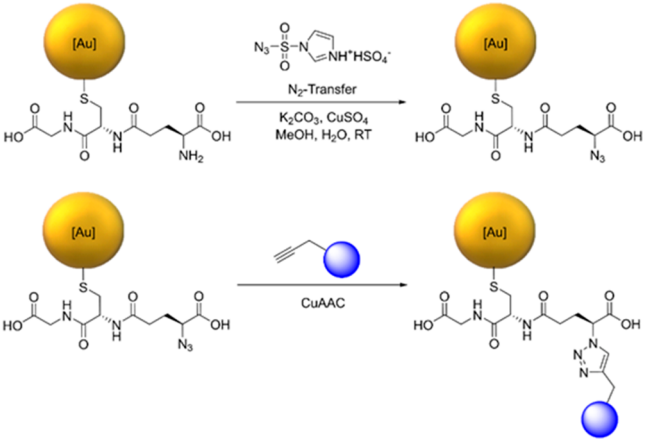


Figure 3. Schematic reaction pathway for the conversion of amino groups of glutathione on the gold nanoparticle surface to azide groups. A covalent coupling of alkyne-terminated ligands (represented by a blue ball) to the gold nanoparticle surface is possible via copper-catalyzed azide-alkyne cycloaddition (CuAAC). Adapted with permission from ref 45. Copyright 2022 Royal Society of Chemistry.

https://doi.org/10.1021/acs.accounts.3c00459
Acc. Chem. Res. 2023, 56, 3369−3378

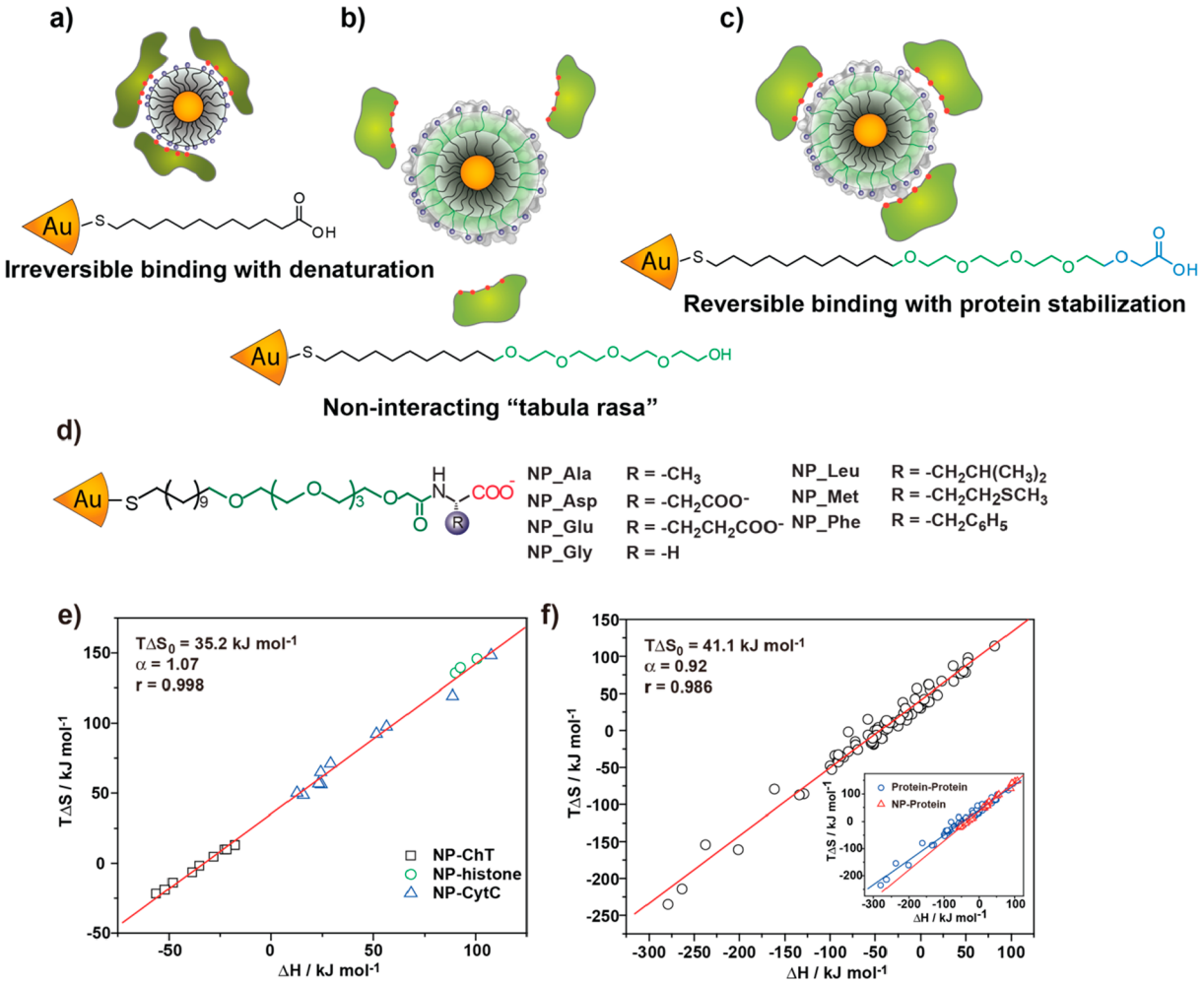


Figure 4. (a) Surfactant-like ligands bind and denature ChT. (b) TEG disrupts protein binding. (c) Appending a carboxylate to the outside of the TEG layer provides ChT binding without denaturation. (d) Gold nanoparticles (2 nm core) with anionic amino acid termini. Quantification of (e) nanoparticle−protein and (f) protein−protein interactions by ITC. Overlap of entropy−enthalpy compensation plots shown in the inset. Adapted from ref 59. Copyright 2007 American Chemical Society.

number of ligands to be determined on the intact nanoparticles (i.e., without detachment of the ligand shell), although the spectra can become crowded and difficult to interpret.[1,42] Functionalization is also possible via 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) conjugation of the carboxy group of a tiopronin ligand attached to gold and the amino group of a functional ligand, e.g., doxorubicin or cadaverine.[52] Another option are thiolated and functionalized macromolecules that efficiently cover the nanoparticle surface.[31,53]

