Nanomedicine

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Engineered Nanoparticles for Drug Delivery in Cancer Therapy

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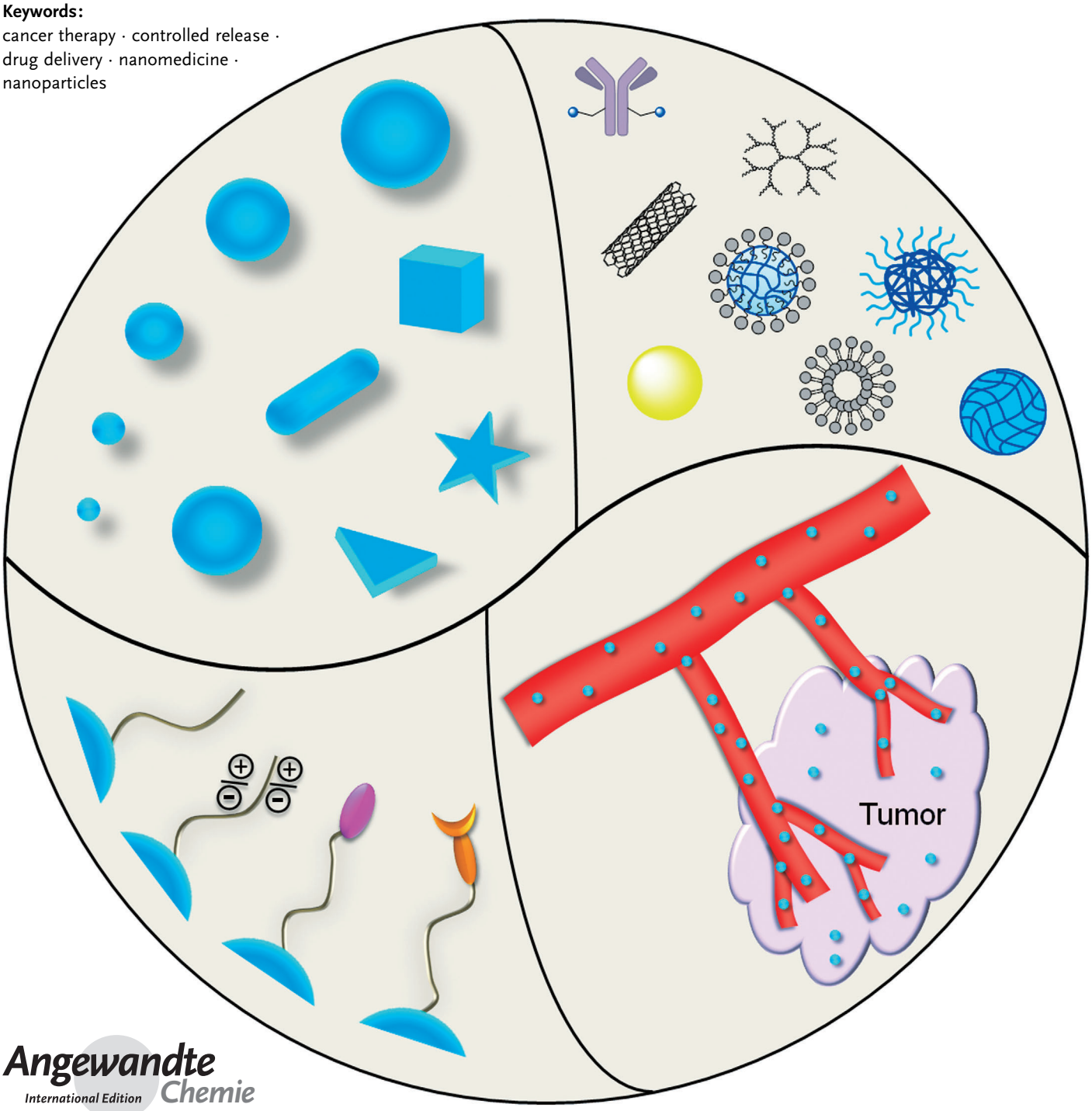

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In medicine, nanotechnology has sparked a rapidly growing interest as it promises to solve a number of issues associated with conventional therapeutic agents, including their poor water solubility (at least, for most anticancer drugs), lack of targeting capability, nonspecific distribution, systemic toxicity, and low therapeutic index. Over the past several decades, remarkable progress has been made in the development and application of engineered nanoparticles to treat cancer more effectively. For example, therapeutic agents have been integrated with nanoparticles engineered with optimal sizes, shapes, and surface properties to increase their solubility, prolong their circulation half-life, improve their biodistribution, and reduce their immunogenicity. Nanoparticles and their payloads have also been favorably delivered into tumors by taking advantage of the pathophysiological conditions, such as the enhanced permeability and retention effect, and the spatial variations in the pH value. Additionally, targeting ligands (e.g., small organic molecules, peptides, antibodies, and nucleic acids) have been added to the surface of nanoparticles to specifically target cancerous cells through selective binding to the receptors overexpressed on their surface. Furthermore, it has been demonstrated that multiple types of therapeutic drugs and/or diagnostic agents (e.g., contrast agents) could be delivered through the same carrier to enable combination therapy with a potential to overcome multidrug resistance, and real-time readout on the treatment efficacy. It is anticipated that precisely engineered nanoparticles will emerge as the next-generation platform for cancer therapy and many other biomedical applications.

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1. Introduction

Cancer is one of the leading causes of death, accounting for 8.2 million deaths worldwide in 2012.[1] Over the past several decades, remarkable breakthroughs have been made in advancing our understanding of how cancer originates and develops, which has in turn led to better methods for both diagnosis and treatment.[2] Although the overall mortality of cancer is showing a declining trend for the first time in five decades, it still remains at a high rate of 20.2%.[3] In the United States alone, for example, cancer resulted in an estimated 580350 deaths out of 1660290 total diagnoses in 2013.[4] A major reason for this high mortality rate lies in our inability to deliver therapeutic agents only to the tumor sites without inducing severe adverse effects on healthy tissues and organs.[5] In addition to surgical intervention, current cancer treatments heavily rely on radiation and chemotherapeutic agents, which also kill "normal" cells and cause toxicity to the patient. Therefore, it would be desirable to develop highly efficient therapeutics, the so-called "magic bullets", that can overcome biological barriers, distinguish between malignant and benign cells, selectively target the cancerous tissues, and "intelligently" respond to the heterogeneous and complex microenvironment inside a tumor for on-demand release of therapeutic agents in the optimal dosage range.[6]

Nanomedicine, the application of nanotechnology to medicine, is anticipated to help us move toward the aforementioned goals. After several decades of technological developments, drug-delivery systems based on engineered nanoparticles have started to show great promise.[7] As shown in Figure 1, the nanoparticles used for drug delivery can be readily fabricated from either soft (organic and polymeric) or hard (inorganic) materials, with their sizes being controlled typically in the range of 1–100 nm and compositions/structures being engineered to load anticancer drugs in a variety of configurations.[8] The physicochemical properties of the nanoparticles can also be finely tuned by tailoring their chemical compositions, sizes, shapes, structures, morphologies, and surface properties.[2,7a,e,9] A number of such delivery systems have been approved for cancer therapy in the clinics, with many more currently under clinical trials or preclinical evaluations (see Table 1 for a list). Nanoparticle-based therapeutics are poised to significantly improve the treatment outcomes for oncological diseases, promising to reshape the landscape of the pharmaceutical industry.[9]

Compared with traditional chemotherapeutics, the delivery of anticancer drugs through a nanoparticle-based plat-

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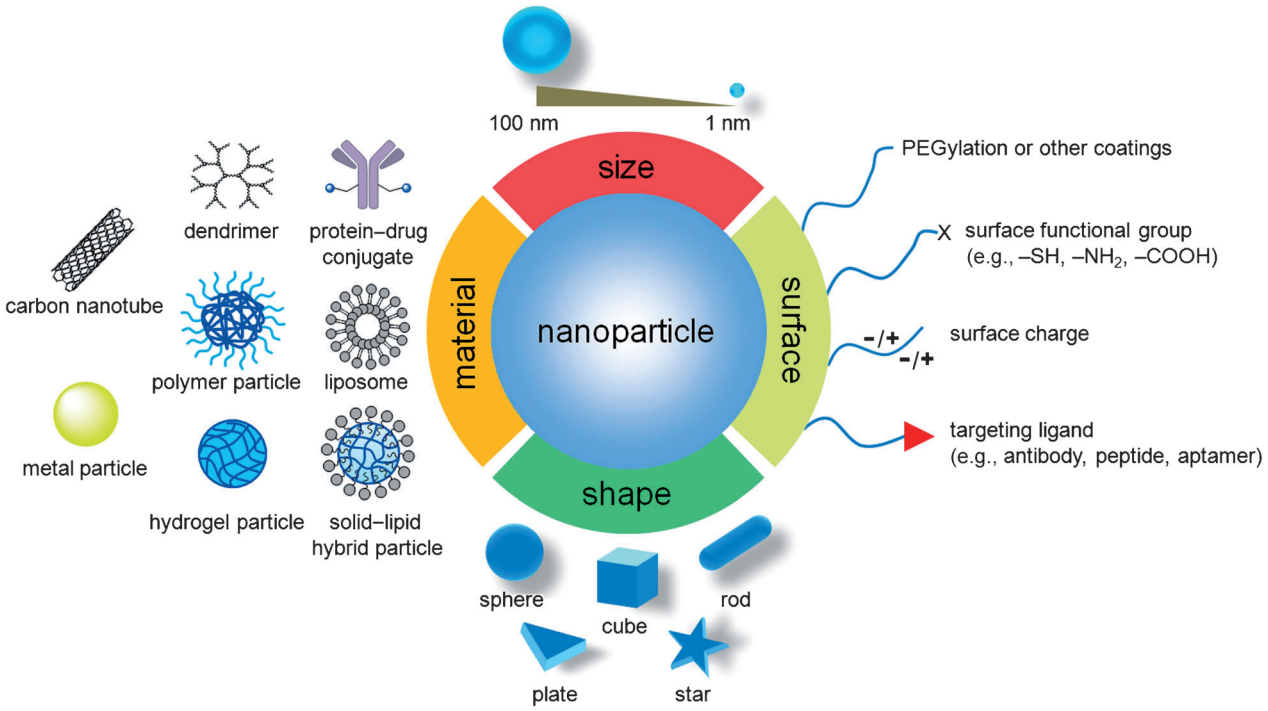

Figure 1. A summary of nanoparticles that have been explored as carriers for drug delivery in cancer therapy, together with illustrations of biophysicochemical properties.

form offers many attractive features, including: 1) improved delivery of drugs that are poorly soluble in water and delivery of a therapeutic agent into cancerous cells at a high dose; 2) better protection of a drug from harsh environments (e.g., the highly acidic environment in the stomach or the lysosomes of a cell, and the high levels of proteases or other enzymes in the blood stream) before they can reach the targets, leading to an extended plasma half-life of the drug in the systemic

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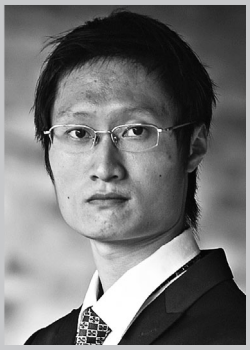

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circulation; 3) targeted delivery of drugs in a cell- or tissue-specific manner so the treatment efficacy can be maximized while systemic side effects are alleviated; 4) controlled release of drugs over a manageable period of time at precise doses and even realization of on-demand release using a more sophisticated, stimuli-responsive system; and 5) co-delivery of multiple types of drugs and/or diagnostic agents (e.g., contrast agents) for combination therapy (which has the potential to overcome multidrug resistance) and real-time readout on the treatment efficacy.[7h,37]

In this Review, we first discuss the critical need for nanoparticle carriers for the delivery of various types (hydrophilic, hydrophobic, and highly charged) of cancer therapeutic agents. We then present a number of strategies based on diffusion, erosion, and stimuli-responsive triggering for regulating the release of drugs from nanoparticle carriers. Following that, we highlight some general challenges in targeted delivery of nanoparticle carriers under in vitro and in vivo conditions, including endocytosis, intracellular transport, biodistribution, biocompatibility, biodegradability, and systemic clearance. We then elaborate on the design, synthesis/fabrication, and functionalization of nanoparticle carriers based on the requirements imposed by various applications. Finally, we illustrate how the concept of nanomedicine has been materialized by focusing on some selected examples of nanoparticle carriers, including protein conjugates, liposomes, dendrimers, as well as those composed of organic polymers, hydrogels, phase-change materials, and inorganic materials.

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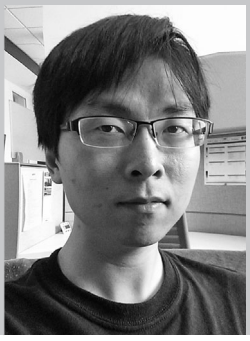

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2. Working with Different Types of Anticancer Drugs

Broadly speaking, cancer therapeutic agents can be classified into two major groups, hydrophobic and hydrophilic, depending on their aqueous solubility. Alternatively, they can be categorized as highly charged or neutral drugs based on their electrostatic properties (Table 2). When choosing or designing nanoparticles to be used as the carrier for a specific type of drug, it is of critical importance to know the properties and behaviors of the drug in order to achieve an optimal encapsulation efficiency and the desired release profile. In this section, we use a set of examples to highlight the challenges faced by the application of various anticancer drugs and then discuss strategies that can potentially overcome these obstacles by using engineered nanoparticles as the carriers.

2.1. Hydrophobic Drugs

The majority of anticancer drugs currently used in the clinic are hydrophobic, including, for example, paclitaxel, which is widely used for treating ovarian, breast, and non-small-cell lung cancers. How to effectively deliver a hydrophobic drug to its target has always been a challenge and a subject of active research. The reason lies in the fact that hydrophobic drug molecules may not be soluble enough to cross the aqueous environment (e.g., the body and tissue fluids in vivo) surrounding a cell and then penetrate the cell membrane to eventually reach intracellular targets. Additionally, their strong tendency to aggregate upon intravenous administration can lead to complications such as embolisms and local toxicity.[51]

One efficient approach to overcome the poor water solubility of a hydrophobic drug is to encapsulate it in a nanoparticle-based carrier. The primary requirement for the carrier is a good loading capacity for the hydrophobic drug, which can be easily met through the use of a hydrophobic or amphiphilic material.[37a] A variety of carriers have thus been developed for the delivery of hydrophobic drugs, including those based on polymer micelles and polymer nanoparticles.[2,52] For example, Allen and co-workers demonstrated that the concentration of ML220 (a highly hydrophobic arylimidazole drug) in an aqueous medium could be increased by a factor of more than 50000 when it was encapsulated in a liposome-based carrier.[53] Park and co-workers found that nanosized micelles based on amphiphilic block copolymers could serve as a carrier for the delivery of drugs poorly soluble in water (such as paclitaxel), significantly increasing the drug concentration in an aqueous medium by a factor of more than 1000.[54] Similar improvement has also been observed with solid nanoparticles made of biocompatible, biodegradable polymers.[55]

2.2. Hydrophilic Drugs

Hydrophilic drugs, including biomacromolecules (e.g., proteins, peptides, and nucleic acids)[56] and many small

Table 1: Nanoparticle-based therapeutics in clinical use and under clinical investigation.

Trade name	Formulation	Drug	Company	Application	Phase of development
Abraxane	albumin-bound nanoparticle	paclitaxel	Abraxis Bioscience, Inc.	metastatic breast cancer[10]	approved
Caelyx	PEGylated liposome	doxorubicin	Schering-Plough	metastatic breast and ovarian cancer, Kaposi sarcoma[11]	approved
DaunoXome	liposome	daunorubicin	Galen Ltd	Kaposi sarcoma[12]	approved
DepoCyt	lipodome	cytarabine	Pacira Pharmaceuticals, Inc.	lymphoma[13]	approved
Doxil	liposome	doxorubicin	Sequus Pharmaceuticals, Inc.	Kaposi sarcoma[14]	approved
Genexol-PM	polymeric micellar nanoparticle	paclitaxel	Samyang Biopharmaceuticals	breast cancer[15]	approved
Marqibo	liposome	vincristine sulfate	Talon Therapeutics, Inc.	lymphoblastic leukemia[16]	approved
Myocet	liposome	doxorubicin	Zeneus Pharma Ltd	metastatic breast cancer[17]	approved
Oncaspar	PEGylated asparaginase	asparaginase	Enzon Pharmaceuticals, Inc.	acute lymphoblastic leukemia[18]	approved
Zinostatin stimalamer	poly(styrene-co-maleic acid)-conjugated neocarzinostatin	neocarzinostatin	Astellas Pharma, Inc.	hepatocellular carcinoma[19]	approved
NK105	micellar nanoparticle	paclitaxel	Nippon Kayaku Co., Ltd	breast cancer[20]	phase III
BIND-014	polymer matrix	docetaxel	BIND Therapeutics, Inc.	prostate cancer[21]	phase II
Genexol-PM	methoxy PEG-PLA	paclitaxel	Samyang Biopharmaceuticals	ovarian and lung cancer[22]	phase II
CRLX101	cylodextrin-PEG micelle	camptothecin	Cerulean Pharma, Inc.	ovarian/tubal/peritoneal cancer, rectal cancer[23]	phase I/II
CYT-6091	gold nanoparticle	tumor necrosis factor α	Cytimmune Sciences, Inc.	pancreatic cancer, melanoma, soft-tissue sarcoma, ovarian, and breast cancer[24]	phase I/II
L-Annamycin	liposome	annamycin	Callisto Pharmaceuticals, Inc.	acute lymphocytic leukemia, acute myelogenous leukemia[25]	phase I/II
NL CPT-11	liposome	irinotecan	University of California, San Francisco	solid tumor[26]	phase I/II
Rexin-G	pathotropic nanoparticle	dominant negative cyclin G1 construct	Epeius Biotechnologies	breast cancer, osteosarcoma[27]	phase I/II
Anti-EGFR immunoliposome	liposome	doxorubicin	University Hospital, Switzerland	solid tumor[28]	phase I
AuroLase	gold nanoparticle		Nanospectra Biosciences, Inc.	lung cancer, head and neck cancer[29]	phase I
BikDD nanoparticle	liposome	proapoptotic Bik gene (*BikDD*)	National Cancer Institute	pancreatic cancer[30]	phase I
CALAA-01	cyclodextrin-containing polymer	siRNA	Calando Pharmaceuticals, Inc.	solid tumor[31]	phase I
CRLX301	cyclodextrin-based polymer	docetaxel	Cerulean Pharma, Inc.	solid tumor[32]	phase I
DEP™-Docetaxel	dendrimer	docetaxel	Starpharma Holdings, Ltd	breast, prostate, lung, and ovarian cancer[33]	phase I
Docetaxel-PNP	polymeric nanoparticle	docetaxel	Samyang Biopharmaceuticals	advanced solid malignancies[34]	phase I
TKM-080301	lipid nanoparticle	siRNA	National Institutes of Health Clinical Center	liver cancer[35]	phase I
C-dots	PEG-coated SiO_2		C-dots Development	melanoma[36]	IND approved

molecules,[57] also play an important role in treating various types of cancers. For example, trastuzumab, a monoclonal antibody that interferes with the human epidermal growth factor receptor 2 (HER2), is now routinely used to treat early-stage and metastatic breast cancer for many years; and gemcitabine, a nucleoside analogue, has been used to treat bladder, pancreas, ovarian, breast, and non-small-cell lung cancers. Nevertheless, successful utilization of hydrophilic drugs has been hindered by a number of obstacles, such as poor uptake by cells because of their inability to cross the