Regulating the Interactions of Ultrasmall Nanoparticles with Biosystems

The ability to decorate nanoparticle surfaces enables us to dictate how they interact with the outside world.[54] Precisely engineered molecular ligands can be employed to control nanoparticle surface properties and chemical architectures.[55] The almost infinite design space of organic ligands yields a toolkit for controlling how nanoparticles interact with biosystems at multiple scales, with broad implications in the field of nanomedicine.[56] Ultrasmall nanoparticles feature sizes commensurate with proteins, making them useful as scaffolds that can avoid nonspecific protein recognition. Interfacing ultrasmall nanoparticles with proteins requires engineering of the particle surface. For instance, simple alkanethiol ligands with anionic termini on ultrasmall nanoparticles were used to bind the positively charged pocket on the surface of chymotrypsin (ChT) (Figure 4a). These surfactant-like ligands bound proteins with nanomolar affinity; however, they rapidly denatured adsorbed ChT.[57] The challenge of denaturation was addressed by appending a short tetra(ethylene glycol) (TEG) layer to these nanoparticles to provide a noninteracting "tabula rasa" particle that behaved like high-molecular weight ethylene glycol (Figure 4b). Attachment of an anionic carboxylate to the terminus of these ligands allowed binding of these particles to ChT without denaturation (Figure 4c).[58] This ligand motif was highly tunable, as shown through interactions of different amino acid-based anionic gold nanoparticles with cationic proteins (ChT, cytochrome C, and histone) quantified using isothermal titration calorimetry (ITC) (Figure 4d−f).[59] These studies showed that ultrasmall nanoparticle−protein interaction affinity can be tuned. Notably, entropy-enthalpy analysis of these interactions was quite similar to that of protein−protein interactions (Figure 4f, inset).

The covalent bond between the metal core and the ligand shell (e.g., Au-S)[48] is stable in dispersion and under cell culture conditions. Thus, the functionalized nanoparticles can be used to target proteins, cells, and tissues. Thiolated ligands can be

https://doi.org/10.1021/acs.accounts.3c00459
Acc. Chem. Res. 2023, 56, 3369−3378

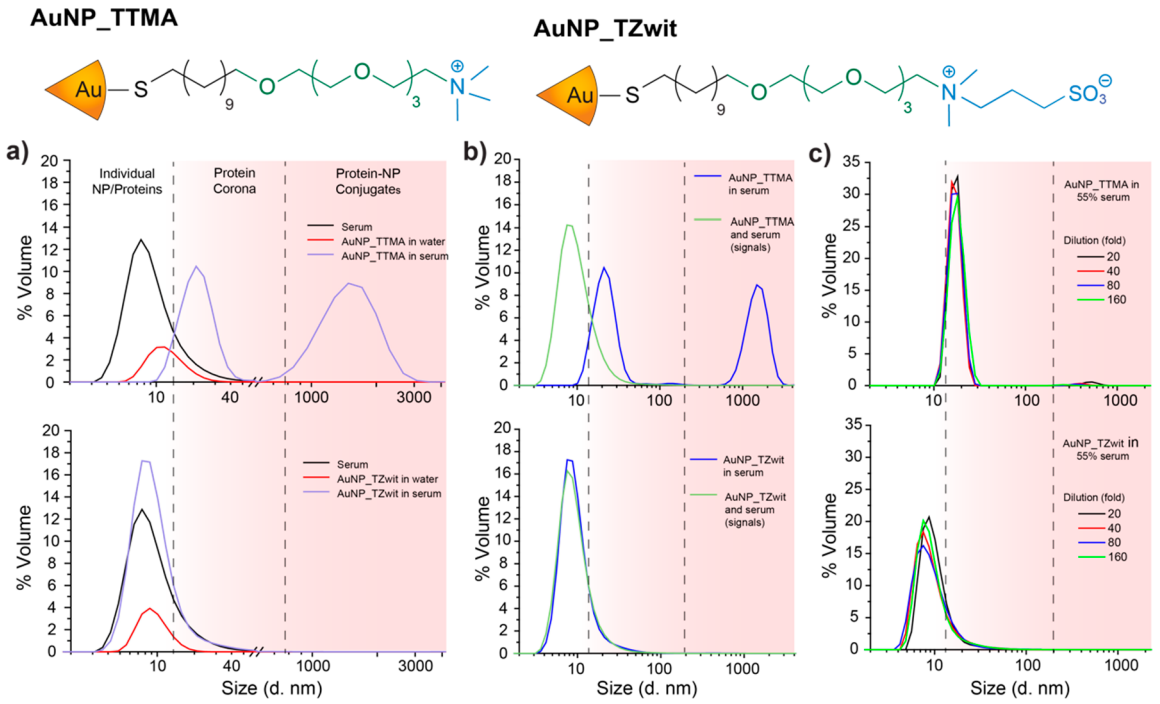


Figure 5. (a) Protein complexation is observed by DLS with AuNP_TTMA but not with AuNP_TZwit. (b) Large aggregates are observed in serum (5%) with AuNP_TTMA but no larger-sized assemblies were observed with AuNP_TZwit. (c) Dilution after incubation in 55% human serum showed no irreversible corona formation with either AuNP_TZwit or AuNP_TTMA, where size increase corresponds to a simple monolayer of protein around the particle. Adapted from ref 2. Copyright 2014 American Chemical Society.

exchanged by other thiolated ligands, i.e., there is a dynamic equilibrium.[49] This exchange has been observed both *in vitro* and *in vivo*, driven by intracellular thiols including cysteine and glutathione.[32]

The surface of ultrasmall nanoparticles can be engineered to evade interactions with proteins as well as foster them. A diverse body of research using >10 nm nanomaterials suggested that protein corona formation was inevitable.[60] This understanding was countered using zwitterionic 2 nm particles with the sulfobetaine motif with varying hydrophobicity.[2] Charged AuNP_TTMA increased in size (signaling corona formation) when mixed with serum, while the hydrodynamic size of AuNP_TZwit and other analogous particles zwitterionic particles was constant. Significantly, neither AuNP_TTMA nor AuNP_TZwit formed an irreversible corona even at physiological serum concentrations (Figure 5).

Ultrasmall gold nanoparticles have been functionalized with peptides[50,51] and organic receptor molecules[1] to target amino acids on the surface of proteins (Figure 6). Suitable methods to demonstrate and quantify this interaction are NMR spectroscopy (of the proteins and the targeting ligands), isothermal titration calorimetry (ITC), and fluorescence polarization (FP) if the protein is fluorescently labeled.[1,50,51]

The surface of ultrasmall nanoparticles can also be decorated by a wide range of functional molecules. As antibodies are much larger (about 12 nm) than ultrasmall nanoparticles, it does not make sense to attach them as targeting moiety to their surface. However, the much smaller nanobodies (about 4 nm)[61] were successfully attached to ultrasmall gold nanoparticles (2 nm; carrying about 4 nanobodies each) and showed highly efficient targeting against the protein Survivin.[62]

The ability to "dial in" nanoparticle-biosystem interactions fully extends to cells. HeLa cells were incubated with gold nanoparticles with 2, 4, and 6 nm cores and cationic, anionic, and

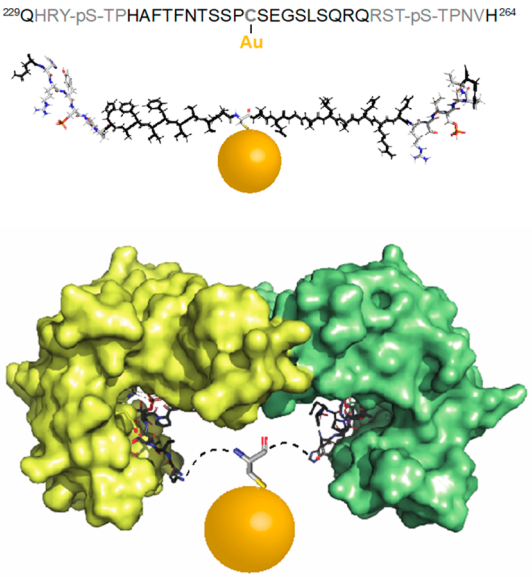


Figure 6. (Top) Schematic view of the modified sequence of the protein CRaf, conjugated to a gold nanoparticle (1.55 nm) by a central cysteine, drawn to scale. (Bottom) Schematic view of CRaf on a gold nanoparticle interacting with the homodimeric protein 14-3-3σ. Only the amino acids that interact with the protein-binding region are shown; the other part of the chain is shown as a dashed line. Adapted with permission from ref 51. Copyright 2021 Wiley-VCH.

zwitterionic ligands (Figure 7a), and the uptake was quantified by inductively coupled mass spectrometry (ICP-MS) (Figure 7b).[30] On the basis of the anionic membrane surface of mammalian cells, it is not surprising that cationic AuNP_TTMA nanoparticles were taken up more efficiently than the corresponding anionic and zwitterionic gold nanoparticles.

https://doi.org/10.1021/acs.accounts.3c00459
Acc. Chem. Res. 2023, 56, 3369−3378

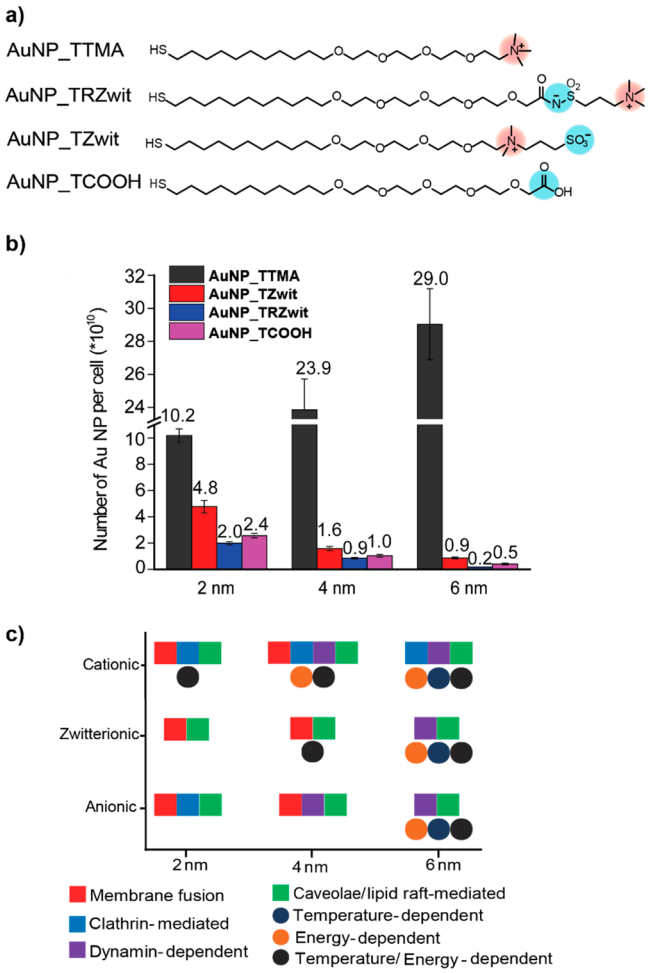


Figure 7. Uptake of 2, 4, and 6 nm core gold nanoparticles by HeLa cells. (a) Nanoparticle monolayers. (b) HeLa cell uptake of gold nanoparticles as quantified by ICP-MS. (c) Observed uptake pathways for gold nanoparticles. Adapted from ref 30. Copyright 2015 American Chemical Society.