Table 2: Anticancer drugs currently in clinical use.[a]

Drug	Clinical use
Hydrophobic	
adriamycin	ALL, AML, Wilms' tumor, neuroblastoma, soft-tissue and bone sarcomas, breast, ovary, urinary bladder, thyroid, gastric, Hodgkin's disease[38]
cisplatin	metastatic testicular tumors, metastatic breast cancer, glioblastoma, lung, lymphoma, anal canal[38]
docetaxel	breast, NSCLC, HRPC, gastric adenocarcinoma, SCCHN[38]
etoposide	SCLC, GCT, lymphoma, Ewing's sarcoma, ovary[39]
methotrexate	gestational choriocarcinoma, ALL, breast, epidermoid cancer of the head and neck, advanced mycosis[38]
paclitaxel	advanced carcinoma of the ovary, breast, lung, stomach, head and neck, GCT, urinary bladder, urothelial[40]
Hydrophilic	
bevacizumab	metastatic CRC, NSCLC, Metastatic breast cancer, glioblastoma, ovary, renal cell carcinoma[41]
cetuximab	metastatic colorectal and non-small-cell lung carcinoma[42]
cyclophosphamide	malignant lymphomas, Hodgkin's disease, lymphocytic lymphoma, CLL, CML, ALL, AML, osteosarcoma, GCT[43]
gemcitabine	breast, NSCLC, pancreas, ovary, gallbladder, lymphoma, urinary bladder[44]
ibritumomab	B-cell non-Hodgkin's lymphoma[45]
L-asparaginase	ALL[46]
panitumumab	metastatic colorectal carcinoma[47]
rituximab	CD20-positive B-cell non-Hodgkin's lymphoma[48]
tositumomab	CD20-positive B-cell non-Hodgkin's lymphoma[49]
transtuzumab	metastatic breast cancer[50]
Highly charged	
DNA	no clinical use
siRNA/miRNA	no clinical use

[a] ALL (acute lymphoblastic leukemia), AML (acute myeloid leukemia), CLL (chronic lymphocytic leukemia), CML (chronic myelogenous leukemia), CRC (colorectal cancer), GCT (granulosa cell tumor), HRPC (hormone refractory prostate cancer), NHL (hon-Hodgkin lymphoma), NSCLC (non-small-cell lung cancer), SCCHN (squamous cell carcinoma of the head and neck), SCLC (small-cell lung cancer).

lipid-rich, hydrophobic cell membranes, low bioavailability arising from their poor stability against proteolytic and hydrolytic degradation, and short half-life in the circulatory system.[56,58]

To circumvent these hurdles, nanoparticles have been actively explored as carriers to encapsulate and deliver hydrophilic drugs. Similar to hydrophobic drugs, loading efficiency is also one of the major issues to consider because the overall dosage has to be increased when nanoparticles with low drug contents are administered.[59] Considering the hydrophobic nature of most materials used for fabricating the carriers, loading of a hydrophilic drug into such a delivery system is not always straightforward, owing to the poor miscibility between these two phases. To this end, a number of approaches have been developed to improve the loading efficiency of a hydrophilic drug. For instance, Hall and co-workers replaced 5-fluorouracil (5-FU, a hydrophilic pyrimidine analogue for cancer treatment) with 1-alkylcarbonyloxymethyl (an amphiphilic prodrug of 5-FU) to significantly increase the drug loading efficiency from 3.68 % to 47.23 %.[60] Fattal and co-workers discovered that adjusting the pH value of the external aqueous phase to the isoelectric point of a protein drug could increase the drug loading.[61] Xu and co-workers demonstrated that the electrostatic and hydrophobic interactions between lipidoids and a protein drug could be enhanced to facilitate the formation of protein–lipidoid complexes and thus intracellular delivery.[59c] Furthermore, McGinity and co-workers found that a less hydrophilic organic solvent in the oil phase could prevent the encapsulated hydrophilic drugs from releasing into the outer water phase.[62] For some of these modifications, the poor dispersion of a hydrophilic drug in nanoparticles, which often results in rapid release of the drug, can also be largely addressed.[63]

2.3. Highly Charged Drugs

Gene therapeutics based on DNA, siRNA, and microRNA represent a special class of hydrophilic drugs with high densities of charges. They were developed over the past few decades and are expected to serve as a powerful molecular therapy for the treatment of various diseases.[64] These drugs have all the characteristics of a hydrophilic drug, such as poor cellular uptake and fast degradation in the physiological milieu. Additionally, systemic administration of these therapeutics is impeded by barriers such as rapid clearance by the mononuclear phagocyte system (MPS) and kidney filtration. It should be pointed out that there has been no clinical success in gene therapy to date. In a sense, efficient delivery of these highly charged agents through proper carriers remains a major obstacle for achieving their therapeutic benefits.[65]

Currently, loading and delivery of gene drugs rely on the electrostatic interactions between the highly charged nucleic acids and nanoparticle carriers. As the nucleic acids are usually negatively charged under physiological conditions, it is reasonable to rely on the use of positively charged carriers, such as liposomes and polymer nanoparticles consisting of cationic building blocks, to improve the loading efficiency.[66] To this end, Wang and co-workers developed a positively charged micelle system composed of amphiphilic and cationic

triblock copolymers, which could effectively immobilize siRNA for intracellular delivery and release.[67] Unfortunately, some of these nanoparticles can cause severe problems associated with toxicity and immune or inflammatory responses,[68] which need to be taken into consideration when designing the next-generation carriers. Direct conjugation of a gene drug to the surface of nanoparticle carrier has been demonstrated as another effective approach.[69] By immobilizing nucleic acids on the surface, the complexity of a loading process may be reduced relative to the encapsulation method. In one example, Mirkin and co-workers initially coated Au nanoparticles with a layer of short DNA chains containing specially designed sequences. Using these DNA-coated Au nanoparticles as the carrier, they were able to further immobilize a target DNA or siRNA of interest for efficient delivery of the therapeutics.[69]

3. Methods for Controlled Release

Placing drug molecules inside or on the surface of a nanoparticle carrier allows for controlled release, which offers multiple benefits compared to the conventional dosing forms based on free drugs. For example, it can improve the temporal and spatial presentations of a drug in the body, protect the drug from physiological degradation or elimination, reduce toxicity to the healthy tissues and organs, and increase patient compliance and convenience. Great strides have been made in the design and development of nanoparticle-based systems for controlled release,[70] and their operation modes can be broadly classified into two major categories: sustained and stimuli-responsive (i.e., smart) release. Here we only discuss the general principles of controlled release that can be readily applied to nanoparticle-based carriers.

3.1. Sustained Release

Sustained release aims to deliver a drug at a predetermined rate over an extended period of time. This mode of release is critical for drugs that are rapidly metabolized and eliminated from the body after administration. The sustained release can maintain the concentration of the drug at a constant level in the plasma or target tissue by matching the rate of drug release with the rate of drug elimination. In the case of cancer therapy, maintaining the concentration of a drug within the therapeutic window is beneficial to the patient.

Once dissolved in the aqueous body fluid, most drugs can be freely transported with the fluid to quickly reach the target receptors. One approach to achieve sustained release is to prevent the drug molecules from entering the aqueous environment for a controllable period of time. As shown in Figure 2, the prevention can be realized by controlling the diffusion of drug molecules through an insoluble polymer shell or matrix, or by simply controlling the degradation rate of a carrier.[71] The release mechanisms and the corresponding mathematical models have been extensively studied and reviewed.[72]

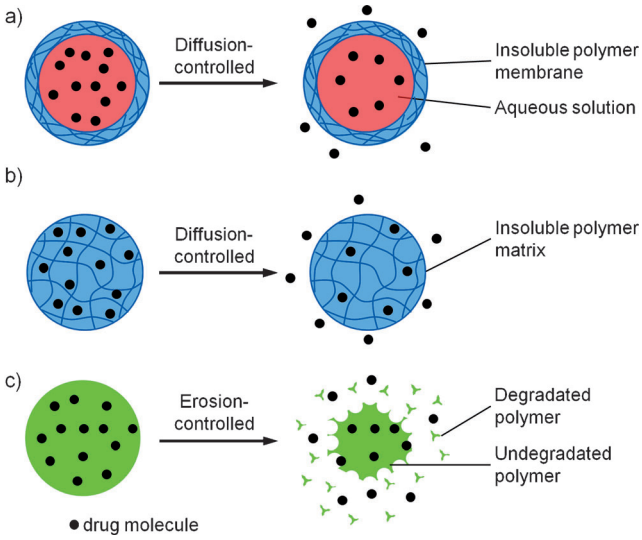

Figure 2. Illustration of three major mechanisms for achieving sustained drug release: a,b) diffusion through an insoluble polymer shell or matrix, and c) erosion of a polymer matrix. Reproduced with permission from Ref. [71b], copyright 2010 Springer.

3.1.1. Diffusion-Controlled Release

In diffusion-controlled release, the drug molecules preloaded inside a nanoparticle are restricted from entering the aqueous environment by a barrier provided by an insoluble material (typically, an organic polymer). In general, diffusion-controlled release can be realized using a reservoir- or matrix-based system.[73] By its name, a reservoir-based system consists of a core reservoir that contains the drug and a membrane surrounding the reservoir (Figure 2a). The concept of this release system can be easily extended to the nanosized carriers by switching to colloidal hollow particles.[74] Typically, the drug molecules initially loaded in the reservoir can only diffuse out through the membrane. The drug release rate is determined by the physicochemical properties of the membrane and the loaded drug, as well as the thickness of the membrane. In spite of their simplicity in release mechanism and their capability to achieve a steady-state release, polymer-membrane-based systems have a critical drawback because of the undesired dose from pinholes and cracks that may form in the membrane.[75] Recently, hollow nanoparticles made of inorganic materials have been developed for drug delivery in cancer therapy.[76] For example, Fe_3O_4 hollow nanoparticles with pores of around 3 nm in size could release cisplatin through the pores by a diffusion-controlled, slow process. The porous shell was mechanically and physiologically stable.[77]

In matrix-based systems (Figure 2b), which have been most extensively explored, the drug molecules are uniformly dispersed in the carrier made of a water-insoluble polymer, such as polyurethane (PUA) and poly(methyl methacrylate) (PMMA). These systems typically display a noticeable release at the initial point (i.e., the so-called burst release) owing to the desorption of drug molecules adsorbed on the surface of the nanoparticles.[78] In the following steps, the release will be retarded because it takes time for the drug molecules inside

the polymer matrix to diffuse to the surface. This retardation effect is more prominent for spherical carriers, as the number of available drug molecules decreases with the distance from the surface.[71b,75] In one study, it was shown that PUA nanoparticles only released 40% of the preloaded doxorubicin during six days of incubation.[79] Similarly, only 35% of the encapsulated docetaxel (Dtxl) was released from the PCL-Tween 80 nanoparticles over a period of 28 days.[80]

3.1.2. Erosion-Controlled Release

Nanoparticle carriers made of erodible or degradable polymers (Figure 2c) have attracted much attention in recent years because they do not require retrieval or further manipulation after the drug is fully released.[80] The pattern of drug release can be controlled by tailoring the erosion kinetics of nanoparticles through careful selection of polymers and encapsulation techniques.[81]

A number of biodegradable polymers, both synthetic and natural, have been used for formulating erodible polymer nanoparticles. Synthetic polymers have the advantage of sustained release of preloaded drugs over periods of days to several weeks. Representative examples include polyesters such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and poly(lactic-*co*-glycolic acid) (PLGA). These polymers are degraded through hydrolytic cleavage of the ester bond between lactic and glycolic acid, and thus can be easily metabolized in the body and eliminated as carbon dioxide and water.[82] During the hydrolytic process, the accessibility of water molecules to polymer matrices (i.e., the hydrophilicity of polymers) determines the erosion rate. Furthermore, the hydrolysis is dependent on the local concentrations of proton donors and acceptors. As the degraded monomers of certain polymers (e.g., PLA, PGA, and PLGA) provide acidic protons, their degradation rate may be self-expedited upon accumulation of these acidic products.[83] The addition of external acidic or basic excipients can also regulate the rate of polymer erosion.

Polymer erosion can proceed through a surface or bulk mechanism.[84] Surface erosion takes place when the rate of erosion is faster than the rate of water permeation into the bulk of the polymer. This is considered to be a preferred mechanism of erosion for drug delivery because a steady-state release of the drug can be reproducibly achieved using a very thin polymer or by keeping the surface area of the carrier constant. One good example of surface-eroding polymers is polyanhydride,[85] whose exceptional hydrophobicity retards water permeation while its highly labile groups lead to rapid hydrolysis when encountering water molecules.

Bulk erosion occurs if water molecules can imbibe into the polymer more rapidly than erosion takes place.[71] In this case, chain scission occurs throughout the matrix, leading to a very complex degradation/erosion process for the polymer. Polyesters, the most commonly used biodegradable polymers for controlled release, work by bulk erosion. The release of drug from a bulk-eroding polymer typically undergoes three stages.[86] In the first stage, drug is released from the surface or from pores that are connected to the surface. During the second stage, the remaining drug is released at a slow to intermediate rate when the polymer gradually degrades. In the last stage, the trapped drug is rapidly released upon complete destruction of the polymer matrix.

In practice, the release of drug molecules from nanoparticles made of an erodible polymer is much more complex than those based on diffusion because various release mechanisms can be simultaneously involved. For nanoparticle formulated with large specific surface areas, bulk and surface erosions have been shown to occur concurrently. A further discussion on this system in cancer therapy can be found in Section 6.3.

3.2. Stimuli-Responsive Release

There are many clinical situations that require treatments beyond sustained, continuous release of drugs.[87] Studies in chronopharmacology indicate that the onsets of certain diseases, such as tumorigenesis and progression of cancer, exhibit strong circadian dependence.[88] Treatment of such diseases requires smart control over the drug release patterns and profiles in response to in vivo physiological conditions or external stimuli.[89] With respect to the biological system, the stimuli used to trigger the release of a drug can be broadly classified as either internal (e.g., variation in pH value or concentrations of ions, small molecules, and enzymes)[70e,90] or external (e.g., light, ultrasound, electric field, magnetic field, and heating, which are also commonly referred to as physical stimuli).[70e,90d,91] In principle, the fast response of a drug-delivery system to a stimulus can be employed for real-time manipulation of drug dosage and further achievement of on-demand drug delivery. A wide spectrum of stimuli-responsive materials and their detailed mechanisms in controlling release have been extensively reviewed elsewhere.[70d,92] In the following sections, we only focus on several representative stimuli-responsive release systems used in cancer therapy.

3.2.1. pH-Sensitive Release

The variation in pH value associated with a pathological situation such as cancer or inflammation has been extensively used to trigger the release of a drug into a specific organ (e.g., gastrointestinal tract or vagina) or intracellular compartment (e.g., endosome or lysosome).[70e] Many anticancer drug-delivery systems have exploited the difference in pH values existing between healthy tissues (ca. 7.4) and the extracellular environment of solid tumors (6.5–6.8). One approach is to use polymers with functional groups that can alter the density of charges in response to pH variation as the nanoparticle carriers. Notable examples include poly(acryl amide) (PAAm), poly(acrylic acid) (PAA), poly(methacrylic acid) (PMAA), poly(methyl acrylate) (PMA), poly(diethylaminoethyl methacrylate) (PDEAEMA), and poly(dimethylaminoethyl methacrylate) (PDMAEMA). With the use of these polymers, the structure and hydrophobicity of the nanoparticle carriers can change as a result of protonation or deprotonation.[93]

Figure 3 schematically illustrates how the polymer chains either extend or collapse in response to the variation in

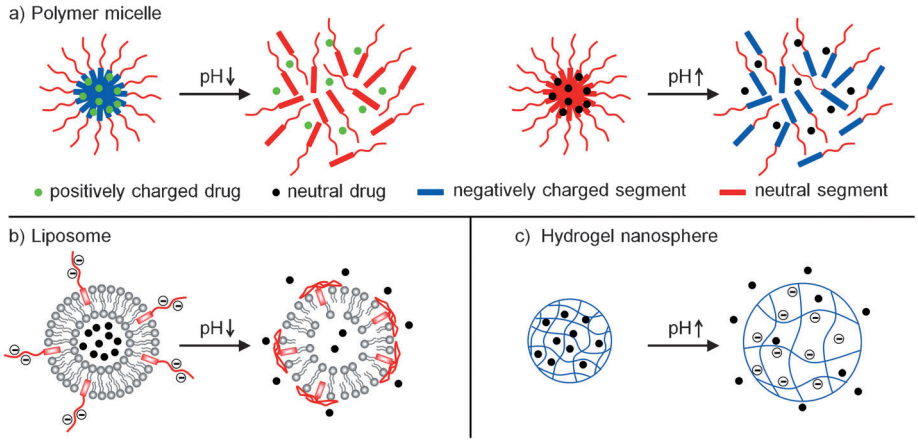

Figure 3. Schematic illustration of drug release from a polymer micelle containing polycarboxylates, a liposome, and a hydrogel nanosphere initiated by a variation in the pH value. a) Protonation (left) or deprotonation (right) results in destruction of the polymer micelle. b) Protonation induces collapse of the polyanion chains, making the liposomal shell leaky and thus promoting efflux of the drug from the liposome. c) Deprotonation leads to swelling of the hydrogel matrix, triggering drug release from the nanosphere. Reproduced with permission from Ref. [98a], copyright 2012 Elsevier.

pH value and thereby cause changes to the electrostatic interactions, allowing for pH-dependent release of drug molecules.[93d,e] For example, micelles derived from block co-polymers can release the preloaded drug when the pH value is varied (Figure 3a). One way to trigger the release is to reduce electrostatic interactions between a positively charged drug (such as doxorubicin) and an oppositely charged block copolymer by protonating the carboxylate groups of the block copolymer.[94] Polymer chains sensitive to the pH value can also be incorporated into liposome to make the permeability of the phospholipid bilayers dependent on pH value (Figure 3b).[93e] For example, the protonation of poly-2-vinylpyridine (P2VP) blocks at pH 4.9 induced rupture of the membrane of PEG-*b*-P2VP polymersomes, triggering the release of the encapsulated drug.[95] Hydrogel nanoparticles can be employed for pH-sensitive release as well, where changes to the degree of protonation/deprotonation lead to swelling and/or shrinking, triggering the release of drug molecules (Figure 3c).[96] For example, Bae and co-workers demonstrated the synthesis of a self-assembled, pH-responsive hydrogel. When loaded with doxorubicin, this system showed an enhancement in toxicity at pH 6.8, which is similar to the pH level inside the tumor tissue.[97] Another approach is to incorporate cleavable bonds into the nanoparticle carrier. The cleavable bonds can be broken to directly release the drug molecules conjugated to or encapsulated in the carrier.[69e,98] Figure 4 shows a partial list of cleavable bonds that can be incorporated into polymer carriers. In the case of polymer–drug conjugates, pH-sensitive linkages such as hydrazone, hydrazide, and acetal have been used to directly attach drug molecules to polymers. To this end, Ríhová and co-workers synthesized *N*-(2-*h*ydroxypropyl) methacrylamide (HPMA), a polymer conjugated with hydrazone groups for the attachment of doxorubicin. The drug was released from the conjugates at pH 5.[99] Similarly, doxorubicin conjugated to a hydrazone-linked dendrimer was released in a pH-sensitive manner. The release was rapidly completed at pH 5, reaching 100% release within 48 h.[100] Kratz and co-workers developed an acid-cleavable doxorubicin prodrug derived from dendritic polyglycerol. Use of the hydrazone linker led to a dramatic change in drug release between pH 5 and 6.[101] A polymer containing an acid-degradable backbone was obtained through terpolymerization of PEG, divinyl ether, and serinol. The pendant amino groups can be used for the conjugation of doxorubicin to the polymer backbone.[102] In another demonstration, acid-degradable linkers such as the pH-labile hydrazone bond was exploited to trigger the release of a drug. Such a linker allows the attachment of doxorubicin to the hydrophobic block of an amphiphilic polymer.[103]

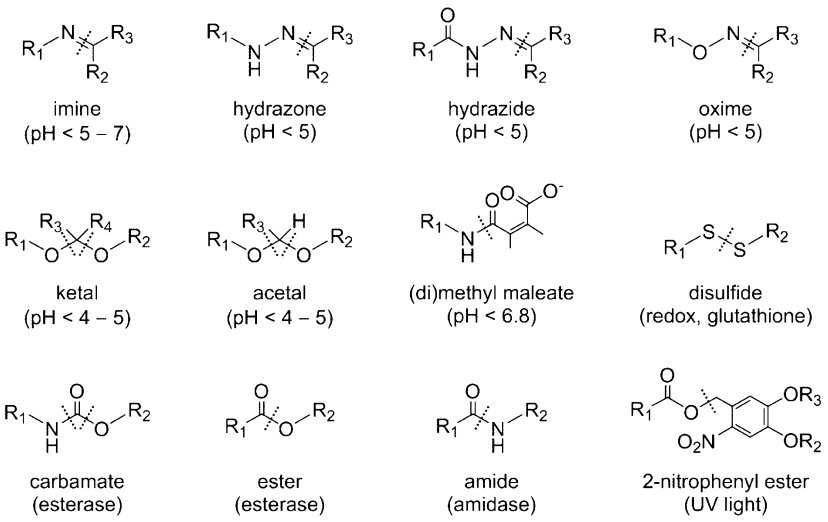

Figure 4. Cleavable linkers that have been used for stimuli-responsive drug release. The dotted line in each molecule indicates the bond that will be broken upon activation by the corresponding stimulus (indicated in parentheses). Reproduced with permission from Ref. [70d], copyright 2012 Elsevier.