The uptake increased with increasing AuNP_TTMA size, presumably due to the increased surface area for interaction with the cell membrane. Uptake efficiency diminished with increasing anionic and zwitterionic particle size, owing presumably to unfavorable particle-membrane interactions (Figure 7b). A rather surprising array of uptake pathways were observed with these particles, with interplay between particle size and surface coverage regulating particle uptake (Figure 7c). Clearly, the ultrasmall nanoparticles can use different endocytosis pathways to enter a cell.[47,52]

In principle, many ultrasmall metallic nanoparticles show autofluorescence.[63] However, this autofluorescence is less efficient than that of organic fluorophores.[64] Fluorescent dye-labeled ultrasmall nanoparticles can be easily traced into and inside cells.[65] Cell penetration of ultrasmall nanoparticles is efficient, in line with results obtained for larger nanoparticles. As with larger nanoparticles, endocytosis is the predominant uptake mechanism.[66]

Ultrasmall nanoparticles can enter the cell nucleus (Figure 8), something larger nanoparticles usually do not do.[1,67] They can also cross the blood-brain barrier in 3D cell culture models, i.e., brain organoids.[68] Consequently, they can act as transporters of drug cargo in the body. In general, ultrasmall nanoparticles are rather mobile in the body and rapidly excreted via the renal

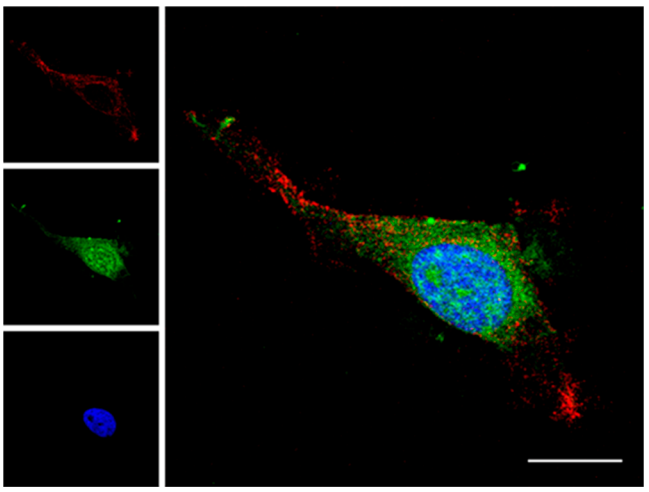


Figure 8. Confocal laser scanning microscopic images (z-stacks across the cells) to demonstrate the uptake of FAM-labeled fluorescing ultrasmall gold nanoparticles (2 nm) by HeLa cells (24 h). The particles easily entered the cell and the cell nucleus. 1×10^4 cells were plated per well, and 2.09×10^{14} ultrasmall gold nanoparticles were present in each well. Scale bar: 20 μm. Red, actin staining of the cell cytoskeleton; blue, nuclear staining by Hoechst33342; and green, FAM-labeled gold nanoparticles. Adapted from ref 70. Copyright 2019 American Chemical Society.

pathway.[4] They are also small enough to enter bacteria, as demonstrated by super-resolution microscopy (SIM and STORM).[69]

Finally, the attachment of small-interference RNA (siRNA) to the nanoparticle surface leads to efficient gene silencing inside eukaryotic cells, also of virus proteins.[45] This is in line with the concept of spherical nucleic acids,[71] i.e., siRNA conjugated to plasmonic gold nanoparticles. However, the mass-specific loading to ultrasmall nanoparticles is much higher due to the higher specific surface area. Eight siRNA molecules were attached to one 2 nm gold nanoparticle, corresponding to about 1.9 siRNA molecules per nm^3.[45] For 13 nm plasmonic gold nanoparticles with 40 attached siRNA molecules, the corresponding number is 0.035 siRNA molecules per nm^3.[71] This underscores the utility of the high specific surface area of ultrasmall nanoparticles.

CONCLUSIONS

With a size smaller than most proteins, antibodies, and plasmid DNA, ultrasmall nanoparticles (1−3 nm) can address biological targets more specifically, and without familiar off-target effects, than conventional (larger) nanoparticles (10−15 nm). Their specific surface functionalization permits a precise control over the interaction with biomolecules and cells. Due to their small size, they can easily penetrate cells, tissues, and biological barriers like the blood-brain barrier. Ultrasmall nanoparticles have a very high cargo-loading efficiency and do not quench fluorescence, therefore they can be easily fluorescently labeled and followed in cells and tissues. As they are small enough to evade the formation of a stable protein corona, their interaction with cells and tissues can be tuned more easily than in the case of larger nanoparticles. As ultrasmall nanoparticles work their way toward the clinic, key issues of biocompatibility and absorption, distribution, metabolism, and excretion (ADME) need to be addressed. As we learn the roadmap for the interaction of these

https://doi.org/10.1021/acs.accounts.3c00459
Acc. Chem. Res. 2023, 56, 3369−3378

particles with biosystems, their real-world utility will dramatically increase.

AUTHOR INFORMATION

Corresponding Authors

Matthias Epple − *Inorganic Chemistry and Centre for Nanointegration Duisburg-Essen (CeNIDE), University of Duisburg-Essen, 45117 Essen, Germany;* orcid.org/0000-0002-1641-7068; Email: matthias.epple@uni-due.de

Vincent M. Rotello − *Charles A. Goessmann Professor of Chemistry and University Distinguished Professor, Department of Chemistry, University of Massachusetts Amherst, Amherst, Massachusetts 01002, United States;* orcid.org/0000-0002-5184-5439; Email: rotello@chem.umass.edu

Kenneth Dawson − *UCD School of Chemistry, Science Centre South, University College Dublin, Dublin 4, Ireland;* orcid.org/0000-0002-0568-6588; Email: kenneth.a.dawson@cbni.ucd.ie

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.accounts.3c00459

Notes

The authors declare no competing financial interest.

Biographies

Matthias Epple obtained his Ph.D. at the Technical University of Brauschweig (Germany) in 1992. Next, he worked as postdoctoral scientist at the University of Washington (Seattle, USA), the University of Hamburg (Germany), and the Royal Institution (London, U.K.). In 2000, he became Associate Professor at the Ruhr-University of Bochum (Germany), and in 2003, he became Full Professor at the University of Duisburg-Essen (Germany). His research interests cover synthesis, characterization, and biomedical application of nanoparticles as well as the synthesis of biomaterials and the investigation of biomineralization.

Vincent M. Rotello started his independent career at the University of Massachusetts at Amherst (USA) in 1993 and is currently a University Distinguished Professor and the Goessmann Professor of Chemistry there. He is a Fellow of the American Association for the Advancement of Science (AAAS) and the Royal Society of Chemistry (U.K.). His research program focuses on nanomedicine, including bioorthogonal catalysis, antimicrobials, diagnostics, and delivery of biologics.

Kenneth A. Dawson is Director of Centre for Bionano Interactions and Chair of Physical Chemistry at University College Dublin (Ireland). He obtained his B.Sc. and M.Sc. degree at Queens University Belfast (Ireland) and D. Phil. at University College Oxford (U.K.). He is seeking to elucidate the mechanisms by which nanostructures are recognized and processed by living organisms and to use that understanding in the targeting of RNA and other nanoscale medicines. He has introduced several concepts, including the bimolecular corona, as a means to understand bionanoscale recognition.

ACKNOWLEDGMENTS

M.E. acknowledges support from the Deutsche Forschungsgemeinschaft (Germany, Ep 22/62-1). V.M.R. acknowledges support from the NIH (USA, EB022641, AI134770, and DK121351). K.D. acknowledges numerous academic collaborators that have helped this understanding, particularly with Professor Yan Yan at University College Dublin (Ireland).

REFERENCES

(1) van der Meer, S. B.; Hadrovic, I.; Meiners, A.; Loza, K.; Heggen, M.; Knauer, S. K.; Bayer, P.; Schrader, T.; Beuck, C.; Epple, M. New tools to probe the protein surface: ultrasmall gold nanoparticles carry amino acid binders. *J. Phys. Chem. B* **2021**, *125*, 115−127.

(2) Moyano, D. F.; Saha, K.; Prakash, G.; Yan, B.; Kong, H.; Yazdani, M.; Rotello, V. M. Fabrication of corona-free nanoparticles with tunable hydrophobicity. *ACS Nano* **2014**, *8*, 6748−6755.

(3) Boselli, L.; Polo, E.; Castagnola, V.; Dawson, K. A. Regimes of biomolecular ultrasmall nanoparticle interactions. *Angew. Chem., Int. Ed.* **2017**, *56*, 4215−4218.

(4) Zarschler, K.; Rocks, L.; Licciardello, N.; Boselli, L.; Polo, E.; Garcia, K. P.; De Cola, L.; Stephan, H.; Dawson, K. A. Ultrasmall inorganic nanoparticles: State-of-the-art and perspectives for biomedical applications. *Nanomedicine* **2016**, *12*, 1663−1701.

(5) Sobhanan, J.; Rival, J. V.; Anas, A.; Sidharth Shibu, E.; Takano, Y.; Biju, V. Luminescent quantum dots: Synthesis, optical properties, bioimaging and toxicity. *Adv. Drug Deliv. Rev.* **2023**, *197*, 114830.

(6) Merinopoulos, I.; Gunawardena, T.; Stirrat, C.; Cameron, D.; Eccleshall, S. C.; Dweck, M. R.; Newby, D. E.; Vassiliou, V. S. Diagnostic applications of ultrasmall superparamagnetic particles of iron oxide for imaging myocardial and vascular inflammation. *JACC-Cardiovasc. Imaging* **2021**, *14*, 1249−1264.

(7) Chang, S. L. Y.; Reineck, P.; Krueger, A.; Mochalin, V. N. Ultrasmall nanodiamonds: Perspectives and questions. *ACS Nano* **2022**, *16*, 8513−8524.

(8) Wang, Y. F.; Zhou, Y.; Sun, J.; Wang, X.; Jia, Y.; Ge, K.; Yan, Y.; Dawson, K. A.; Guo, S.; Zhang, J.; Liang, X. J. The Yin and Yang of the protein corona on the delivery journey of nanoparticles. *Nano Res.* **2023**, *16*, 715−734.

(9) Dreaden, E. C.; Alkilany, A. M.; Huang, X.; Murphy, C. J.; El-Sayed, M. A. The golden age: gold nanoparticles for biomedicine. *Chem. Soc. Rev.* **2012**, *41*, 2740−2779.

(10) Guo, C.; Yarger, J. L. Characterizing gold nanoparticles by NMR spectroscopy. *Magn. Reson. Chem.* **2018**, *56*, 1074−1082.

(11) Kang, X.; Li, Y. W.; Zhu, M. Z.; Jin, R. C. Atomically precise alloy nanoclusters: Syntheses, structures, and properties. *Chem. Soc. Rev.* **2020**, *49*, 6443−6514.

(12) Liu, Z. H.; Wu, Z. N.; Yao, Q. F.; Cao, Y. T.; Chai, O. J. H.; Xie, J. P. Correlations between the fundamentals and applications of ultrasmall metal nanoclusters: Recent advances in catalysis and biomedical applications. *Nano Today* **2021**, *36*, 101053.

(13) Moyano, D. F.; Duncan, B.; Rotello, V. M. Preparation of 2 nm gold nanoparticles for in vitro and in vivo applications. *Methods Mol. Biol.* **2013**, *1025*, 3−8.

(14) Pelaz, B.; Alexiou, C.; Alvarez-Puebla, R. A.; Alves, F.; Andrews, A. M.; Ashraf, S.; Balogh, L. P.; Ballerini, L.; Bestetti, A.; Brendel, C.; Bosi, S.; Carril, M.; Chan, W. C. W.; Chen, C.; Chen, X.; Chen, X.; Cheng, Z.; Cui, D.; Du, J.; Dullin, C.; Escudero, A.; Feliu, N.; Gao, M.; George, M.; Gogotsi, Y.; Grünweller, A.; Gu, Z.; Halas, N. J.; Hampp, N.; Hartmann, R. K.; Hersam, M. C.; Hunziker, P.; Jian, J.; Jiang, X.; Jungebluth, P.; Kadhiresan, P.; Kataoka, K.; Khademhosseini, A.; Kopecek, J.; Kotov, N. A.; Krug, H. F.; Lee, D. S.; Lehr, C. M.; Leong, K. W.; Liang, X. J.; Ling Lim, M.; Liz-Marzan, L. M.; Ma, X.; Macchiarini, P.; Meng, H.; Möhwald, H.; Mulvaney, P.; Nel, A. E.; Nie, S.; Nordlander, P.; Okano, T.; Oliveira, J.; Park, T. H.; Penner, R. M.; Prato, M.; Puntes, V.; Rotello, V. M.; Samarakoon, A.; Schaak, R. E.; Shen, Y.; Sjöqvist, S.; Skirtach, A. G.; Soliman, M. G.; Stevens, M. M.; Sung, H. W.; Tang, B. Z.; Tietze, R.; Udugama, B. N.; Van Epps, J. S.; Weil, T.; Weiss, P. S.; Willner, I.; Wu, Y.; Yang, L.; Yue, Z.; Zhang, Q.; Zhang, Q.; Zhang, X. E.; Zhao, Y.; Zhou, X.; Parak, W. J. Diverse applications of nanomedicine. *ACS Nano* **2017**, *11*, 2313−2381.