3.2.2. *Enzyme-Sensitive Release*

Enzymes perform a vast array of important functions inside our body. For example, they can covalently link polymer chains together to generate self-assembled struc-

tures. They can also break certain bonds, causing disassembly or destruction of the structures.[104] The latter enzymatic reactions can be utilized to trigger the release of a drug. More significantly, the altered expression of a specific enzyme associated with a pathological condition can be employed to achieve enzyme-mediated drug release at the desired biological target only.[69e]

An enzyme-sensitive release system includes either a structure scaffold that is susceptible to the degradation by a specific enzyme or a linker between the drug and the carrier as the product of an enzymatic reaction.[69d] Hydrolases are the most widely used enzymes for such an application, which can break covalent bonds or modify certain chemical groups by altering the balance between electrostatic, hydrophobic, and van der Waals forces, π–π interactions, or hydrogen bonding.[104b,105] For example, proteases can induce the release of a drug linked to a carrier through a peptide bond; glycosidases can trigger the release from a polysaccharide-based carrier; lipases can facilitate the drug release by hydrolyzing the phospholipid building blocks in a liposome; and hydrolases can be used to maneuver the assembly and disassembly of inorganic nanoparticles, as well as the degradation of a gatekeeping material that blocks the pores of a carrier.[104b,106] In addition, kinases and phosphatases have been used to reversibly break/form covalent bonds, achieving the release of a drug in an "on–off" manner.[107]

Enzyme-responsive drug-delivery systems have been designed and fabricated in the form of vesicles (micelles and liposomes), hydrogel nanoparticles, and porous silica nanoparticles with an enzyme-sensitive polymer coating as the gatekeeper.[105a,106,108] All of them have shown promise for attaining specific release at the inflammation site and inside a tumor cell. In recent studies, short peptides with sequences cleavable by matrix metalloproteinases (MMPs) have been used as linkers between poly(ethylene glycol) (PEG) chains and liposomes,[109] polymeric nanoparticles,[110] or iron oxide nanoparticles.[111] Cleavage of the PEG shell in the tumor microenvironment led to the exposure of a surface bioactive ligand, which enhanced intracellular penetration of the nanosized carrier. This approach allowed for the systemic administration of siRNA-loaded nanoparticles at a 70% gene-silencing efficiency in tumor-bearing mice.[112] Mesoporous silica nanoparticles (MSNs) grafted with polysaccharide derivatives have been demonstrated for the specific delivery of doxorubicin through lysosome-mediated cleavage of the glycoside bonds and reduction of the polysaccharide chain lengths.[69e,113] Gu and co-workers recently fabricated nanoparticles from peptide dendrimers, which were conjugated with doxorubicin through an enzyme-responsive tetra-peptide linker, Gly-Phe-Leu-Gly (GFLG). The nanoparticles showed better in vivo antitumor efficacy over free doxorubicin at equal dose.[114]

3.2.3. Thermoresponsive Release

Thermoresponsive release is among the most extensively investigated strategies for smart drug delivery because it can take advantage of the local temperature increase caused by the pathological condition (e.g., tumor, inflammation, or infection). Thermoresponsive release relies on a sharp change to the physical properties of a temperature-sensitive material. Such a sharp response can trigger the release of drug in the event of variation to the local temperature around the carrier. The range of temperatures, within which the drug-delivery system is activated, should be kept between 37 and 42 °C, because temperatures beyond this range will cause protein denaturation or function disruption.[69d,115]

The thermoresponsive drug-delivery systems are usually based on liposomes or nanoparticles composed of thermosensitive polymers. For liposomes, thermoresponsiveness usually arises from the conformational/structural changes associated with the constituent lipids to induce variations to the permeability of the lipid bilayers.[116] Thermosensitive liposomes (TSLs) represent an advanced system for drug-delivery applications related to cancer therapy. Doxorubicin-loaded TSLs (ThermoDox, Celsion Corporation) are under investigation in phase II trial for the treatment of breast cancer and colorectal liver metastasis, and have reached phase III trial for the treatment of hepatocellular carcinoma.[69e] More recently, advanced liposomal formulations have been demonstrated to release their payloads at the onset of hyperthermia (ca. 40–45 °C).[117] The thermoresponsive, bubble-generating liposomal system is a promising example.[117c–e] This system uses the generation of CO_2 bubbles through quick decomposition of ammonium bicarbonate (NH_4HCO_3) upon heating to 40 °C. The generated CO_2 bubbles produce a disruptive force to percolate the lipid-bilayer membranes and trigger release of the encapsulated payloads such as proteolytic enzymes[117e] and doxorubicin.[117c,d] On the other hand, thermosensitive polymers experience coil-to-globule transition at either a lower critical solution temperature (LCST) or an upper critical solution temperature (UCST) as a result of change to the efficiency of hydrogen bonding between the polymer chains and water molecules.[118] The LCST and UCST refer to critical temperatures below and above which the polymer is completely miscible with the solvent, respectively. When the temperature is above the LCST, the polymer will become hydrophobic and change its conformation from the expanded (soluble) to the globular (insoluble) state.[118] If the polymer is used as a chemically cross-linked network, a thermally reversible swelling/shrinking of the network will lead to on-demand release of the encapsulated drug as a consequence of controlled changes to the porosity.[119] Thermosensitive polymers can be integrated with other nanosized carriers such as liposomes and inorganic nanoparticles to introduce or enhance the thermoresponsiveness (Figure 5). Examples of the commonly used thermosensitive polymers include: poly(*N*-isopropylacrylamide) (PNIPAAm), poly(*N*,*N*-diethylacrylamide) (PDEAAm), poly(methyl vinylether) (PMVE), poly(*N*-vinylcaprolactam) (PVCL), and poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO, also known as Pluronics).[104b,120]

3.2.4. Photosensitive Release

The use of light as a stimulus to trigger drug release has been actively explored owing to its convenience for remote

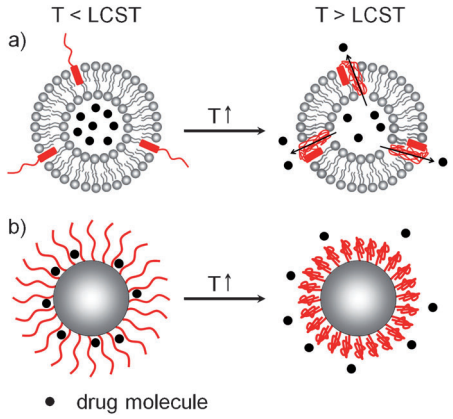

Figure 5. Drug release from two different types of thermosensitive carriers: a) a liposome containing a thermosensitive polymer and b) a nanoparticle coated with a thermosensitive block copolymer. Upon heating, the thermoresponsive component undergoes conformational change, initiating or accelerating the drug release. Panel (a) is reproduced with permission from Ref. [116], copyright 2013 Elsevier. Panel (b) is reproduced with permission from Ref. [121d], copyright 2013 Royal Society of Chemistry.

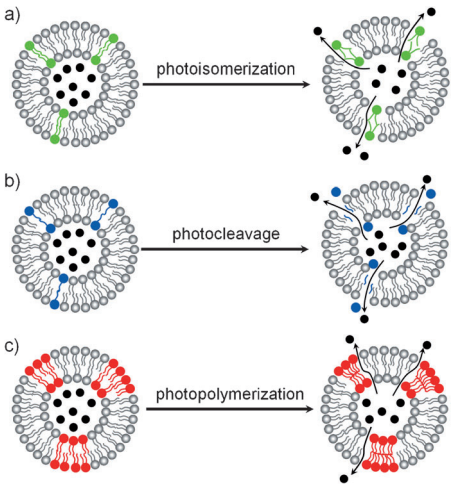

Figure 6. Photosensitive liposomes constructed through incorporation of light-responsive units into the lipid bilayers with an aim to control the drug release with optical irradiation. The release can be achieved through a) photoisomerization, b) photocleavage, and c) photopolymerization. Reproduced with permission from Ref. [129d], copyright 2012 Ivyspring.

control and its potential of high spatiotemporal resolution (Figure 6).[69d,e,121] Photosensitivity is often introduced to nanoparticles through functional groups that can change their conformations or other properties upon irradiation by light with a proper wavelength.[122] Notable examples include azobenzene, pyrene, nitrobenzene, cinnamoyl, and spirobenzopyran.[104b,123] The reversible molecular switching of the azobenzene group (and its derivatives), from *trans* to *cis* when irradiated by UV light (300–380 nm) and from *cis* to *trans* by visible light, allows for photoregulated control of drug release.[69e] This has been realized by functionalizing the interior pores of MSNs with an azobenzene-containing compound,[124] by generating an azobenzene-based molecular valve at the entrance of each pore,[125] and by inducing light-controlled host–guest recognition between the cavity in cyclodextrin and an azobenzene derivative.[126] The hydrophobic–hydrophilic transition as a result of *trans–cis* switching has also been used for this purpose.[127] Besides these systems involving conformational/structural changes, UV light has also been employed to trigger the release of biological effectors by breaking chemical bonds in a system widely known as caged compounds.[128] Despite many reports on this and related systems, the shallow penetration depth (< 200 μm) caused by the strong scattering of soft tissues has limited the use of UV-triggered systems for in vivo applications.[69f] Even for in vitro applications, the UV light may also cause damages to the cells.

Near-infrared (NIR, 700–900 nm) irradiation can penetrate more deeply into soft tissues than UV and visible light without significantly damaging/heating the area of application. The capability of plasmonic nanoparticles to transduce the absorbed NIR light into heat has been used to trigger the release of drug molecules.[129] For example, doxorubicin-loaded Au nanocages could be used for controlled release by irradiation at 808 nm, improving anticancer activity and potentially reducing systemic toxicity.[129a] A brief discussion on this system can be found in Section 6.8. The photothermal effect of Au nanorods upon exposure to NIR irradiation can also cause rapid rise in local temperature, which has been exploited to induce dehybridization of DNA helices conjugated to the surface and thereby release doxorubicin molecules bound to consecutive cytosine–guanine base pairs.[129b] However, inorganic nanoparticles are usually not biodegradable and raise concerns in their potential long-term toxicity.[130] Recently, organic transducers that can convert absorbed NIR light into heat have attracted significant attention.[131] Among them, indocyanine green (ICG), an FDA-approved fluorescent dye, is the most widely used molecule for cancer therapy.[132] For example, PLGA-based nanoparticles loaded with doxorubicin and ICG showed strong temperature responsiveness and faster doxorubicin release under NIR irradiation.[133]

4. In vitro and in vivo Delivery

Nanoparticles, when empowered with either passive or active targeting capability, can enhance the concentration of drugs inside a tumor, while reducing systemic toxicity in healthy tissues. Although the use of nanoparticles as a drug-delivery system offers many advantages, a number of issues remain to be addressed, including their instability during blood circulation, low renal clearance, limited accumulation in cancer tissues, and inadequate uptake by cancerous cells. To find solutions to these problems, it is of great importance to have a comprehensive understanding of the responses to nanoparticles by biological systems at the levels of cell, tissue, organ, and body.

4.1. *In vitro Delivery*

Understanding the in vitro delivery of nanoparticles is a critical initial step toward their successful applications. On one hand, the nanoparticles to serve as a drug carrier must be first evaluated in vitro at the cellular level before they are further tested in vivo at the tissue, organ, and body levels. On the other hand, only with sufficient knowledge of nanoparticle–cell interactions can one start to engineer the properties of nanoparticles for optimal delivery in vivo and effective cancer therapy.

Whenever a nanoparticle encounters a cell, it will be quickly internalized into the cell through endocytosis. Afterwards, it will be transferred to various organelles, including endosomes, lysosomes, Golgi apparatus, mitochondria, endoplasmic reticulum (ER), and nucleus. During the intracellular transport process, the nanoparticle has to be degraded or disassembled to allow for quick release of its payload. Here we only focus on the major steps involved in the in vitro delivery of a nanoparticle. At the end of this section, we also briefly elaborate on the issue of multidrug resistance.

4.1.1. *Endocytosis*

In order to deliver the drug to a subcellular target and execute therapeutic functions, the nanoparticle carrier must first cross the plasma membrane of a cell through endocytosis, an energy-dependent process defined as the internalization of cargo into a cell by engulfment. The endocytosis process can be initiated by either highly selective binding between the ligand attached to the nanoparticle and the receptor present on the cell membrane, or nonselective binding based on hydrophobic or electrostatic interactions. Depending on the size and surface properties of the cargos to be internalized, four major pathways have been identified:[134] 1) clathrin-mediated endocytosis (also known as receptor-mediated endocytosis, or RME), which involves the inward budding from the plasma membrane of vesicles (ca. 100 nm in size) containing receptors specific to the cargos being internalized; such vesicles possess a crystalline coating made of a protein complex associated with the cytosolic protein clathrin that is recruited to assist the internalization process; 2) caveolae-mediated endocytosis, where extracellular molecules are internalized upon binding to specific receptors in caveolae, the flask-shaped pits (ca. 50 nm in size) in the plasma membrane that exist on the surface of many, but not all types of cells; 3) pinocytosis (also known as cell drinking), a process that begins with the formation of a pocket through the invagination of the cell membrane in a highly ruffled region; the pocket is subsequently pinched off into the cell to generate a vesicle (0.5–5 μm in size) that is nonspecifically filled with a large volume of extracellular fluid, together with substances and small particles present in the fluid; and 4) phagocytosis, a process by which cells actively bind to and internalize particles larger than about 250 nm in diameter, such as small dust particles, cell debris, microorganisms, and even apoptotic cells. The last two processes involve the uptake of much larger areas of cell membrane than the clathrin- and caveolae-mediated pathways. While pinocytosis can occur in all types of cells, phagocytosis can only be performed by a small set of specialized mammalian cells, such as macrophages, monocytes, and neutrophils.[135]

Figure 7 shows a summary of the endocytosis pathways that have been reported for a number of nanoparticles. For

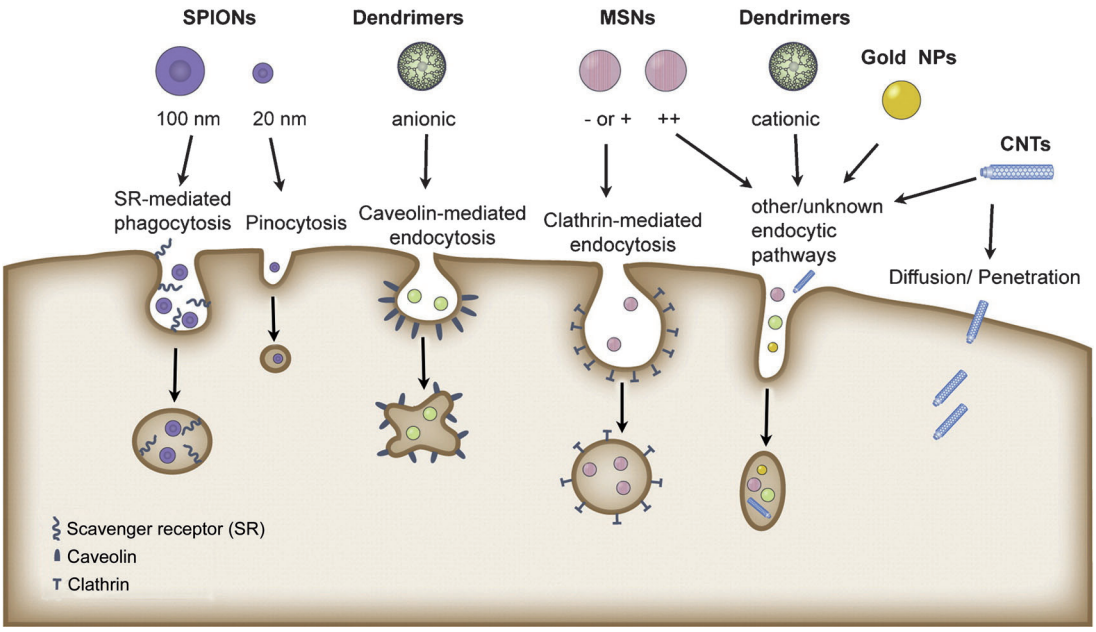

Figure 7. Pathways for the cellular internalization of different types of nanoparticles. The pathway is mainly determined by the size and surface properties of the nanoparticles, as well as the type (e.g., macrophages vs. endothelial cells) and activation status of the cells. Despite the significant progress in recent years, the details of uptake routes for some nanoparticles remain elusive. CNT, carbon nanotube; MSN, mesoporous silica nanoparticle; SPION, superparamagnetic iron oxide nanoparticle. Modified reproduction with permission from Ref. [134a], copyright 2011 Elsevier.

a specific cell type, the internalization pathway of nanoparticles is largely determined by their size and surface properties (hydrophobicity vs. hydrophilicity, the sign/density of charge, and the type/density of a ligand). While large particles are internalized through phagocytosis and pinocytosis, small particles have to rely on clathrin- and caveolae-mediated (or occasionally, independent of clathrin and caveolae) pathways.[136] The geometry or aspect ratio of nanoparticles is another factor affecting the mode of cellular uptake.[137] For example, Lehr and co-workers found that clathrin-mediated endocytosis is the primary uptake mechanism for spherical nanoparticles. In comparison, nanoparticles with a high aspect ratio may have one dimension fall within the clathrin limit but the other dimension within the limits of pinocytosis and phagocytosis, and therefore the uptake mechanism of these nanoparticles would actually depend on their orientation on the surface of the cells.[137b] Interestingly, simulation further showed that the endocytic rate of spherical nanoparticles is dependent on their size, whereas the endocytosis of spherocylindrical nanoparticles may proceed in a sequence of laying-down-then-standing-up if the particles are docked on the membrane plane in an initial upright position.[137c]

Using superparamagnetic iron oxide nanoparticles (SPIONs) as a model system, Gupta and co-workers demonstrated that the endocytic pathways of these magnetic nanoparticles were strongly correlated with their surface properties.[138] For example, they investigated the internalization of SPIONs and pullulan-coated SPIONs (Pn-SPIONs) by cells using transmission electron microscopy (TEM) and found that fewer Pn-SPIONs entered the cells as compared to SPIONs. This reduction in uptake for Pn-SPIONs can be attributed to the hydrophilicity of pullulan, which prevented Pn-SPIONs from interacting with the cell membranes.[138,139] Mirkin and co-workers reported a scavenger-receptor-mediated endocytosis pathway for the cellular uptake of DNA-coated Au nanoparticles.[140] They found that serum proteins hampered the cellular uptake of DNA-coated Au nanoparticles and the highest uptake was achieved for Au nanoparticles under serum-free cultures. The cellular uptake of MSNs was also dependent on the particle size and surface charge.[141] While MSNs with a low density of positive charges on the surface were internalized through clathrin-mediated endocytosis, particles with a high density of positive charges on the surface underwent a charge-dependent endocytosis pathway, whose mechanism is yet to be elucidated.[141a]

The endocytosis of nanoparticles is also affected by the type and physiological condition of the cells. For example, Chan and co-workers compared the uptake of transferrin-coated Au nanoparticles by three different cell lines: STO mouse embryonic cancer fibroblasts, HeLa human cervical cancer cells, and SNB19 human astrocytoma cells. They found that HeLa cells and STO cells exhibited the fastest and slowest uptake rates, respectively, for the Au nanoparticles, and the uptake of all Au nanoparticles occurred through clathrin-mediated endocytosis, regardless of the size of the nanoparticles.[142] Finally, the internalization of nanoparticles was known to be temperature dependent.[143] When incubating PLGA nanoparticles with cells at 4 °C, a condition under which energy-dependent endocytosis was halted, the uptake of the nanoparticles by the cells decreased dramatically when compared with the regular cultures performed at 37 °C.

4.1.2. *Intracellular Transport*

After internalization, the nanoparticles enveloped by vesicles will be transported along the endolysosomal network to other organelles or sometimes even be exocytosed (excretion from the cell). As shown in Figure 8, the intra-

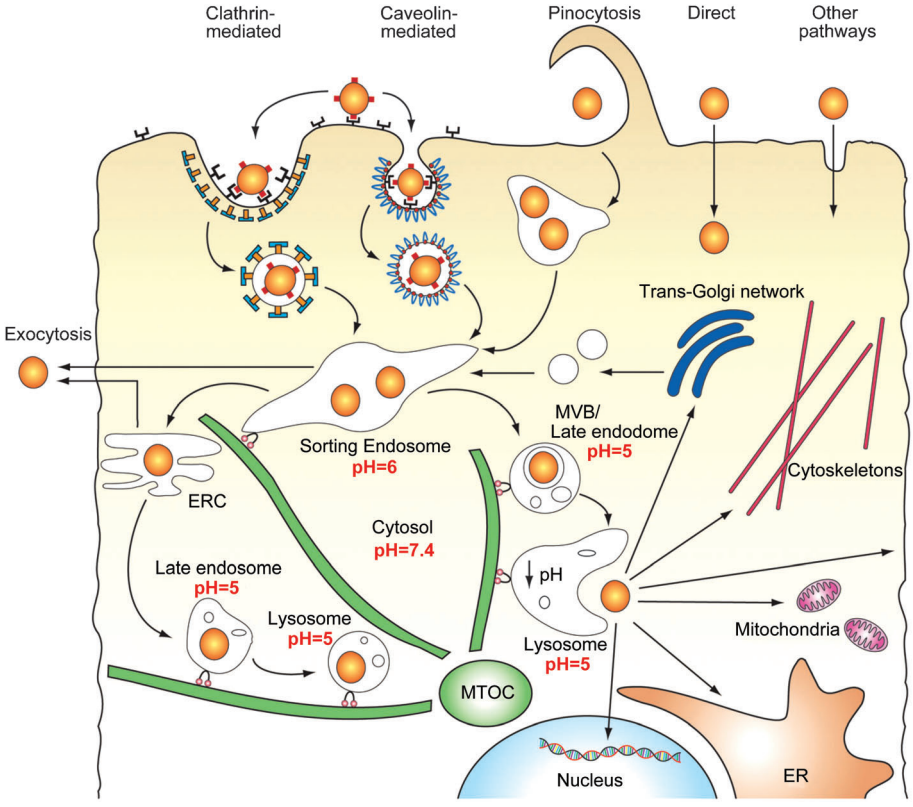

Figure 8. Intracellular transport of nanoparticles. After internalization, the nanoparticle is trafficked along the endolysosomal network in vesicles with the aid of motor proteins and cytoskeletal structures. Note the difference in pH values between different intracellular compartments. ER, endoplastic reticulum; ERC, endocytic recycling compartment; MTOC, microtubule-organizing center; MVB, multivesicular bodies. Modified reproduction with permission from Ref. [143b], copyright 2011 the Royal Society of Chemistry.

cellular trafficking of nanoparticles is a very complex process that involves motor proteins shuttling the nanoparticle-loaded vesicles along cytoskeletal structures (e.g., microtubules) within a cell.[143b]

In a recent study, Saltzman and co-workers used rhodamine-loaded PLGA nanoparticles to explore the intracellular transport processes in three different types of epithelial cells (HBE bronchial epithelial cells, Caco-2 epithelial colorectal adenocarcinoma cells, and OK kidney epithelial cells).[143a] After internalization, the PLGA nanoparticles could escape endolysosomal degradation, and be sequentially transported to the Golgi apparatus and ERs. Based on these results, an endocytosis–exocytosis pathway was proposed, by which the PLGA nanoparticles encounter endosomes first and then escape from the compartment, followed by interactions with exocytic organelles (i.e., ER, Golgi apparatus, and other secretory vesicles) in the cell.

Nie and co-workers used Tat-peptide-conjugated quantum dots (Tat-QDs) to examine the intracellular transport of nanoparticles in HeLa cells. They took advantage of dynamic confocal imaging and found that Tat-QDs were internalized through pinocytosis, followed by entrapment in the cytoplasmic organelles. The vesicles loaded with Tat-QDs were found to be actively transported along microtubule tracks by molecular machines, and finally to the microtubule-organizing center (MTOC), which is located outside the cell nucleus.[144]

Interestingly, the surface of nanoparticles can be functionalized with a ligand to target a specific organelle in the cell. For example, adding a nuclear localization signal (NLS) peptide motif to the surface of nanoparticles could lead to effective nuclear targeting. As reported by Mao and co-workers, liposome protamine/DNA complexes termed lipoplexes (LPDs) were accumulated in the nuclei of cells after their internalization when the surface of the particle was derivatized with NLS peptides.[145] Compared to LPDs with no nucleus-targeting ligand on the surface, the gene expression level was significantly elevated when DNA was delivered into the nuclei of the cells.

4.1.3. *Intracellular Escape and Degradation of Nanoparticles*

For successful delivery of therapeutic agents, the nanoparticles also need to be designed with an ability to escape from the endolysosomal network and enter the cytosol, which is the typical working site for most drugs (Figure 8). To achieve this goal, several strategies have been explored. For example, a type of virus-like nanoparticles was reported, which were capable of fusing with endosomal membranes and transporting drugs from endosomes to the cytoplasm.[146] Alternatively, nanoparticles were coated with a polymer (typically with amine groups) that has a buffering capacity between pH 5.2–7.0 to enable endosomal escape through the "proton-sponge effect".[147] Once these cationic nanoparticles were engulfed into an acidic endolysosomal compartment, the amino groups could continuously sequester protons pumped inwards by the v-ATPase (i.e., the proton pump), leading to accumulation of water molecules inside the compartment.[147] Eventually the swelling resulted in the rupture of the endolysosome. As an example, QDs with a surface coating of PEG-grafted polyethylenimine (PEI-*g*-PEG) were capable of penetrating cell membranes and then disrupting endolysosomal organelles, owing to the highly positive surface charges provided by the multiple amine groups.[148] Labhasetwar and co-workers fabricated PLGA nanoparticles that were responsive to pH change and studied the internalization of these nanoparticles using vascular smooth muscle cells (VSMCs). Once internalized, the PLGA nanoparticles underwent rapid endolysosomal escape because of the reversal of surface charges on the nanoparticles from anionic to cationic under the low pH value in endolysosomes and the subsequent induction of the "proton-sponge effect".[149]

Rapid escape/release of nanoparticles from endolysosomes can also be induced when their surfaces are modified with a pH-sensitive peptide capable of physically interacting with endolysosomal membranes. GALA, a pH-sensitive fusion peptide composed of 30 amino acids with repeating units of glutamic acid-alanine-leucine-alanine, could perturb the lipid bilayer and facilitate nanoparticles to escape from endosomes at low pH values.[150] When the pH value decreased from 6 to 5 in the endosome, the negative charges on GALA decreased, causing a conformational change from random coil to amphipathic α-helix. This change allowed GALA to bind to the endosomal membrane, causing membrane disruption.

In many cases, nanoparticles in the endolysosomes or the cytoplasm must be destructed to a certain extent to allow for proper release of the payloads. For polymer nanoparticles, several strategies can be utilized to improve the efficiency of disassembly, including the use of enzyme-active linkers, acid-labile cross-linkers, pH-sensitive detergent, thermal-sensitive liposomes, and disulfide cross-linkers that are sensitive to a reducing environment.[151] In one example, Ithakissios and co-workers studied the use of nanoparticles based on PLGA-PEG copolymers for the delivery of cisplatin.[152] They found that the intracellular degradation of PLGA-PEG nanoparticles was dependent on their composition. With higher PEG content, the degradation rate of nanoparticles increased, resulting in a faster release of the encapsulated cisplatin. In another example, Tasciotti and co-workers reported a drug-delivery system based on porous silicon nanoparticles, the so-called multistage nanovectors (MSVs).[153] According to their results, the decomposition of MSVs was largely determined by the pore size, and MSVs with larger pore sizes were degraded more rapidly. Along with the tunable drug-loading capacity of MSVs, the controlled release of a drug could be realized through engineering of the pore size of MSVs.

4.1.4. *Multidrug Resistance*

Multidrug resistance (MDR) is a major problem encountered in chemotherapy that negatively impacts the treatment efficacy of chemotherapeutics.[154] A number of mechanisms have been reported for MDR, including increased efflux pumping of drugs by the overexpressed ATP-binding cassette (ABC) transporters, reduced intracellular accumulation of drugs by non-ABC drug transporters, blocked apoptosis, repair of drug-induced DNA damage, metabolic modifica-

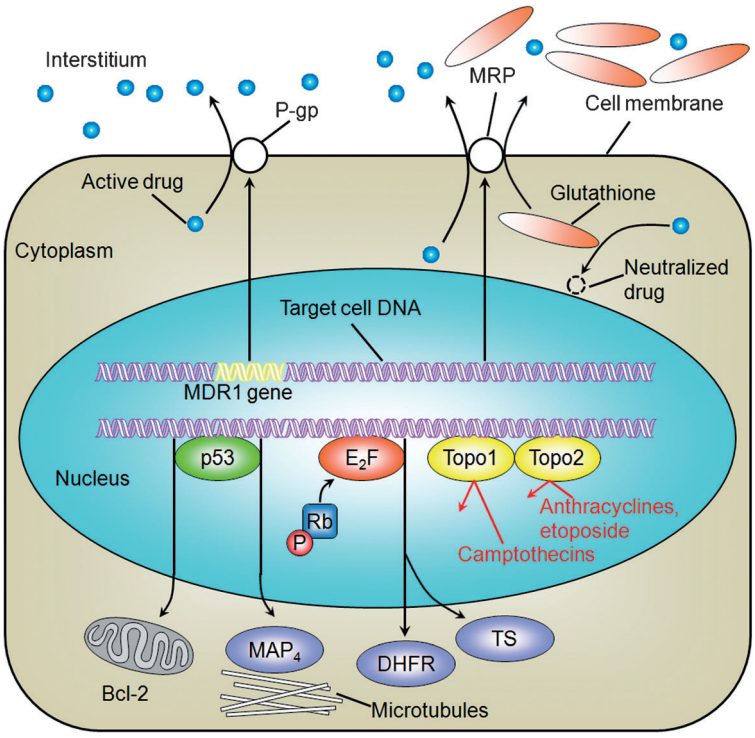

Figure 9. Mechanisms of multiple-drug resistance in cancer cells: drug efflux caused by multidrug resistance protein (MRP) (e.g., P-glycoprotein or P-gp), down-regulation of the sensitivity to drug by the tumor suppressor protein p53, reduction in sensitivity to methotrexate and fluorouracil by phosphorylation of the retinoblastoma protein (Rb), and production of resistance to camptothecins through down-regulation expression or mutations in topoisomerases (Topo).

tion, and detoxification by drug-metabolizing enzymes (Figure 9).[155] Among these mechanisms, the overexpression of plasma membrane P-glycoprotein (P-gp, or ABCB1), a member of the ABC superfamily, is one of the most common causes of MDR. P-gp is capable of extruding a number of positively charged xenobiotics out of the cell, including some of the commonly used anticancer drugs.[154] The overexpression of other ABC transporters, such as MDR proteins and breast cancer resistance protein (BCRP), has also been identified as a primary cause of MDR.

To suppress MDR of cancerous cells and maximize the cytotoxic efficacy of anticancer drugs, a general strategy is to co-administrate one drug (e.g., a gene) to inhibit ABC transporters and promote apoptosis together with another anticancer drug for the actual treatment. To this end, nanoparticle carriers based on liposomes and polymers have been utilized to encapsulate the dual components and at the same time ensure precise delivery to the targeted sites.[154] In one example, Amiji and co-workers demonstrated the use of PEO-modified poly(ε-caprolactone) (PCL) nanoparticles as a multi-drug-delivery system for the apoptosis modulator ceramide and the chemotherapeutic drug paclitaxel.[156] Their results indicate that the dual-drug-delivery system could greatly improve chemosensitivity of ovarian cancer cells exhibiting MDR by bypassing P-gp drug efflux. Alternatively, Shi and co-workers reported a pH-responsive multi-drug-delivery system based on MSNs that could overcome MDR.[157] In their study, DOX was used as a chemotherapeutic drug and cetyltrimethylammonium bromide (CTAB), a surfactant commonly used in the synthesis of Au nanorods, as an MDR inhibitor. The MSNs co-loaded with DOX and CTAB showed high therapeutic efficacy against both drug-resistant MCF-7/ADR cells and drug-sensitive MCF-7 cells. The MDR-evading mechanism was shown to involve a synergistic effect between the cell cycle arrest and the apoptosis-inducing effect from the chemosensitization of CTAB.

Liposomes represent another class of delivery vehicles commonly used for overcoming MDR in cancer therapy. Galactosylated pluronic P123 (Gal-P123) modified liposomes loaded with mitoxantrone have been used to treat hepatocellular carcinoma (HCC). An improved therapeutic efficacy against HCC cells was reported, which can be attributed to the ability of the liposome to reverse BCRP-mediated MDR.[158] Propylene glycol liposomes loaded with epirubicin (EPI-PG-liposomes) were also demonstrated with a potential to overcome MDR in breast cancers.[159] Nevertheless, it is interesting to mention that EPI-PG-liposomes were not observed to interfere with P-gp, indicating that this type of nanoparticles might be able to overcome MDR using a mechanism that does not involve the inhibition of P-gp.

4.2. In vivo Delivery

While drug delivery in vitro is mainly concerned about nanoparticle–cell interactions, the application in vivo emphasizes more on how to send the nanoparticle carrier to the target lesion from the site of administration. Upon introduction into the body, the carrier needs to reach the target lesion and be accumulated there before any treatment can take place. As a result, one has to deal with many additional issues related to the transport of nanoparticles, as well as immune response, selectivity and efficiency in targeting, biodistribution, biodegradation, clearance, and toxicity at the organ and system levels. Ideally, the nanoparticles that serve as the carrier of a drug-delivery system should have the following attributes: 1) a good targeting efficiency to ensure selective deposition of drug in the target lesion while maintaining low concentrations in healthy tissues/organs; 2) consisting of biocompatible and/or biodegradable materials only; and 3) clearance from the body within a predetermined time frame.[160] In reality, however, it is almost impossible to satisfy all these requirements.

With the advances in nanotechnology, many new materials and techniques have emerged to help us realize the aforementioned goals. For example, the physicochemical properties of nanoparticles, including composition, size, shape, morphology, surface charge, and surface coating, can all be tailored to improve their performance in vivo.[161] An interesting example can be found in the use of QDs for cancer diagnostics and therapeutics . The long-term toxicity caused

by the elements (e.g., cadmium, selenium, and tellurium) commonly used for the synthesis of QDs is difficult to avoid because these elements are essential to the optical properties of most QDs.[162] However, through surface modifications such as PEGylation and conjugation with targeting agents, it is feasible to reduce their accumulation in and toxicity to major organs to an acceptable level by increasing their circulation half-life and reducing their accumulation in organs in a less nonspecific manner.[163]

Intravenous injection represents the most commonly used route for the administration of nanoparticle-based therapeutics as it bypasses the barriers in the epithelial absorption process by directly entering the circulatory system.[164] Upon injection, the nanoparticles are immediately subjected to clearance through a joint force of the MPS,[165] the renal system,[166] and the immune system.[167] During circulation, the size, shape, and surface properties of the nanoparticles can all strongly affect their behaviors/performance with respect to targeting and clearance.[136,168] Figure 10 shows how the behavior and fate of nanoparticles in the body are dependent on their size, surface charge, and hydrophobicity. In general, nanoparticles with a size smaller than 6 nm will be rapidly filtered out and cleared by the kidneys. Nanoparticles larger than 8 nm cannot undergo glomerular filtration; instead, they will either accumulate in a lesion or be cleared by the MPS.[165,169] A positive surface charge on the nanoparticles will lead to high systemic toxicity because of complications such as hemolysis and platelet aggregation, and the nanoparticles tend to be quickly cleared from the blood by the MPS.[169] Negatively charged nanoparticles have longer circulation half-life than their positive counterparts while the blood half-life of neutral nanoparticles is the longest. Altering the surface chemistry of a nanoparticle will cause changes to its hydrodynamic size and surface charge, as well as reactivity (e.g., binding affinity). Although complete clearance is eventually desired when the treatment is completed, the nanoparticles must be able to avoid rapid clearance in order to achieve the desired targeting efficiency. In general, the circulation half-life of nanoparticles should be prolonged to allow them to pass by a lesion multiple times, giving them increased opportunities to accumulate in the lesion.[170]

To understand the opportunities and challenges in drug delivery in vivo, we discuss this subject from two different angles: how do nanoparticles reach the targeted lesion and how are they cleared from the body. After analyzing several biological barriers for the in vivo delivery of nanoparticles, we focus on how to optimize their pharmacokinetics and biodistribution.