(15) Khurana, A.; Allawadhi, P.; Khurana, I.; Allwadhi, S.; Weiskirchen, R.; Banothu, A. K.; Chhabra, D.; Joshi, K.; Bharani, K. K. Role of nanotechnology behind the success of mRNA vaccines for COVID-19. *Nano Today* **2021**, *38*, 101142.

(16) Germain, M.; Caputo, F.; Metcalfe, S.; Tosi, G.; Spring, K.; Åslund, K. K. O.; Pottier, A.; Schiffelers, R.; Ceccaldi, A.; Schmid, R.

https://doi.org/10.1021/acs.accounts.3c00459
Acc. Chem. Res. 2023, 56, 3369−3378

Delivering the power of nanomedicine to patients today. *J. Controlled Release* **2020**, *326*, 164−171.
(17) van der Meel, R.; Sulheim, E.; Shi, Y.; Kiessling, F.; Mulder, W. J. M.; Lammers, T. Smart cancer nanomedicine. *Nat. Nanotechnol.* **2019**, *14*, 1007−1017.
(18) Leifert, A.; Pan-Bartnek, Y.; Simon, U.; Jahnen-Dechent, W. Molecularly stabilised ultrasmall gold nanoparticles: synthesis, characterization and bioactivity. *Nanoscale* **2013**, *5*, 6224−6242.
(19) Jin, R.; Zeng, C.; Zhou, M.; Chen, Y. Atomically precise colloidal metal nanoclusters and nanoparticles: fundamentals and opportunities. *Chem. Rev.* **2016**, *116*, 10346−10413.
(20) Du, Y.; Sheng, H.; Astruc, D.; Zhu, M. Atomically precise noble metal nanoclusters as efficient catalysts: A bridge between structure and properties. *Chem. Rev.* **2020**, *120*, 526−622.
(21) Kent, R. D.; Vikesland, P. J. Dissolution and persistence of copper-based nanomaterials in undersaturated solutions with respect to cupric solid phases. *Environ. Sci. Technol.* **2016**, *50*, 6772−6781.
(22) Zeng, C. J. Precision at the nanoscale: On the structure and property evolution of gold nanoclusters. *Pure Appl. Chem.* **2018**, *90*, 1409−1427.
(23) Yang, J.; Jin, R. C. New advances in atomically precise silver nanoclusters. *ACS Mater. Lett.* **2019**, *1*, 482−489.
(24) Li, Y. W.; Jin, R. C. Seeing ligands on nanoclusters and in their assemblies by X-ray crystallography: atomically precise nanochemistry and beyond. *J. Am. Chem. Soc.* **2020**, *142*, 13627−13644.
(25) Loza, K.; Heggen, M.; Epple, M. Synthesis, structure, properties, and applications of bimetallic nanoparticles of noble metals. *Adv. Funct. Mater.* **2020**, *30*, 1909260.
(26) Wolff, N.; Loza, K.; Heggen, M.; Schaller, T.; Niemeyer, F.; Bayer, P.; Beuck, C.; Oliveira, C. L. P.; Prymak, O.; Weidenthaler, C.; Epple, M. Ultrastructure and surface composition of glutathione-terminated ultrasmall silver, gold, platinum, and alloyed silver-platinum nanoparticles (2 nm). *Inorg. Chem.* **2023**, *62*, 17470−17485.
(27) Zhou, S.; Zhao, M.; Yang, T. H.; Xia, Y. N. Decahedral nanocrystals of noble metals: Synthesis, characterization, and applications. *Mater. Today* **2019**, *22*, 108−131.
(28) Du, X. S.; Jin, R. C. Atomic-precision engineering of metal nanoclusters. *Dalton Trans.* **2020**, *49*, 10701−10707.
(29) Barnard, A. S. Direct comparison of kinetic and thermodynamic influences on gold nanomorphology. *Acc. Chem. Res.* **2012**, *45*, 1688−1697.
(30) Jiang, Y.; Huo, S.; Mizuhara, T.; Das, R.; Lee, Y. W.; Hou, S.; Moyano, D. F.; Duncan, B.; Liang, X. J.; Rotello, V. M. The interplay of size and surface functionality on the cellular uptake of sub-10 nm gold nanoparticles. *ACS Nano* **2015**, *9*, 9986−9993.
(31) van der Meer, S. B.; Seiler, T.; Buchmann, C.; Partalidou, G.; Boden, S.; Loza, K.; Heggen, M.; Linders, J.; Prymak, O.; Oliveira, C. L. P.; Hartmann, L.; Epple, M. Controlling the surface functionalization of ultrasmall gold nanoparticles by sequence-defined macromolecules. *Chem. Eur. J.* **2021**, *27*, 1451−1464.
(32) Elci, S. G.; Yesilbag Tonga, G.; Yan, B.; Kim, S. T.; Kim, C. S.; Jiang, Y.; Saha, K.; Moyano, D. F.; Marsico, A. L. M.; Rotello, V. M.; Vachet, R. W. Dual-mode mass spectrometric imaging for determination of in vivo stability of nanoparticle monolayers. *ACS Nano* **2017**, *11*, 7424−7430.
(33) Wetzel, O.; Prymak, O.; Loza, K.; Gumbiowski, N.; Heggen, M.; Bayer, P.; Beuck, C.; Weidenthaler, C.; Epple, M. Water-based synthesis of ultrasmall nanoparticles of platinum group metal oxides (1.8 nm). *Inorg. Chem.* **2022**, *61*, 5133−5147.
(34) Wetzel, O.; Hosseini, S.; Loza, K.; Heggen, M.; Prymak, O.; Bayer, P.; Beuck, C.; Schaller, T.; Niemeyer, F.; Weidenthaler, C.; Epple, M. Metal-ligand interface and internal structure of ultrasmall silver nanoparticles (2 nm). *J. Phys. Chem. B* **2021**, *125*, 5645−5659.