4.2.1. *The EPR Effect and Passive Tumor Targeting*

Nanoparticle therapeutics directly administrated into the circulatory system[164] need to extravasate through the vascular walls into the target lesion and then release the payload. Unlike small molecules, nanoparticles cannot go through the tight junctions between endothelial cells on normal vascular linings, owing to their relatively large sizes (Figure 11, left panel). However, the vessels inside a tumor region are well-known for their leaky walls (Figure 11, right panel), allowing nanoparticles with the right sizes to pass through efficiently.[171] As the lymphatic system inside a tumor is largely absent or dysfunctional, the insufficient drainage facilitate

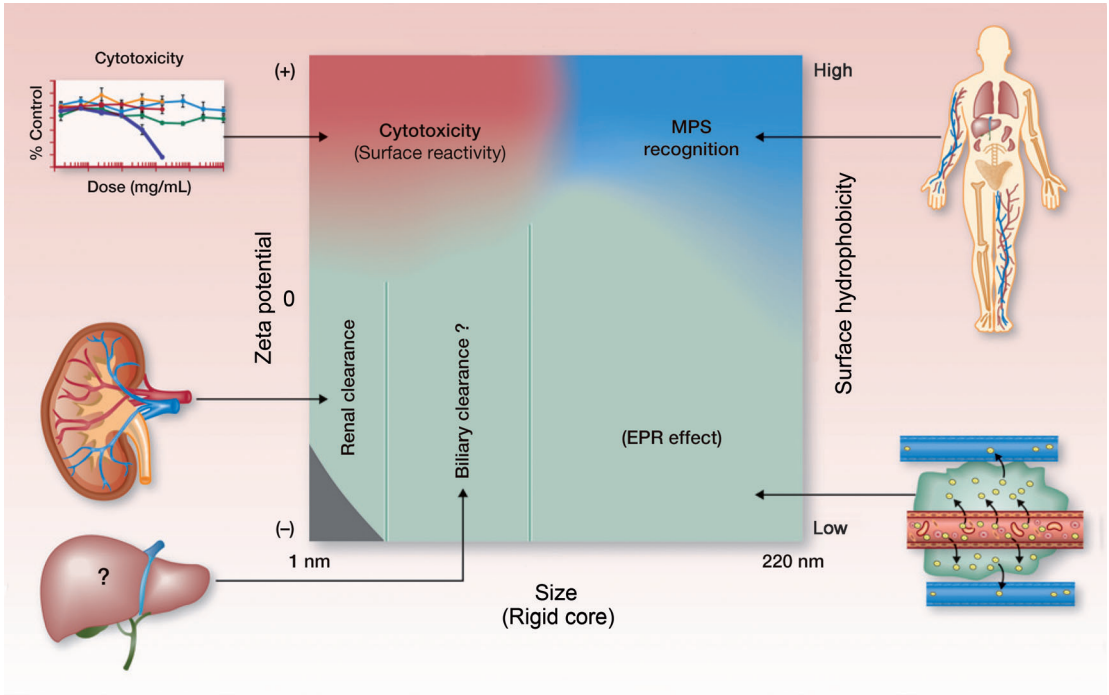

Figure 10. The surface charge (zeta potential), size, and surface hydrophobicity of nanoparticle can affect their cytotoxicity (surface reactivity), recognition by the mononuclear phagocyte system (MPS), clearance (renal or biliary), and enhanced permeability and retention (EPR) effect in tumor targeting. Modified reproduction with permission from Ref. [201], copyright 2012 American Association for Cancer Research.

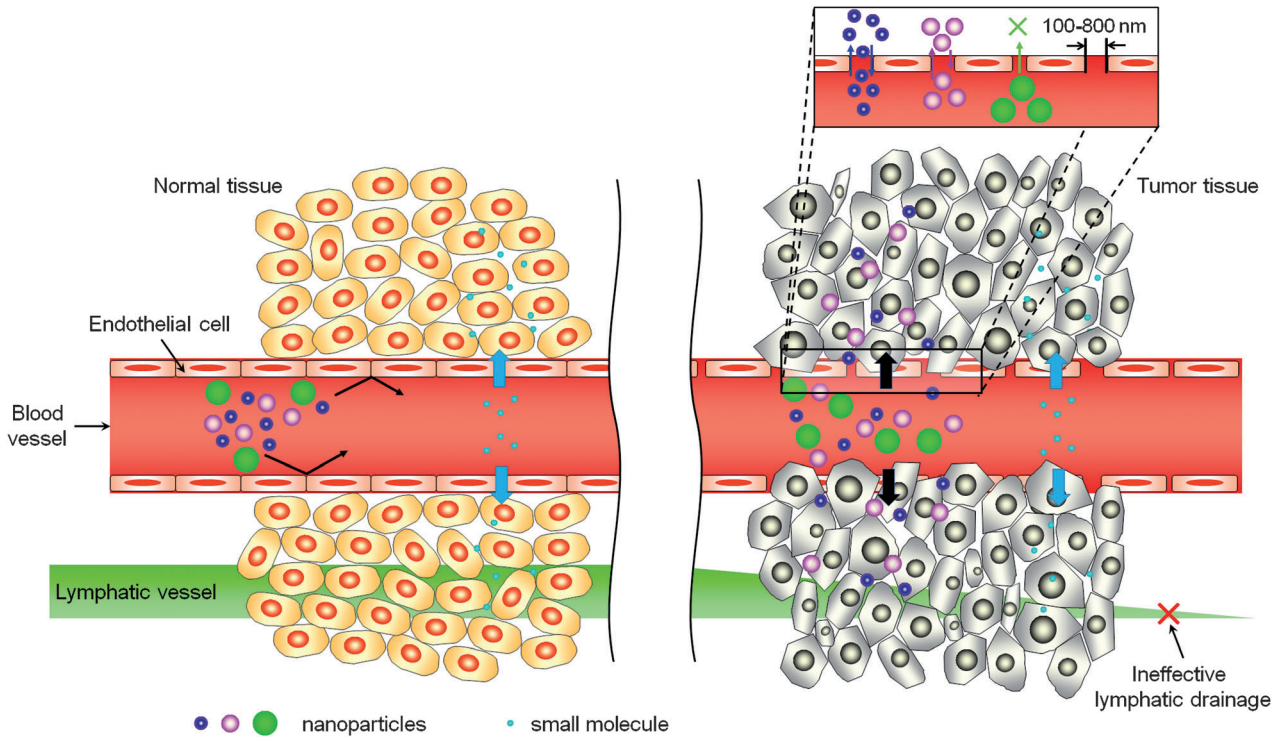

Figure 11. Transport of nanoparticles with different sizes and small molecules through normal (left) and cancerous (right) tissues. The enhanced permeability and retention (EPR) effect is a unique feature of most tumors, allowing nanoparticles of appropriate sizes to accumulate more in cancerous tissues than in normal tissues.

accumulation of nanoparticles in the tumor tissue.[171,172] This phenomenon has been widely known as the EPR effect of nanoparticles, which is the basis for passive tumor targeting.

The EPR effect was first noticed by Maeda and co-workers in studying the inflammation induced by microbial infections.[173] In 1986, Matsumura and Maeda further provided experimental evidences to support the concept of the EPR effect in tumor targeting for the first time.[37a] Because of the EPR effect, both macromolecular drugs and nanoparticle therapeutics can target tumors more efficiently than small-molecule drugs, as the extravasation of nanosized objects occurs in a tumor-selective manner. Over the past several decades, utilization of the high permeability of tumor tissues for nanoparticle delivery has become an important strategy for the design and development of new therapeutics for cancer treatment.

The EPR effect is one of the most important features and results of tumor angiogenesis. Solid tumors rely on rapid angiogenesis to maintain sufficient supplies of nutrients and oxygen.[174] The rapid proliferation of endothelial cells during angiogenesis usually results in a reduced density of endothelial cells and thus loss of tight junctions and formation of large gaps between the cells. The presence of large gaps between the endothelial cells on the tumor vascular walls has been confirmed by direct visualization through optical and electron microscopy.[175] The underlying basement membrane of the blood vessels is mostly abnormal or missing.[176] In addition, tumor blood vessels lack pericytes and smooth muscle cell layers, making them more vulnerable to the high interstitial pressure and rapidly shifting blood flow.[177] Depending on the tumor type, the openings in the tumor vasculature are typically in the size range of 100–800 nm.[177] A functional pore size of 1200–2000 nm has also been reported in MCa-IV mouse mammary carcinoma, which is likely at the high end of size range for tumor vessel leakage.[175]

Among various parameters, the size of a nanoparticle plays the most important role in EPR-based tumor targeting. As shown in Figure 11, only nanoparticles with a size smaller than the gap between adjacent endothelial cells can extravasate from the vasculature. As described above, the cutoff size typically varies from 100 to 800 nm, depending on the type and stage of a tumor. Particles smaller than this cutoff size can extravasate from the blood vessels into the tumor interstitium. When liposomes of different mean sizes were tested, it was shown that the cutoff size for extravasation into tumors was approximately 400 nm,[178] whereas particles with diameters smaller than 200 nm were found to work most effectively.[5] This is because particles larger than 200 nm were cleared from the blood stream more rapidly by the spleen (see Section 4.2.3). Once extravasated, the penetration of the nanoparticles into the tumor tissues is a diffusion-mediated process, which is inversely correlated with the particle size. On the other hand, the nanoparticles extravasated into the tumor interstitium can also travel back into the blood vessels through the gaps in vascular walls and then get cleared by the MPS or kidneys.[5] For nanoparticles relatively small in size, an equilibrium in particle distribution across the vasculature can be rapidly established. In general, nanoparticles with sizes in the range of 30–200 nm show better retention by the tissue resistance, shifting the equilibrium toward extravasation and

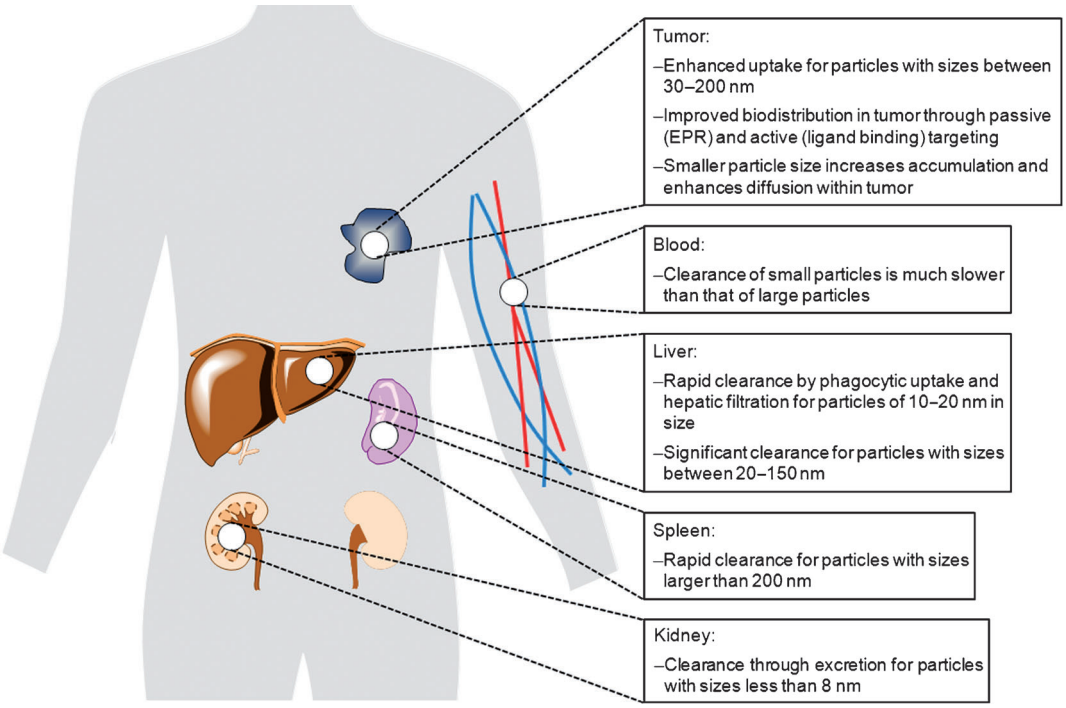

Figure 12. Biodistribution and clearance of nanoparticles from the human body. Tissue defects as well as the size, targeting ligand, and stealth properties of the nanoparticles are some of the major factors that affect the biodistribution and clearance of nanoparticles.

leading to enhanced accumulation. Taken together, nanoparticles with sizes between 30–200 nm are believed to be optimal for passive targeting of most types of solid tumors by capitalizing on the EPR effect (Figure 12).[179]

The tumor interstitium also plays an important role in determining the passive targeting efficiency. The tumor interstitium is composed of an elastic network of collagen fibers filled with hydrophilic fluid.[180] Unlike the normal tissue, there exists a high interstitial pressure in the tumor interstitium, especially in the central portion of the tumor, which tends to work against the extravasation of nanoparticles. In general, the transport of nanoparticles into the interstitium is driven by a net force between the extravasation and interstitial pressure, as well as the gradient in concentration.[177,181] Interestingly, the shape of the nanoparticles was also found to play a role in EPR-based tumor targeting.[182] Both simulation and experimental results have shown that nanoparticles with a spherical shape tend to follow a laminar flow pattern so that only those particles that move near the surface of the vascular wall will be able to extravasate into the tumor.[7c,183] In contrast, rod- and bar-shaped nanoparticles are hydrodynamically more unstable and sometimes fail to follow the flow pattern as they travel in the blood stream.[184] These hydrodynamic features provide more opportunities to finely tune the geometrical parameters of nanoparticles and thereby enhance their chance to cross the gaps on the vascular wall.[184]

The surface properties (including functional groups and charges) may affect the efficiency of extravasation and retention as they affect the hydrodynamic radius, plasma reactivity, circulation half-life, and "stealth" capability of the nanoparticles. For nanoparticles with prolonged circulation half-life and "stealth" capability, they generally have less protein adsorption and thus improved tumor accumulation as a result of significant reduction in clearance by the MPS.[170]

To achieve a maximum therapeutic effect in treating solid tumors, nanoparticles need to uniformly penetrate deeply into the tumors and then release their payloads. Cellular uptake of nanoparticles is also needed to increase the drug-delivery efficiency while reducing nonspecific accumulation and the associated issues such as multidrug resistance (discussed in Section 4.1). Despite its widespread use in the clinic, the passive targeting strategy has many limitations as the vessels formed through angiogenesis are not evenly distributed in a solid tumor and the permeability may not be homogeneous throughout the tumor. For a small tumor or metastatic lesion that does not exhibit strong angiogenesis, the passive targeting efficiency based on the EPR effect will be rather limited.[5] Active targeting will help address some of these issues.

4.2.2. Active Tumor Targeting

To better utilize the biochemical properties of cells to be targeted, ligands such as small molecules, peptides, antibodies and antibody fragments, and nucleic acids (e.g., aptamers) have been added to the surface of nanoparticles to improve their targeting efficiency. This new targeting mode that involves molecular recognition is known as active targeting, in which ligand–receptor binding allows the nanoparticle to selectively and strongly bind to the surface of a specific type of cells. This strategy has proven to be effective in vitro and to a certain extent in vivo.[5,185] For example, when conjugated with a targeting ligand, the nanoparticles often show

enhancement in internalization as this process is dominated by receptor-mediated endocytosis.[186] The conjugated ligand will increase the affinity of binding and thereby induce receptor-mediated endocytosis more effectively. For targeting solid tumors in vivo, the nanoparticles with an active targeting ligand on the surface still need to rely on the EPR effect to pass through the gaps in vascular walls.

Currently, the improvement in tumor accumulation achieved through the introduction of an active targeting ligand is still under debate.[187] It is believed that the accumulation of nanoparticles in tumor tissue is dominated by the passive process, which is time dependent and requires a long circulation half-life. As discussed in the previous section, the efficiency of this accumulation process is largely determined by the physicochemical properties of nanoparticles other than the active targeting ligand. Even without using any targeting ligand, it is always possible to increase the accumulation of nanoparticles in a tumor by engineering their size, shape, and surface chemistry.[188] In the presence of a targeting ligand, the retention and uptake of nanoparticles by cancer cells can indeed be augmented as a result of receptor-mediated endocytosis, but only after the nanoparticles have extravasated from the vasculature.[189] In this way, active targeting can help achieve a higher intracellular drug concentration, even though there is only a modest improvement in tumor accumulation.[187] The escalation in intracellular drug concentration can drastically increase cellular cytotoxicity and improve the therapeutic efficacy of drugs that work with intracellular targets.[189,190] Some experimental results suggest that active targeting would increase both tumor accumulation and cell uptake,[191] but these results are likely caused by the discrepancy in surface properties of the particles, nonuniformity of tumor models, and the variance of targeting ligands.[160a] On the other hand, it is argued that active targeting may anchor nanoparticles to tumor cells next to the leaky vessels, decreasing the efficiency of diffusion, depth of penetration, and uniformity of distribution.[7h] To overcome these potential drawbacks, the density of the targeting ligand presented on the nanoparticle's surface needs to be carefully tuned to optimize the balance between penetration depth and binding affinity.

Active vascular targeting has been demonstrated as a promising alternative for tumor targeting. By targeting and killing the endothelial cells of the tumor vessels, nanomedicine can be used to eradicate tumor cells by cutting off their supplies of oxygen and nutrients.[192] In the case of active vascular targeting, the targeting ligand is of critical importance as tumor accumulation is no longer determined by the EPR effect, but by the binding affinity to blood vessels. Nanoparticles have been developed for targeting various moieties on the vascular walls, including fibronectin extradomain B, large tenascin-C isoforms, integrins, annexin A1, as well as vascular endothelial growth factors (VEGFs) and their receptors.[192]

Currently, the targeting of solid tumors remains a challenging task and represents a bottleneck for the development of future cancer therapeutics. Even those "successful" drug-delivery systems based on nanoparticles still show significant accumulation in major organs. In general, the therapeutic effect depends on the properties of both the tumor (e.g., the type and degree of angiogenesis and degree of tumor vascularization) and the nanoparticles (e.g., the size, shape, charge, and surface chemistry).[160a] The toxicity profiles and therapeutic effects of most nanoscale therapeutics still need to be improved to meet the minimum requirements for clinical applications.

4.2.3. Clearance by the MPS

The clearance of a foreign substance or object from the body may involve organs such as liver, spleen, and kidneys, as well as the immune and complement systems (Figure 12). It is a natural process that helps maintain our body in a healthy state. The original targets of these systems for clearance are mostly pathogens such as bacteria and viruses, but they also have the capability to handle other exogenous particles. The force for clearance is so strong that most nanoparticles administrated intravenously will be removed from the blood in as little as a few minutes to hours. Upon injection, the clearing process will immediately "compete" with the targeting process (either passive or active) for the nanoparticles, resulting in an unfavorable distribution between the tumor site and organs such as the liver. The rapid clearance can dramatically offset the desired targeting effect and present a major barrier for the development of effective nanomedicine.[160a]

The MPS and renal clearances represent two major routes for the removal of nanoparticles from our body. One of the physiological functions of the MPS is to actively capture and eliminate viruses and other relatively small objects.[193] MPS involves organs such as the liver, spleen, and bone marrow that are rich in phagocytic cells such as macrophages, Kupffer cells, and monocytes. These cells are able to engulf and digest nanoparticles. Studies have shown that the MPS is responsible for the clearance of most nanoparticles larger than 10 nm, regardless of their shape and surface chemistry.[165]

When nanoparticles enter the plasma, opsonization (i.e., the adsorption of serum proteins) will occur immediately on their surfaces.[5,160a] Through opsonization, foreign organisms or particles will be coated with nonspecific proteins known as opsonins to generate a corona and make the particles more visible to the phagocytic cells in the MPS. Opsonins typically contain complement proteins and immunoglobins (usually IgG) along with albumins, fibronectins, fibrinogens, and apolipoproteins.[194] Studies have shown that the corona has a layered architecture. It starts with an inner layer of proteins that strongly adsorb onto the surface, with $K_d \approx 10^{-6}$ to 10^{-8} M, to form the hard corona, which is then surrounded by a layer of soft corona formed by weak interactions.[169,195] The primary driving forces for opsonization are based on hydrophobic and electrostatic interactions, together with entropic and conformational changes for the adsorbed proteins.[196] Depending on the charge and hydrophobicity of the nanoparticles, opsonization can occur within minutes. Experimental results suggest that a charged surface tends to be covered by proteins more rapidly than their counterparts with a neutral surface.[160a]

Upon opsonization, the nanoparticles will be rapidly internalized by phagocytic cells in MPS organs. As a result, the fate of nanoparticles is determined by their surface properties in conjunction with opsonization.[197] Phagocytic cells have ciliated borders and stellate branches, which provide them with an efficient mechanism for trapping and removing foreign particles.[198] These cells possess numerous membrane receptors, including those for the complement proteins and for the Fc portion of IgG that is deposited on the nanoparticles.[198] After binding of the nanoparticles to the receptors on phagocytic cells, receptor-mediated endocytosis will be initiated, followed by enzymatic degradation. Segments that remain after enzyme breakdown will be retained in the cells and be accumulated in organs such as the liver and spleen (which is typically the situation for noble-metal nanoparticles).[198]

The liver plays the most important role in cleaning nanoparticles that do not undergo renal clearance, as it contains numerous Kupffer cells whose natural function is to eliminate foreign substances through phagocytosis. Hepatocytes can also take up and process nanoparticles, providing the critical function of biliary excretion, which can expunge certain nanoparticles permanently from the body.[198] All nanoparticles excreted through the biliary system must be broken down by hepatocytes before secretion, so clearance from the biliary system is considered to be an active process, even though hepatocytes are not a part of the MPS. It is worth mentioning that the processing of nanoparticles by hepatocytes (i.e., the hepatic process), and the biliary excretion process are relatively slow, although hepatic uptake of nanoparticles always occurs quickly.[193] Different from the hepatic process, nanoparticles processed by Kupffer cells and phagocytic cells will always stay in the MPS organs. In general, there is always a large number of nanoparticles accumulated in the liver and other MPS organs, which could potentially induce long-term side effects.

Intrinsically a cleaning organ, the spleen has a blood filtration system composed of a tight reticular mesh (ca. 200 nm wide) made of interendothelial cells.[199] Nanoparticles larger than 200 nm are preferentially cleared by spleen. By choosing an appropriate size and surface chemistry, rigid nanoparticles with long circulation half-life can accumulate in the spleen at a high percentage.[200] Experiments showed that about 50% of polystyrene nanoparticles with a diameter of 250 nm and coated with poloxamine 908 accumulate in the spleen within 24 h after injection because of the physical filtration effect.[199,200] Such a feature mainly exists in nanoparticles with long plasma half-life, as other types of nanoparticles tend to be captured by the MPS in the liver because of their high phagocytic activity after opsonization. In addition to the active capture by the MPS, clearance of large nanoparticles (>150 nm) also utilizes physical filtration to trap particles in the spleen or liver,[199] making clearance by the major MPS organs a hybrid of active and passive processes.

To avoid clearance by the MPS, the surface of nanoparticles needs to be carefully engineered to prevent or at least mitigate opsonization. This will help prolong their plasma half-life, reduce MPS clearance and enhance the targeting efficacy. If possible, the surface of the nanoparticles should be kept neutral, as a neutral surface attracts the least amount of serum proteins. The presence of negative charges on the surface will induce opsonization and thus MPS uptake, but at a reduced rate relative to a positively charged surface.[201] Surface modifications such as PEGylation are widely used to reduce opsonization and consequently increase the circulation half-life of nanoparticles. Known as a "stealth" property, PEGylated nanoparticles are less recognized by phagocytic cells as a result of lower opsonization and tend to accumulate in tumors more effectively.

Recent studies have demonstrated that the opsonization process could be much more complicated than simple protein adsorption. Nanoparticles could be actively targeted by the immune system through antibodies. It has been shown that up to 25% of patients developed anti-PEG antibodies after treatment with PEGylated nanoparticles.[167] This finding is critically important as PEGylation is heavily relied upon to provide the nanoparticles with a "stealth" property. Further studies indicate that the balance between Th1-Th2 cytokines and M1-M2 macrophages also affects the rate and amount of nanoparticle clearance, because Th1-prone mice cleared nanoparticles at a slower rate than Th2-prone mice.[202] All these results suggest that the immune system may actually affect the uptake and clearance of nanoparticles in a more profound way than what is currently understood.

4.2.4. *Renal Clearance*

Renal clearance is based on physical filtration (dialysis) rather than cell uptake. Unlike the clearance of nanoparticles from circulation by the MPS system, the renal system removes the nanoparticles from the body through the urine rather than having them accumulated in related organs. As the removal of nanoparticles by bile is a relatively long process, renal clearance is an optimal method for expelling nanoparticles from the body with minimal side effects.

Renal clearance of nanoparticles is a passive process, involving glomerular filtration and tubular secretion. After entering the glomerular capillary bed, nanoparticles are either filtered out through the glomerular capillary or remained within the vasculature, depending on their properties (Figure 13). During extraction, the nanoparticles need to be filtered through the fenestrated endothelium, the glomerular basement membrane (GBM), and the glomerular epithelial cells.[203] Although these layers of cells are known to have pores with sizes up to 43 nm, the functional or physiologic pore size is only around 5 nm if we take into consideration the combined effect of all layers in the glomerular capillary wall.[203,204] As a passive process, the filtration of particles is highly dependent on their sizes.[203] Typically, nanoparticles with a hydrodynamic diameter of less than 6 nm are rapidly filtered out, whereas those with hydrodynamic diameters of 6–8 nm are removed less efficiently, and particles larger than 8 nm generally cannot escape.

The difference in particle properties can often result in distinct renal handling. Several studies using QDs and Au nanoparticles have been conducted to evaluate the effect of these characteristics on the size threshold for filtration.[160b,166]

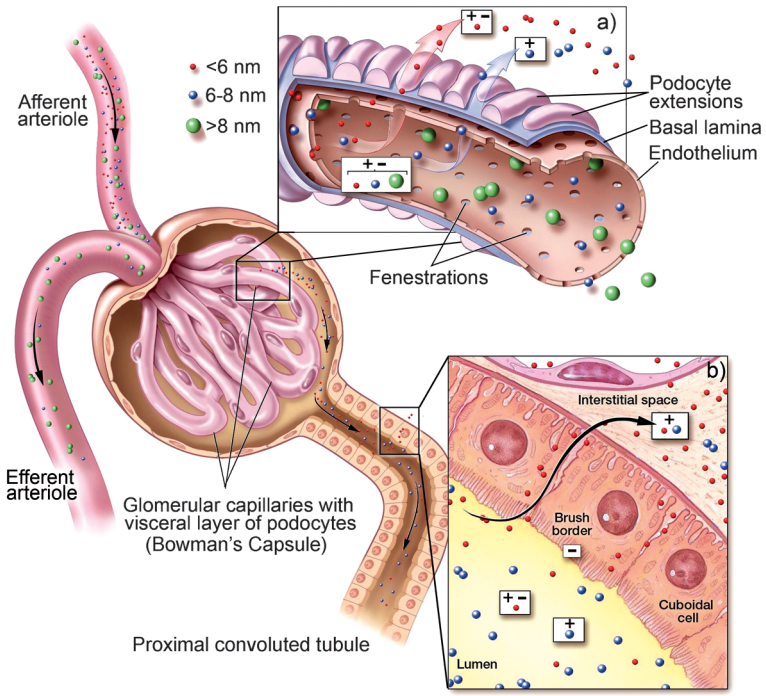

Figure 13. Renal handling of nanoparticles with different sizes and charges. a) The glomerular capillary wall contains a filtration slit, which has a physiologic pore size of 4.5–5 nm. Nanoparticles <6 nm (red) are small enough to be freely filtered, irrespectively of surface charge. However, positively charged nanoparticles of 6–8 nm (purple) are more readily filtered than equally sized but negatively charged nanoparticles. Nanoparticles >8 nm cannot undergo glomerular filtration. b) As the brush border of the proximal tubule epithelial cells is negatively charged, positively charged nanoparticles are more readily resorbed than negatively charged nanoparticles with comparable sizes. Modified reproduction with permission from Ref. [165], copyright 2008 Future Medicine.

Clearance studies with QDs showed efficient renal excretion for particles with hydrodynamic diameters less than 6 nm (4.36–5.52 nm).[166] QDs with a hydrodynamic diameter larger than 8 nm (8.65 nm) did not go through renal filtration, but instead were cleared from the circulation by the MPS.[166] Other studies indicate that Au nanoparticles with a spherical shape and a size of 2 nm were rapidly extracted, with only 3.7% accumulation in the liver. In comparison, 4.0% and 27.1% of Au nanoparticles of 6 nm in size were found in the urine and liver, respectively, whereas 0.5% and 40.5% of Au nanoparticles of 13 nm in size ended up in the urine and liver, respectively.[160b] Polyamidoamine (PAMAM) dendrimers with a near-spherical shape were also used to evaluate the threshold, and dendrimers of 5.4 nm in size demonstrated effective glomerular extraction.[165] These experimental results indicate that the size threshold for renal filtration is comparable to that of most macromolecules.

In addition to the size, the surface charge is another factor that can influence renal handling of nanoparticles. As the surface charge of a particle increases, the adsorption of serum proteins will likewise increase, resulting in a larger in vivo hydrodynamic diameter.[166] Moreover, the surface charges on the nanoparticle can interact with the charges on the glomerular capillary wall.[203] As a result, QDs with a neutral surface charge were shown to have the highest chance to pass through, while both positively and negatively charged nanoparticles adsorbed more serum proteins,[166] increasing their apparent hydrodynamic diameters and dramatically reducing their ability to go through renal filtration.[166] PEGylation has been proven effective in preventing protein adsorption, but the PEGylation process itself increases the hydrodynamic diameter, thus negatively impacting its usefulness in renal filtration.[166] For nanoparticles with a size of around 6–8 nm, the surface charge and surface chemistry are especially important, as these particles are no longer small enough for charge-independent filtration.[203]

Before draining into urine, nanoparticles may still have a chance to be resorbed from the tubular fluid. Studies have shown that some nanoparticles based on polyamine dendrimers may undergo proximal tubule resorption.[165] Further studies are still needed to evaluate the resorption of nanoparticles as the capability of nanoparticles to be resorbed is still an open question.

It is reasonable to conclude that nanoparticles with hydrodynamic diameters smaller than 8 nm can be cleared through the renal pathway when the surface chemistry and charge of the nanoparticle are optimized. Nanoparticles larger than 8 nm can also undergo renal clearance if they can be somehow broken down into fragments smaller than 6 nm after drug release.

4.2.5. Pharmacokinetics and Biodistribution

As many anticancer drugs are highly toxic, it is desirable to have the drug deployed to the tumor site only and then purged within a certain period of time to minimize the side effects. For nanoparticle-based therapeutics, the pharmacokinetics and biodistribution are the results of multiple entangling factors, including the targeting efficiency and clearance of the nanosized carriers, as well as the release profile and related properties of the loaded drug. In general, the pharmacokinetics and biodistribution of such a therapeutic agent are largely determined by the nanoparticle carrier itself up until the drug molecules are released.[205] This makes it more complicated to manipulate the pharmacokinetics and biodistribution of nanomedicine, because both the distribution of nanoparticles and the release profile of the drug need to be taken into consideration.[201] For intravenously administrated nanoparticles, their distribution in the body is largely controlled by a limited set of organs and the diseased lesion (Figure 12). To a certain extent, the pharmacokinetics and biodistribution of a nanosized therapeutic agent would be determined by the amount of nanoparticles distributed among these sites as a function of time. Although guidelines have been established for maximizing or minimizing the accumulation of nanoparticles at these sites, it remains a grand challenge to achieve the optimal conditions for the accumulation/clearance at all these sites. At the current stage of development, the majority of the administered nanoparticles end up in healthy organs and tissues, even

with the assistance of effective targeting,[201,206] and the off-target toxicity is still unacceptable for most nanoparticle-based therapeutics.[201]

Animal models can affect pharmacokinetic studies in many ways. For different kinds of laboratory animals, the primary MPS organs of nanoparticle sequestration are species dependent.[201] For different animal xenograft models, the functional pore sizes of tumor vasculature can also vary significantly.[175] As the EPR-based targeting process is highly dependent on the vascular pore size, it is important that the feature of tumor vasculature should resemble the clinical cases as much as possible.[178] In general, the pharmacokinetics and biodistribution of nanomedicine must be systematically studied case by case. It has been found that formulations that were successful in multiple xenograft models were generally found to have better therapeutic effects clinically.[205]

It is worth noting that several patient-related factors can also affect the pharmacokinetics and biodistribution, thus causing pharmacokinetic variability. For example, clinical studies show that the age, body composition, gender, and presence/absence of a tumor in the liver can all alter the pharmacokinetics of PEGylated liposomal agents.[201] A study involving PEGylated liposomal doxorubicin (Doxil) and PEGylated liposomal CKD-602 (S-CKD602) indicates that the clearance was dramatically lower in patients over 60 years old,[207] and female patients tended to show a lower clearance of drugs encapsulated in PEGylated liposomes. Population pharmacokinetic studies further suggested that patients with primary or metastatic tumors in their livers, along with refractory solid tumors, had a higher clearance for S-CKD602.[208]

4.2.6. *Biocompatibility and Biodegradation*

Biocompatibility has been a subject of extensive research ever since the first foreign material was implanted into the human body. As nanoparticles and bulk materials have different properties, the responses of the body can be drastically different even for those with the same composition, resulting in different levels of toxicity. For example, Au has been extensively used in dentistry for centuries, and it is widely known to be bioinert and biocompatible.[209] Conversely, Au nanoparticles may exhibit toxicity because of their ability to induce the generation of reactive oxygen species (ROS).[210]

According to Kohane and Langer, the biocompatibility of a material can be generally considered as "an expression of the benignity of the relation between a material and its biological environment".[211] Top issues concerning biocompatibility include acute and long-term toxicity, and also the response from the whole body and the functionality of the tissues/organs involved. In general, biologically inert nanoparticles that do not induce unacceptable toxic, immunogenic, thrombogenic, and carcinogenic responses tend to show a high level of biocompatibility.

Several factors must be kept in mind when the biocompatibility issue is evaluated: 1) The biocompatibility of nanoparticles is strongly correlated with the type and anatomical features of the surrounding tissue. Tissues with different anatomical features may react differently to the presence of the same nanoparticles.[212] Nanoparticles engineered for certain applications in specific tissues may not be suited for other applications involving other types of tissues. 2) The intrinsic properties associated with the material of a nanoparticle are not necessarily a major factor in determining the biocompatibility of the nanoparticles.[212] 3) When assessing the biocompatibility, it is necessary to gauge benefits versus risks. Hazards such as inflammation may not damage neighboring tissues and will heal over time.[212]

Among biocompatibility issues, toxicity is the most important one and rightfully garners the most attention. The toxicity of various types of nanoparticles, including those made of polymers, magnetic materials, noble metals, and semiconductors, has been summarized and reviewed elsewhere.[213] The in vivo toxicity profiles that have been investigated include skin sensitization, dermal toxicity, ocular toxicity, inhalation toxicity, oral toxicity, neurotoxicity, developmental toxicity, reproduction toxicity, and genotoxicity.[214] The intrinsic toxicity of materials, including those of the nanoparticle and its payload, the corresponding responses from the body, and any reactive species generated may all play important roles in determining the toxicity in vivo. It is worth noting that long-term studies (months to years) still need to be conducted for most nanoparticles.[160a] It is possible that some nanoparticles considered to be non- or minimally toxic may induce a higher degree of toxicity during their degradation or processing by the organism.[160a]

It is also worth pointing out that the toxicity can be caused by chemical residues left from a synthesis. Complete removal of these residues is often difficult and sometimes impossible. For instance, CTAB is widely used in the synthesis of Au nanoparticles, in particular, nanorods. Complete removal of CTAB is difficult and may lead to aggregation of the nanoparticles if completely removed. The positive charge of CTAB attached to the surface of nanoparticles can induce cytotoxicity and rapid opsonization, followed by MPS clearance.[215] For this reason, new protocols of synthesis involving reagents extracted from natural sources as stabilizers and reducing agents have been developed to produce Au and Ag nanoparticles, as well as CdSe QDs.[216] Although the results are promising, greater effort is needed for such syntheses to better control the size, shape, and uniformity of the nanoparticles.

Biodegradability is another important issue in nanomedicine. In general, it is desirable for the nanoparticles to be completely broken down and expunged from the body after the payload has been released. To this end, nanoparticles have been developed from a number of biodegradable polymers such as PLA, PGA, PLGA, PCL, poly(alkyl cyanoacrylates) (PAC), chitosan, and gelatin.[217] A large array of anticancer drugs, including 9-nitrocamptothecin, paclitaxel, cisplatin, xanthones, Rose Bengal, triptorelin, and dexamethasone, have all been encapsulated in those biodegradable nanoparticles.[59a] To demonstrate biodegradability and acquire the drug release profile while evaluating any therapeutic improvement, several types of tests have been designed and conducted both in vivo and in vitro for these systems.[213b] It was shown that these systems could indeed be broken down to

small molecules, which could then be processed by catabolic mechanisms in the body.[165] Although made of biodegradable materials, these nanoparticles still exhibited toxicity to a certain extent because of nonspecific accumulation and thus release of their toxic payloads in healthy tissues.[218]

Gold nanoparticles have been actively explored for biomedical applications, including drug delivery, imaging contrast enhancement, and cancer treatment.[219] These nanoparticles are normally considered not to be biodegradable as the nanoparticles cannot be readily digested and the resultant metal ions can be highly toxic.[220] However, Sokolov and coworkers recently demonstrated the synthesis of Au nanoparticles through the assembly of Au clusters, and such nanoparticles are potentially biodegradable in vivo.[221] The Au clusters used in this synthesis had an average diameter of 4 nm, which fulfills the requirement for rapid renal clearance. By carefully tuning the ratio of Au clusters to a polymeric stabilizer, the product with a size of 83 nm showed good biodegradability in vitro.[221]

In summary, the biocompatibility of nanoparticles depends on their structure and surface properties, and many other factors. At the current stage of development, long-term assessment (months to years) are missing for most samples. Therefore, the long-term fate and toxicity of nanoparticles are essentially unknown.[222] This is an especially important issue for nanoparticles made of non-biodegradable materials.[223] In addition, the evaluation of biocompatibility must be carried out case by case with a systematic methodology and a long-term mindset.