(35) Banerjee, S.; Liu, C. H.; Lee, J. D.; Kovyakh, A.; Grasmik, V.; Prymak, O.; Koenigsmann, C.; Liu, H.; Wang, L.; Abeykoon, A. M. M.; Wong, S. S.; Epple, M.; Murray, C. B.; Billinge, S. J. L. Improved models for metallic nanoparticle cores from atomic pair distribution function (PDF) analysis. *J. Phys. Chem. C* **2018**, *122*, 29498−29506.
(36) Abraham, A.; Mihaliuk, E.; Kumar, B.; Legleiter, J.; Gullion, T. Solid-state NMR study of cysteine on gold nanoparticles. *J. Phys. Chem. C* **2010**, *114*, 18109−18114.
(37) Zan, X. F.; Li, Q. Z.; Pan, Y. T.; Morris, D. J.; Zhang, P.; Li, P.; Yu, H. Z.; Zhu, M. Z. Versatile ligand-exchange method for the synthesis of water-soluble monodisperse AuAg nanoclusters for cancer therapy. *ACS Appl. Nano Mater.* **2018**, *1*, 6773−6781.
(38) Salassa, G.; Burgi, T. NMR spectroscopy: A potent tool for studying monolayer-protected metal nanoclusters. *Nanoscale Horiz.* **2018**, *3*, 457−463.
(39) Marbella, L. E.; Millstone, J. E. NMR techniques for noble metal nanoparticles. *Chem. Mater.* **2015**, *27*, 2721−2739.
(40) Rees, G. J.; Orr, S. T.; Barrett, L. O.; Fisher, J. M.; Houghton, J.; Spikes, G. H.; Theobald, B. R. C.; Thompsett, D.; Smith, M. E.; Hanna, J. V. Characterisation of platinum-based fuel cell catalyst materials using 195Pt wideline solid state NMR. *Phys. Chem. Chem. Phys.* **2013**, *15*, 17195−17207.
(41) Klein, K.; Loza, K.; Heggen, M.; Epple, M. An efficient method for covalent surface functionalization of ultrasmall metallic nanoparticles by surface azidation, followed by copper-catalyzed azide-alkyne cycloaddition. *ChemNanoMat* **2021**, *7*, 1330−1339.
(42) Ruks, T.; Beuck, C.; Schaller, T.; Niemeyer, F.; Zähres, M.; Loza, K.; Heggen, M.; Hagemann, U.; Mayer, C.; Bayer, P.; Epple, M. Solution NMR spectroscopy with isotope-labelled cysteine (^{13}C, ^{15}N) reveals the surface structure of L-cysteine-coated ultrasmall gold nanoparticles (1.8 nm). *Langmuir* **2019**, *35*, 767−778.
(43) Yu, R.; Liz-Marzan, L. M.; Garcia de Abajo, F. J. Universal analytical modeling of plasmonic nanoparticles. *Chem. Soc. Rev.* **2017**, *46*, 6710−6724.
(44) Malola, S.; Lehtovaara, L.; Enkovaara, J.; Häkkinen, H. Birth of the localized surface plasmon resonance in monolayer-protected gold nanoclusters. *ACS Nano* **2013**, *7*, 10263−10270.
(45) Wolff, N.; Kollenda, S.; Klein, K.; Loza, K.; Heggen, M.; Brochhagen, L.; Witzke, O.; Krawczyk, A.; Hilger, I.; Epple, M. Silencing of proinflammatory NF-κB and inhibition of herpes simplex virus (HSV) replication by ultrasmall gold nanoparticles (2 nm) conjugated with small-interfering RNA. *Nanoscale Adv.* **2022**, *4*, 4502−4516.
(46) Perez-Potti, A.; Lopez, H.; Pelaz, B.; Abdelmonem, A.; Soliman, M. G.; Schoen, I.; Kelly, P. M.; Dawson, K. A.; Parak, W. J.; Krpetic, Z.; Monopoli, M. P. In depth characterisation of the biomolecular coronas of polymer coated inorganic nanoparticles with differential centrifugal sedimentation. *Sci. Rep.* **2021**, *11*, 6443.
(47) Muraca, F.; Boselli, L.; Castagnola, V.; Dawson, K. A. Ultrasmall gold nanoparticle cellular uptake: influence of transient bionano interactions. *ACS Appl. Bio. Mater.* **2020**, *3*, 3800−3808.
(48) Häkkinen, H. The gold-sulfur interface at the nanoscale. *Nat. Chem.* **2012**, *4*, 443−455.
(49) Smith, A. M.; Marbella, L. E.; Johnston, K. A.; Hartmann, M. J.; Crawford, S. E.; Kozycz, L. M.; Seferos, D. S.; Millstone, J. E. Quantitative analysis of thiolated ligand exchange on gold nanoparticles monitored by ^{1}H NMR spectroscopy. *Anal. Chem.* **2015**, *87*, 2771−2778.
(50) Ruks, T.; Loza, K.; Heggen, M.; Prymak, O.; Sehnem, A. L.; Oliveira, C. L. P.; Bayer, P.; Beuck, C.; Epple, M. Peptide-conjugated ultrasmall gold nanoparticles (2 nm) for selective protein targeting. *ACS Appl. Bio Mater.* **2021**, *4*, 945−965.
(51) Ruks, T.; Loza, K.; Heggen, M.; Ottmann, C.; Bayer, P.; Beuck, C.; Epple, M. Targeting the surface of the protein 14−3-3 by ultrasmall gold nanoparticles (1.5 nm), carrying the specific peptide CRaf. *ChemBioChem* **2021**, *22*, 1456−1463.
(52) Sokolova, V.; Ebel, J. F.; Kollenda, S.; Klein, K.; Kruse, B.; Veltkamp, C.; Lange, C. M.; Westendorf, A. M.; Epple, M. Uptake of functional ultrasmall gold nanoparticles in 3D gut cell models. *Small* **2022**, *18*, 2201167.
(53) Seiler, T.; Lennartz, A.; Klein, K.; Hommel, K.; Figueroa Bietti, A.; Hadrovic, I.; Kollenda, S.; Sager, J.; Beuck, C.; Chlosta, E.; Bayer, P.; Juul-Madsen, K.; Vorup-Jensen, T.; Schrader, T.; Epple, M.; Knauer, S. K.; Hartmann, L. Potentiating tweezer affinity to a protein interface