5. Perspectives on the Design of Nanoparticle Carriers

The first attempted development of a nanoparticle-based therapeutic can be traced back to the synthesis of a polymer–drug conjugate in the 1950s.[224] Ever since, nanoparticle carriers have been prepared and tested using a wide variety of materials, including proteins, polysaccharides, synthetic polymers, metals, and many other organic/inorganic materials. As a major requirement for the design of nanoparticle carriers for drug-delivery applications, the composition, size, shape, surface properties, biocompatibility, and degradation profile all need to be precisely engineered and optimized to achieve site-specific release of drugs at therapeutically optimal rates and dose regimes.

5.1. Natural versus Synthetic Materials

The use of natural materials is attractive because of their abundance, good biocompatibility, and the potential to be modified through chemical/biochemical reactions.[175] Naturally occurring materials offer many advantages over their synthetic counterparts. For example, the biological system can easily recognize and metabolically process natural materials through established pathways, while synthetic materials may induce toxicity, chronic inflammation, and clearance issues. However, natural materials are also plagued by a number of drawbacks, including the lack of dedicated optical/electric/magnetic properties, lot-to-lot variability, immunogenicity, inadequate biomechanical properties, and structural complexity.[225]

As a major advantage, natural materials can be readily metabolized by and cleared from a biological system through enzymatic or hydrolytic degradation.[225] While this may not be desirable for permanent and long-term implants, such as hip replacements, it is a clear advantage when timed biological resorption is desired. Natural materials can be chemically modified and cross-linked to adjust degradation rates for specific drug-delivery applications.[226] The most frequent concern about natural materials is the immunogenic response, which can rapidly occur upon introduction into the body. This response stems from the fact that the introduced materials, although similar to endogenous host extracellular matrix components, may not be identical and in fact often contain antigenic contaminants. This issue occurs most commonly among protein-derived materials and is typically less severe for polysaccharides such as chitosan.[225] This immunogenic effect can be reduced through either chemical modification or purification to remove the immunogenic components. However, the complex structures of natural materials can complicate the modification processes that are relatively simple to perform with synthetic materials. Despite this, many groups have demonstrated successful procedures for the modification (and purification) of natural materials. Another common issue arising from naturally derived materials is lot-to-lot variations in molecular structure as a result of different animal sources. The inconsistency arises not only from interspecies variations, but also at the tissue level, which can complicate processing and quantification of these materials.[225] Recently, bacterial recombinant techniques have been used to produce several natural materials, including hyaluronic acid and collagens, effectively addressing the variability and immunogenicity issues associated with these materials.

Currently, drug delivery is dominated by nanoparticles based on synthetic materials because they offer precise control over the physicochemical properties of the formulations. To deliver anticancer drugs to the tumor sites in vivo, the nanoparticles must be stable, biologically inert, and nontoxic. Concurrently, they must remain in the bloodstream for a sufficiently long period of time to reach the target site and even pass by the target site multiple times. The nanoparticles can induce the formation of a corona of serum proteins around the surface, so highly charged nanoparticles are phagocytosed by the MPS more quickly than neutral particles.[195,223b,227] Using synthetic materials, the surface charges and hydrophobicity of nanoparticles can be conveniently adjusted and optimized to increase their circulation half-life. In addition, their surface functionality can be readily engineered to maximize their affinity toward the targeted receptors.

5.2. Size and Shape

The size and shape, as well as the uniformity, are two important parameters of a drug-delivery system based on

nanoparticles, as they determine the in vivo distribution, toxicity, and targeting ability.[228] Additionally, they can influence drug loading, drug release, and in vitro and in vivo stability. For example, smaller particles have a greater risk of aggregation during storage and incubation in vitro, but typically have a longer circulation half-life in vivo. The degradation of polymer nanoparticles can be strongly affected by their size as a result of water availability and removal of degradation products.

Many studies have demonstrated that nanoparticles have a number of advantages over their micrometer-sized counterparts with sizes in the range of 0.1–100 μm for drug-delivery applications.[229] Generally, nanoparticles have relatively higher intracellular uptake and broader availability to a range of biological targets owing to their small sizes and increased mobility. For example, Amidon and co-workers found that nanoparticles of 100 nm in size had a 2.5 times greater uptake than microparticles of 1 μm in size and 6 times greater uptake than microparticles of 10 μm in size for Caco-2 human epithelial colorectal adenocarcinoma cells.[230] In a subsequent study, nanoparticles were found to penetrate through the submucosal layers in a rat in situ intestinal loop model, whereas micrometer-sized particles were largely localized in the epithelial lining.[231] It was also reported that nanoparticles can cross the blood–brain barrier (BBB) by passing the openings of tight junctions treated with a hyperosmotic mannitol solution. Nanoparticles coated with Tween 80 have also been shown to cross the BBB.[232] As such, nanoparticles may provide sustained delivery of therapeutic agents for difficult-to-treat diseases such as brain tumors.[233] In fact, some cell lines were found to only take up nanoparticles, while rejecting larger ones.[234]

The shape of nanoparticles is of equal importance as their size in drug delivery. While spherical nanoparticles are good candidates for drug delivery, anisotropic structures can sometimes provide higher efficiencies because of their larger ratios of surface area to volume, as illustrated in Figure 1. The anisotropy in structure may allow the carrier to take a more favorable configuration for binding with the cell, although the sharp edges and corners can potentially induce injuries to blood vessels.[235] The mechanisms by which nanoparticles cross the cell membranes has been a subject of extensive research in recent years, because an understanding and control of cellular uptake is important for the development of more effective nanomedicine.[235,236] For a more detailed discussion, please refer to Section 4.1.

5.3. Surface Properties

In addition to both size and shape, the surface characteristics of nanoparticles represent another critical parameter in determining their drug-loading efficiency and release profile, circulation half-life, tumor targeting, and clearance from the body. Ideally, the nanoparticles should have a hydrophilic surface to resist the adsorption of plasma proteins and thus escape the uptake by macrophages.[237] This can be achieved in two ways: coating the surface of nanoparticles with a hydrophilic polymer such as PEG, or directly fabricating nanoparticles from block copolymers containing both hydrophilic and hydrophobic segments.[238] Studies by Elsabahy and Wooley suggested that the surface chemistry of nanoparticles can greatly impact their toxicity, immunogenicity, and biodistribution; excess positive charges tend to result in rapid opsonization and clearance.[239]

5.3.1. Surface Charges

The zeta potential of a nanoparticle is commonly used to characterize its surface charge.[240] This variable reflects the electrostatic potential of a particle and is influenced by the composition of the particle as well as the medium in which the nanoparticle is suspended. Nanoparticles with a zeta potential above 30 mV (either positive or negative) have been shown to be stable in suspensions, as repulsion forces originating from the surface charges can prevent the particles from aggregation. Additionally, the inner surface of blood vessels and the surfaces of cells contain various types of negatively charged species, which repel negatively charged nanoparticles. When the surface charge of nanoparticles becomes higher (either positive or negative), they will become more easily scavenged by macrophages, resulting in greater clearance by the MPS. Therefore, control over the surface charge can help minimize the nonspecific interactions between nanoparticles and the MPS, preventing the loss of nanoparticles in undesired locations.[241] Complete exclusion of nonspecific interactions, however, is currently unattainable.

5.3.2. PEGylation

In order to increase the tumor targeting efficiency, it is necessary to prolong the circulation of nanoparticles in the bloodstream by minimizing opsonization. The most commonly used approach to achieve this goal is to coat the surface of nanoparticles with a hydrophilic brush made of PEG chains.[228]

Studies have shown that the conformation of PEG on the nanoparticle's surface is of the utmost importance in repelling opsonins. While PEG coatings with a brush-like configuration reduce phagocytosis and complement activation, those in a mushroom-like configuration are potent complement activators to induce phagocytosis.[237] Since the initial use of PEG in extending the circulation half-life of a protein,[242] PEGylation has been widely adopted to protect nanoparticles such as liposomes,[243] polymer nanoparticles,[63a] and micelles[244] from premature clearance during circulation. The PEG chains form a hydrated shell that allows the nanoparticle to evade opsonization and subsequent phagocytosis.[245] However, this protective shell can interfere with the interactions between a nanoparticle and the target cell.[246] For example, PEGylated liposomal doxorubicin showed a prolonged plasma half-life, which is believed to correlate with better therapeutic efficacy. However, the formulation resulted in lower tumor accumulation than the same liposomes with no PEG coating, indicating a counterproductive effect of PEGylation.[247] A recent publication also noted that PEGylated, multifunctional envelope-type nanodevices were less effective in delivering genes to liver cells in vivo than those with no

PEG coating because of the ineffective uptake of the PEGylated nanodevice by hepatocytes.[248] Using a computational model, Bunker and co-workers further demonstrated that some targeting moieties could lose their functionality because of steric hindrance from the PEG layer.[249] The increased stability of nanoparticles by PEGylation can also hinder the endosomal escape, a critical step for effective intracellular delivery of gene drugs and other therapeutics.[250] These obstacles have prompted a search for new strategies and compounds to disguise the nanoparticles. Examples of recent efforts include the use of different polymers of synthetic or natural origin, biomimetic coatings, and the conditional removal of PEG layers.[251]

5.3.3. Polysaccharides

As a major class of natural polymers, polysaccharides have been widely used in drug delivery and tissue engineering because of their good biocompatibility, availability, and easy modification.[252] As a result of their capability to avoid the complement system and opsonization, some polysaccharides, such as dextran and heparin, have also been recognized as stealth-coating materials.[253] Some studies have shown that the polysaccharides, such as chitosan and hyaluronic acid, even display certain ligand activities of their own. Nanoparticles coated with these polysaccharides show more efficient cellular uptake than other nanoparticles because of specific interactions with various receptors on the surface of target cells.[254] Therefore, polysaccharides have gained increasing interest in the development of nanomedicine as an effective surface modification strategy.

Nanoparticles incorporating polysaccharides can be prepared using many different methods, which have been extensively reviewed.[253a,255] Polysaccharides can be applied as surface coatings on nanoparticles through electrostatic interactions or directly incorporated into the nanoparticles during synthesis. Alternatively, hydrophilic polysaccharides can be grafted to hydrophobic molecules, such as cholesterol, and then used to form nanoparticles through self-assembly, which can also encapsulate hydrophobic drugs in the core. Furthermore, nanoparticles can be prepared through conjugation of polysaccharides to synthetic polymers. For example, grafting polysaccharides to the side chains of a linear hydrophobic polymer can generate a branched copolymer, and conjugation of the polysaccharide terminus to a linear hydrophobic polymer can generate a linear diblock copolymer.

Several hurdles must be surpassed before polysaccharides can be effectively applied as drug carriers. First, most polysaccharides are of natural origin, and there is a high degree of variability with respect to the molecular weight and structure depending on the source. These properties critically determine the biological activities of polysaccharides, and alternative methods need to be established to produce polysaccharides with consistent properties. Second, the desired effect of the polysaccharides may be counteracted by the biologically active contaminants of polysaccharide, such as endotoxins and pathogens. More effective methods for purifying polysaccharides are urgently needed.[225] Third, the exact mechanisms of the biological actions of most polysaccharides are still unclear. A subtle difference in molecular weight, the arrangement of monomers, and the degree of branching can all result in significant differences in biological activities. A complete understanding of the mechanisms of biological effects is the prerequisite for successful introduction of polysaccharides into nanomedicine applications.

5.3.4. Conjugation with Targeting Ligands

Many techniques and tools are currently available to armor nanoparticles for active targeting of cancerous cells. Traditionally, monoclonal antibodies have been used to target epitopes on the surface of cells, but the extensive screening of peptide and aptamer libraries has greatly expanded the repertoire of ligands available for targeted delivery.[6] The currently used targeting ligands include antibodies, antibody fragments, peptides (e.g., RGD for $\alpha_v\beta_3$ integrin), aptamers (e.g., those for prostate-specific membrane antigen and VEGF), oligosaccharides, and even small molecules (folate and SV-119),[185c,d] as long as they can specifically recognize and bind to an overexpressed target on the cell surface. Here we only provide a brief discussion on these ligands:

1) Monoclonal antibodies (MAbs) are macromolecules widely used as targeting ligands because of their immediate availability and their high affinity and specificity for molecular targets. These ligands usually possess a molecular weight of approximately 150 kDa and exhibit high binding affinities. To date, MAbs have been conjugated to essentially all different types of nanoparticles, such as SPIONs,[255] QDs,[256] liposome,[257] and Au nanocages,[258] to give them site-specific targeting ability. However, the bulky size and redundant constant region may cause some major issues in the use of MAbs as targeting ligands because of their immunogenicity and size increase (i.e., the overall size of nanoparticles will dramatically increase). The use of antibody fragments, affibodies, and peptides may help overcome this shortcoming.

2) Single-chain variable fragments (scFv) are fusion proteins of the variable regions of the heavy and light chains of an antibody (VH and VL) connected with a short linker peptide of 10–25 amino acids. The molecular weight of an scFv is about 27 kDa. By engineering the MAbs to cut down the redundant parts of the scFv, the size and immunogenicity of the original antibody can be largely reduced.

3) Affibodies are small, stable Z-domain scaffolds consisting of 58 amino acids and derived from the IgG binding domain of staphylococcal protein A. The binding pocket is composed of 13 amino acids and is able to bind to a variety of targets, depending on the randomization of the amino acids. In contrast with IgGs, the small size (6–15 kDa) of affibodies enables penetration into tumor tissue. Affibodies possess a high receptor affinity, which mimics the active portion of the Fab region of the corresponding antibody. Their short plasma half-life makes them good candidates as tumor imaging probes, but less ideal for tumor targeting, where long circulation half-life is required.[259]

4) Peptides represent a viable targeting moiety with several advantageous characteristics, including low molecular

weight (ca. 1 kDa), tissue penetration capability, lack of immunogenicity, ease of production, and relative flexibility in chemical conjugation processes.[259] Various peptides that can recognize cancer-specific epitopes overexpressed on tumor cells and vasculature have been used as targeting moieties for drugs and drug carriers. For example, RGD peptides showed a high affinity in binding toward integrin,[260] which are typically overexpressed by the endothelium during tumor angiogenesis. By conjugating RGD to the surface of SPIONs, the nanoparticles showed superior targeting affinity and specificity.[261] One possible disadvantage is that peptides sometimes exhibit a lower binding affinity to receptors as compared to MAbs, but this can be compensated by increasing the coverage density of peptides.

5) Aptamers are short, single-stranded, synthetic nucleic acid oligomers, DNA or RNA, that can form complex three-dimensional structures with a capability to bind to surface markers with high affinity and specificity.[262] Advantages of aptamers include availability, ease of chemical synthesis, low molecular weight, and lack of immunogenicity. Many publications have reported the conjugation of aptamers to polymer nanoparticles as targeting ligands.[190b,262]

6) Endogenous ligands, such as folic acid, epidermal growth factor (EGF), and transferrin, are attractive for tumor targeting because they can bind to their respective receptors with low immunogenicity and high affinity. Several protocols have been reported to conjugate folic acid,[263] EGF,[264] and transferrin[265] to various types of nanoparticles.

In summary, the choice of a targeting ligand revolves around numerous considerations, including availability, easiness of production, diversity, affinity, protocols for conjugation, immunogenicity, and cost. All of these parameters should be carefully thought over when designing nanoparticles with a maximum targeting capacity while minimizing the cost.

5.4. Drug Loading

Theoretically, a successful drug-delivery system based on nanoparticles should have a high drug-loading capacity to minimize the quantity of materials needed for administration. Loading of drug molecules into the nanoparticles can be achieved in two different ways: 1) incorporation at the time of nanoparticle formation, and 2) absorption (as well as adsorption) of the drug after the formation of nanoparticles by incubating them with a highly concentrated drug solution.

The efficiency of drug loading and entrapment in a nanoparticle is determined by the properties of both the drug molecules and the carrier material. The properties of the material include its molecular weight, polymer composition, drug–polymer interaction, and the functional groups (e.g., carboxy or ester) at both ends of each polymer chain.[266] A macromolecule or protein has the greatest loading efficiency when the drug loading is performed at or near its isoelectric point, which gives it the minimum solubility and maximum absorption. For small molecules, the use of electrostatic interactions between the drug and matrix material is an effective way to increase the drug-loading efficiency.[267]

Many recent studies have examined different techniques for the fabrication of polymer nanoparticles, including polyelectrolyte complex formation, double emulsion and solvent evaporation, and emulsion polymerization.[268] An oppositely charged polymer can be used to entrap drugs in the polymeric matrix of a nanoparticle, which then releases the drug through a combination of drug diffusion and polymer degradation. The double emulsion and solvent evaporation techniques involve dissolution of the polymer and drug in an organic solvent, followed by emulsification in an aqueous solution. The organic solvent diffuses from the polymer phase to the aqueous phase, and evaporates from the aqueous phase, leaving behind drug-loaded polymer nanoparticles. The drawback of this method lies in the poor uniformity of the nanoparticles that are produced. By contrast, the emulsion polymerization approach is able to generate uniform, nanosized particles based on the polymerization of monomers in emulsified droplets. However, only a few kinds of materials can be used for the fabrication of nanoparticles using this method.

6. Case Studies

Since the concept of nanoparticle therapeutics was conceived in 1955, many different types of carrier systems have been demonstrated or developed. While most of them are still limited to benchtop investigations, some of them have either entered into the market[269] or are currently undergoing different phases of clinical trials (Table 1). In this section, we use a set of selected examples to highlight a number of such carriers, including those based on protein–drug conjugates, liposomes, dendrimers, hydrogels, as well as nanoparticles made of biodegradable polymers, phase-change materials, and various inorganic materials.