https://doi.org/10.1021/acs.accounts.3c00459
Acc. Chem. Res. 2023, 56, 3369−3378

with sequence-defined macromolecules on nanoparticles. *Biomacromolecules* **2023**, *24* (8), 3666−3679.
(54) Huang, R.; Luther, D. C.; Zhang, X.; Gupta, A.; Tufts, S. A.; Rotello, V. M. Engineering the interface between inorganic nanoparticles and biological systems through ligand design. *Nanomaterials* **2021**, *11*, 1001.
(55) Kelly, P. M.; Aberg, C.; Polo, E.; O'Connell, A.; Cookman, J.; Fallon, J.; Krpetic, Z.; Dawson, K. A. Mapping protein binding sites on the biomolecular corona of nanoparticles. *Nat. Nanotechnol.* **2015**, *10*, 472−479.
(56) Luther, D. C.; Huang, R.; Jeon, T.; Zhang, X. Z.; Lee, Y. W.; Nagaraj, H.; Rotello, V. M. Delivery of drugs, proteins, and nucleic acids using inorganic nanoparticles. *Adv. Drug Deliv. Rev.* **2020**, *156*, 188−213.
(57) Fischer, N. O.; McIntosh, C. M.; Simard, J. M.; Rotello, V. M. Inhibition of chymotrypsin through surface binding using nanoparticle-based receptors. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 5018−5023.
(58) Hong, R.; Fischer, N. O.; Verma, A.; Goodman, C. M.; Emrick, T.; Rotello, V. M. Control of protein structure and function through surface recognition by tailored nanoparticle scaffolds. *J. Am. Chem. Soc.* **2004**, *126*, 739−743.
(59) De, M.; You, C. C.; Srivastava, S.; Rotello, V. M. Biomimetic interactions of proteins with functionalized nanoparticles: A thermodynamic study. *J. Am. Chem. Soc.* **2007**, *129*, 10747−10753.
(60) Dawson, K. A.; Yan, Y. Current understanding of biological identity at the nanoscale and future prospects. *Nat. Nanotechnol.* **2021**, *16*, 229−242.
(61) Shen, Z.; Sang, Z.; Shi, Y. Nanobodies as a powerful platform for biomedicine. *Trends Mol. Med.* **2022**, *28*, 1006−1007.
(62) Stahl, P.; Kollenda, S.; Sager, J.; Schmidt, L.; Schroer, M. A.; Stauber, R. H.; Epple, M.; Knauer, S. K. Tuning nanobodies' bioactivity: Coupling to ultrasmall gold nanoparticles allows the intracellular interference with survivin. *Small* **2023**, *19*, 2300871.
(63) Huang, Y. Y.; Fuksman, L.; Zheng, J. Luminescence mechanisms of ultrasmall gold nanoparticles. *Dalton Trans.* **2018**, *47*, 6267−6273.
(64) Wey, K.; Epple, M. Ultrasmall gold and silver/gold nanoparticles (2 nm) as autofluorescent labels for poly(D,L-lactide-co-glycolide) nanoparticles (140 nm). *J. Mater. Sci. Mater. Med.* **2020**, *31*, 117.
(65) Klein, K.; Hayduk, M.; Kollenda, S.; Schmiedtchen, M.; Voskuhl, J.; Epple, M. Covalent attachment of aggregation-induced emission molecules to the surface of ultrasmall gold nanoparticles to enhance cell penetration. *Molecules* **2022**, *27*, 1788.
(66) Patel, S.; Kim, J.; Herrera, M.; Mukherjee, A.; Kabanov, A. V.; Sahay, G. Brief update on endocytosis of nanomedicines. *Adv. Drug Deliv. Rev.* **2019**, *144*, 90−111.
(67) Zhang, X.; Shastry, S.; Bradforth, S. E.; Nadeau, J. L. Nuclear uptake of ultrasmall gold-doxorubicin conjugates imaged by fluorescence lifetime imaging microscopy (FLIM) and electron microscopy. *Nanoscale* **2015**, *7*, 240−251.
(68) Sokolova, V.; Mekky, G.; van der Meer, S. B.; Seeds, M. C.; Atala, A. J.; Epple, M. Transport of ultrasmall gold nanoparticles (2 nm) across the blood-brain barrier in a six-cell brain spheroid model. *Sci. Rep.* **2020**, *10*, 18033.
(69) Bialas, N.; Sokolova, V.; van der Meer, S. B.; Knuschke, T.; Ruks, T.; Klein, K.; Westendorf, A. M.; Epple, M. Bacteria (E. coli) take up ultrasmall gold nanoparticles (2 nm) as shown by different optical microscopic techniques (CLSM, SIM, STORM). *Nano Select* **2022**, *3*, 1407−1420.
(70) van der Meer, S. B.; Loza, K.; Wey, K.; Heggen, M.; Beuck, C.; Bayer, P.; Epple, M. Click chemistry on the surface of ultrasmall gold nanoparticles (2 nm) for covalent ligand attachment followed by NMR spectroscopy. *Langmuir* **2019**, *35*, 7191−7204.
(71) Zhang, W. L.; Meckes, B.; Mirkin, C. A. Spherical nucleic acids with tailored and active protein coronae. *ACS Centr. Sci.* **2019**, *5*, 1983−1990.

https://doi.org/10.1021/acs.accounts.3c00459
Acc. Chem. Res. 2023, 56, 3369−3378