6.1. Protein–Drug Conjugates

This class of drug-delivery system is based on the direct conjugation of drug molecules to proteins for targeted drug delivery. When an antibody is used, the system is also known as antibody–drug conjugate (ADC).[270] The linker between the protein and the drug is often biodegradable, capable of setting both parts free upon appropriate stimulation. The simplicity of this system lends both pros and cons to itself. The biggest advantage arises from the small size (ca. 10 nm) of such conjugates, which gives them relatively long circulation half-lives,[271] and makes their extravasation into tumor sites much easier compared to nanoparticles with larger sizes, even for conjugates lacking ligands for active targeting. As for the shortcomings, not all drugs can be readily conjugated to proteins, as the structural sensitivity of certain drugs may exclude them from any chemical modifications. In addition, the stability of drug–protein linkers can be a matter of concern as the linkers tend to be rapidly degraded by proteases and redox-altering agents during plasma circulation.[271,272] It is generally believed that protease-cleavable linkers are more stable than disulfides or other linkers,

although the stability of all of them can be tailored.[271,273] Furthermore, the ability to induce multiple functionality to the conjugates is somewhat limited. Despite these disadvantages, technological advancement in the development of new protein carriers (e.g., monoclonal antibodies), drugs, and especially linkers has greatly improved the capability of ADCs as potent therapeutics. Newer generations of products have been shown with greatly improved systemic stability, where the linkers can be held in place until the conjugates arrive at the targeted site, allowing for more precise and controllable delivery of cytotoxic agents.[270c,274]

One example of these advancements involves two drugs: tumor-necrosis-factor-related apoptosis-inducing ligand (TRAIL) and monomethyl auristatin E (MMAE, Figure 14a).[275] TRAIL can induce programmed cell death by interacting with the death receptors DR4 and DR5.[276] Although this drug has been proven effective, many tumor cells, especially breast tumors and melanomas, are resistant to TRAIL treatment.[277] Interestingly, upon binding to tumor cells, TRAIL is always internalized through endocytosis, although this internalization process is independent of TRAIL-induced apoptosis. By taking advantage of this feature, Chen and co-workers added MMAE, a synthetic antimitotic agent that can inhibit cell division by blocking the polymerization of tubulin,[278] to TRAIL through a linker consisting of valine and citrulline (vc; Figure 14b). The linker is highly stable in body fluids, but cleavable by cathepsin (a protease overexpressed in several types of human cancers)[279] upon entering tumor cells. The authors found that a conjugate of N109C (an active mutant of TRAIL) and vcMMAE had a greatly reduced IC_{50} (the half maximal inhibitory concentration of a therapeutic agent) of 62.5 nM toward TRAIL-resistant MCF-7 breast tumor cells. In comparison, the IC_{50} values for other controls all exceeded 1000 nM, including S96C–vcMMAE (where S96C is an inactive mutant of TRAIL), and the fully active N109C and TRAIL by themselves. They further tested the in vivo biodistribution of N109C–vcMMAE in nude mice bearing NCI-H460 human lung tumors. Intriguingly, unlike the control based on bovine serum albumin (BSA), which tended to accumulate in several organs, the N109C–vcMMAE that remained in the body of a mouse 96 h after injection stayed almost entirely in the tumor region (Figure 14c,d). This strong targeting capability of N109C–vcMMAE conjugates, even in the absence of an antibody or other ligand, can be attributed to their small size and the EPR effect at the tumor site, and the targeting efficiency can be further improved by conjugation with an antibody.

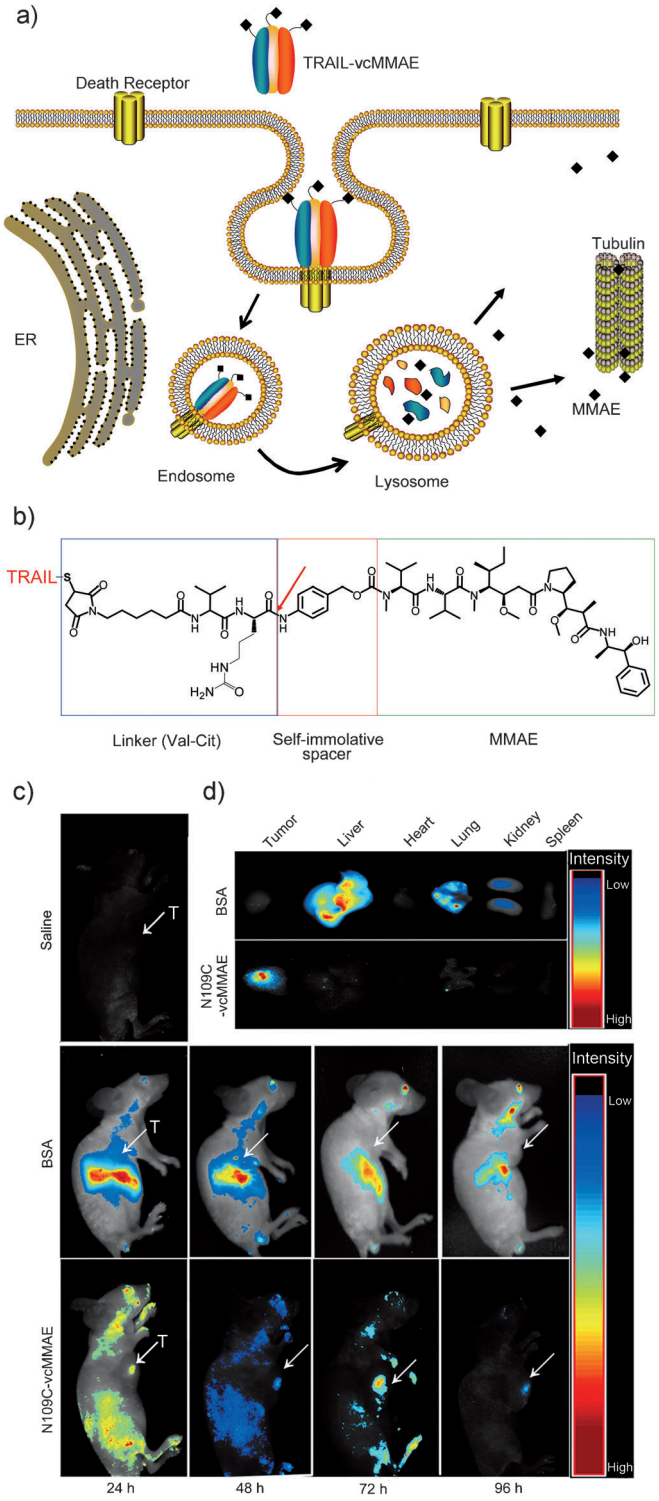

Figure 14. a) Delivery of MMAE to cytoplasm through a TRAIL–vcMMAE conjugate by targeting to the death receptor on tumor cell. b) Chemical structure of TRAIL-vcMMAE. The arrow indicates the cathepsin cleavage site of the linker. c) The Cy5-labeled N109C-vcMMAE and Cy5-BSA were injected into NCI-H460 tumor-bearing nude mice through the tail vein. Saline was used as a negative control. The mice were monitored for fluorescence every 24 h using a Maestro in vivo imaging system. d) At 96 h, the mice were sacrificed and the organs were harvested and imaged. The pseudocolor image represents the spatial distribution of Cy5-labeled N109C-vcMMAE or Cy5-BSA in the organs. Reproduced with permission from Ref. [275], copyright 2013 Wiley-VCH.

6.2. Liposomes

Liposomes refer to spherical vesicles (typically, 50–500 nm in diameter) consisting of a lipid bilayer, which are formed when lipids are emulsified in an aqueous medium.[280] The formation of liposomes is a spontaneous process enabled by the interactions between water molecules and amphiphilic lipid molecules. Upon completion, an aqueous volume becomes trapped within the core of each liposome. This

gives liposomes the capability to selectively sequester solutes for encapsulation, forming the basis for drug delivery. This drug-delivery system was first demonstrated in the 1960s,[281] and it represents one of the few systems that have been successfully translated into the clinic.[7h]

The lipids commonly used to form liposomes include phosphatidylcholine-enriched phospholipids, either natural (e.g., cholestoral and egg phosphatidylcholine) or synthetic (e.g., 1,2-dioleoyl-sn-glycero-3-phosphocholine, DOPC). The properties of a liposome, such as permeability, surface charge and hydrodynamics, are mainly determined by the phospholipid compositions of the bilayer. The initially devised and most commonly used method of drug loading is based on the involuntary encapsulation of hydrophilic drugs dissolved in the aqueous medium during a fabrication process. Other methods have also been demonstrated depending on the properties of the drugs. For example, lipophilic/amphiphilic drugs can be directly mixed with the lipids prior to liposome fabrication, leading to the trapping of the drugs within the lipid bilayers.[282] The loading of these drugs can also be achieved through the use of an exchange mechanism involving organic solvents, after fabrication of the liposomes.[283] However, unlike hydrophilic solutes, which cannot easily pass through the lipid bilayer once encapsulated, lipophilic/amphiphilic drugs may not be efficiently retained in a liposome because they can easily diffuse across the lipid bilayer. In some cases, the different pH value in the interior of liposomes can effectively protonate/deprotonate a neutral drug, making the bilayer membrane no longer permeable to the drug.[284] Using this approach, drugs such as doxorubicin have been successfully encapsulated in preformed liposomes with high loading efficiency.[285]

Liposomes can be stabilized sterically by reinforcing the bilayer with an amphiphilic, long-chain polymer containing PEG at one end, which can concurrently reduce opsonization and prolong the plasma circulation time. Polymers with proper end groups for conjugation with antibodies or ligands can also be inserted into the lipid bilayer, thus making targeted delivery possible. Despite the apparent simplicity in the functionalization, the most interesting feature of liposomes, which differentiates them from other nanoparticle-based drug-delivery systems, is their mechanism of intracellular delivery. As the bilayers of liposomes closely mimic those of cells, they can be directly fused with the plasma membrane. If they are internalized by cells through endocytosis, the lipid bilayer will be disrupted because of the acidic environment of certain intracellular compartments (e.g., endosomes and lysosomes), or the bilayer can be fused with the membranes of intracellular compartments. The fusion process may not occur if the liposomes are functionalized with certain compounds to eliminate a direct contact between the bilayer of a liposome and that of a cell, prompting the need for additional endosomal escape mechanisms.

In a recent study, Ping and co-workers devised a novel type of multistage pH-responsive liposomes (known as $HHG2C_{18}$-L) for anticancer drug delivery.[286] The key component was 1,5-dioctadecyl-L-glutamyl 2-histidyl-hexahydrobenzoic acid ($HHG2C_{18}$), a zwitterionic oligopeptide lipid. The synthetic lipid $HHG2C_{18}$ can be mixed with soy phosphatidylcholine (SPC) and cholesterol to generate the smart liposomes. Under physiological pH values (7.2–8.0), $HHG2C_{18}$-L had a strongly negative surface charge (−22.8 mV), which reduced opsonization and prolonged circulation time. Upon entering the tumor where the pH value was slightly reduced to about 6.5, the zeta potential changed sharply to +6.3 mV owing to the presence of hexahydrobenzoic acid (HBA). Interestingly, a second-stage pH response could occur when the liposomes entered endosomes and/or lysosomes (pH value in endolysosomes: 5.5–4.5) after endocytosis. This endocytosis process led to two outcomes: 1) The imidazole group of histidine in $HHG2C_{18}$

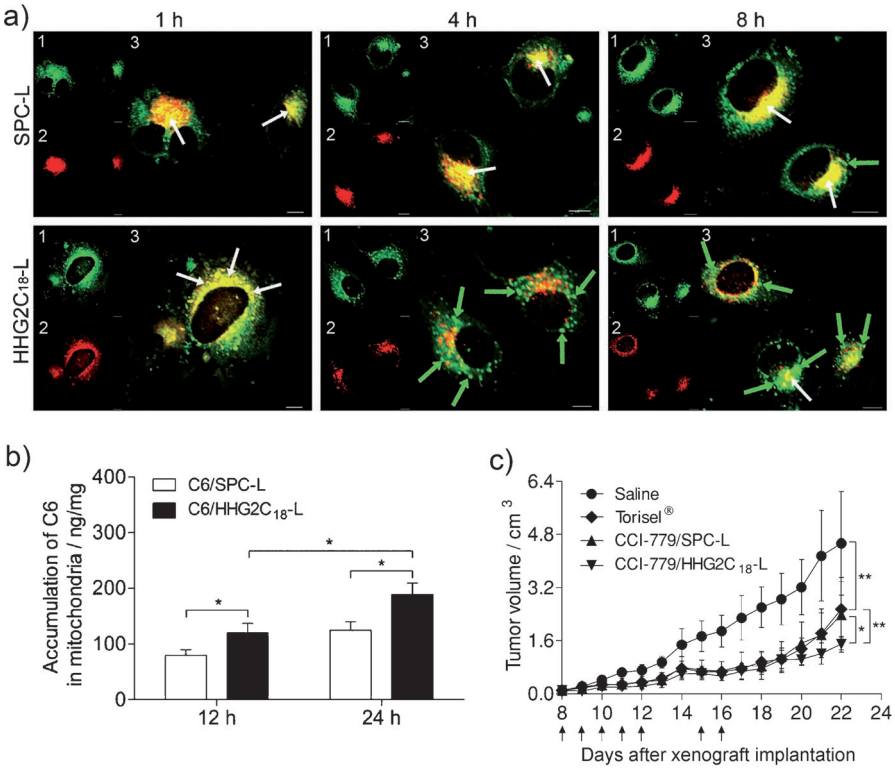

Figure 15. a) Intracellular delivery of the smart liposomes ($HHG2C_{18}$-L) encapsulating coumarin 6 (C6, a green fluorescent dye) in A498 cells at different time points observed by confocal microscopy. The late endosomes and lysosomes were stained by LysoTracker Red. b) C6 content in mitochondria isolated from A498 cells incubated with C6/$HHG2C_{18}$-L and C6/SPC-L for 12 h and 24 h. $*P<0.05$. c) Antitumor efficacy against Renca xenograft tumor after intravenous administration of different formulations of CCI-779 (10 mg kg^{-1}). $*P<0.05$, $**P<0.01$. Reproduced with permission from Ref. [286], copyright 2012 Wiley-VCH.

facilitated proton influx to endolysosomes, resulting in an even more positive surface charge (+15–25 mV) and endolysosomal bursting, and 2) HBA was cleaved in the environment with the low pH value, preventing charge reversion from positive to negative when HHG2C_{18}-L escaped from the endolysosomes to the cytoplasm. In addition, HHG2C_{18}-L has the ability to target mitochondria through electrostatic interactions because it carries a strong, positive surface charge.

The ability of HHG2C_{18}-L to respond in multiple stages was subsequently verified experimentally (Figure 15a). The uptake amount of HHG2C_{18}-L by A498 human renal carcinoma cells was significantly higher at pH 6.5 than at pH 7.4 (Figure 15b, while control liposomes without pH responsiveness (SPC-L) did not show any apparent differences between the two conditions. After endocytosis by the cells, HHG2C_{18}-L could quickly escaped from endolysosomes within a couple of hours, then gradually accumulated in mitochondria. In contrast, most SPC-Ls were trapped in endolysosomes, and only a small amount was accumulated in mitochondria, even at 8 h after cellular uptake. The authors then encapsulated CCI-779, an inhibitor of cell proliferation, within the cavities of HHG2C_{18}-L, and used these liposomes carrying an anticancer drug for both in vitro and in vivo testing. As expected, CCI-779/HHG2C_{18}-L showed a much higher efficiency in reducing the viability of A498 cells at pH 6.5 than at pH 7.4. When injected in vivo into rats bearing Renca (kidney renal adenocarcinoma) tumors, CCI-779/HHG2C_{18}-L also exhibited better inhibition of tumor growth in comparison with CCI-779/SPC-L, torisel (the commercial formulation of CCI-779), or a saline control (Figure 15c).

6.3. Polymer Nanoparticles

Polymer nanoparticles are probably some of the most extensively investigated carrier systems for drug delivery. Here we only discuss synthetic polymers that are hydrophobic and biodegradable (e.g., PLA, PGA, PLGA, and PCL), as well as their copolymers.[7b,59b,c,61b,287] The greatest advantage of these synthetic polymers is that their properties, such as molecular weight, hydrophobicity, biodegradability, can all be varied in a controllable fashion to allow for further functionalization. The fabrication of nanoparticles from these polymers is straightforward because a large number of techniques have been demonstrated, including emulsification, coacervation, nanoprecipitation, and electrospray.[59b,c,287a,288] This versatility makes the encapsulation of drugs relatively easy and highly efficient. Hydrophobic drugs can be directly dissolved in the solvent together with the polymer prior to nanoparticle formation,[289] and hydrophilic drugs can be encapsulated using multiple methods: 1) in the same manner as for hydrophobic drugs, but suspended in the solvent; 2) with techniques such as double emulsions to produce hollow nanoparticles with hydrophilic drugs trapped in the core;[290] and 3) being loaded onto the surface of nanoparticles after fabrication. Because polymer nanoparticles usually contain dense matrices with well-defined degradation profiles, the use of these carriers can result in a much better control over the release of drugs compared with other nanoparticle systems.[291]

As described in one report, it is possible to attain simultaneous delivery of siRNA and paclitaxel by polymer nanoparticles based on a biodegradable triblock copolymer, PEG-*b*-PCL-*b*-poly(2-aminoethylethylene phosphate) (mPEG$_{45}$-*b*-PCL$_{80}$-*b*-PPEEA$_{10}$), for synergistic tumor suppression (Figure 16a).[292] The triblock copolymer is amphiphilic and can self-assemble into nanoparticles with PCL as the hydrophobic core, PPEEA as the cationic shell, and PEG as the hydrophilic sheath. As a result, the hydrophobic anticancer drug paclitaxel can be readily encapsulated in the core during the formation of nanoparticles, and negatively charged siRNA can be entangled with the cationic PPEEA molecules in the shell. Simultaneous delivery of siRNA and paclitaxel was successfully demonstrated by a high degree of intracellular colocalization as shown by confocal microscopy (Figure 16b). When loaded with polo-like kinase 1 (Plk1)-specific siRNA (siPlk1), which knocks down the overexpression of the mitosis-related gene Plk1 in tumor cells, the nanoparticles efficiently inhibited the expression of Plk1 mRNA in MDA-MB-435s human melanocytes (this cell line was previously described as ductal carcinomas until recent genetic verification[292]) in vitro in a dose-dependent manner (Figure 16c). These "two-in-one" nanoparticles also showed improvement in antitumor effect compared with those carrying only one agent and the blank control (Figure 16d).

The ability to control the physical properties of polymer nanoparticles has provided researchers with a great capacity to probe the effects of different factors on the cellular delivery of nanoparticles. It is worth pointing out that most of the polymer nanoparticles fabricated using emulsion and related techniques are limited to the spherical shape, and often plagued by polydispersity in size. Recently, a technique known as particle replication in nonwetting templates (PRINT)[293] was introduced to fabricate polymer nanoparticles with uniform, controllable sizes, shapes, aspect ratios, and elasticity properties. All these parameters were found to affect the amount, rate, and/or pathway of cellular uptake.[294] More systematic investigations are still needed to establish a comprehensive understanding of the drug-delivery system based on polymeric nanoparticles.

6.4. Polymer–Lipid Hybrid Nanoparticles

While liposomes and polymer nanoparticles have been successfully used separately, a novel class of hybrid nanoparticles taking advantage of both systems has also been developed. These hybrid nanoparticles possess high drug-encapsulation yields, precisely controlled drug-release profiles, and excellent targeting capabilities. To this end, Farokhzad and co-workers reported a platform for fabricating sub-100 nm targeted polymer–lipid hybrid nanoparticles through a combination of self-assembly and precipitation.[295] As shown in Figure 17a,b, the nanoparticles were comprised of 1) a biodegradable, hydrophobic polymer core that can be loaded with water-insoluble drugs with sustained release

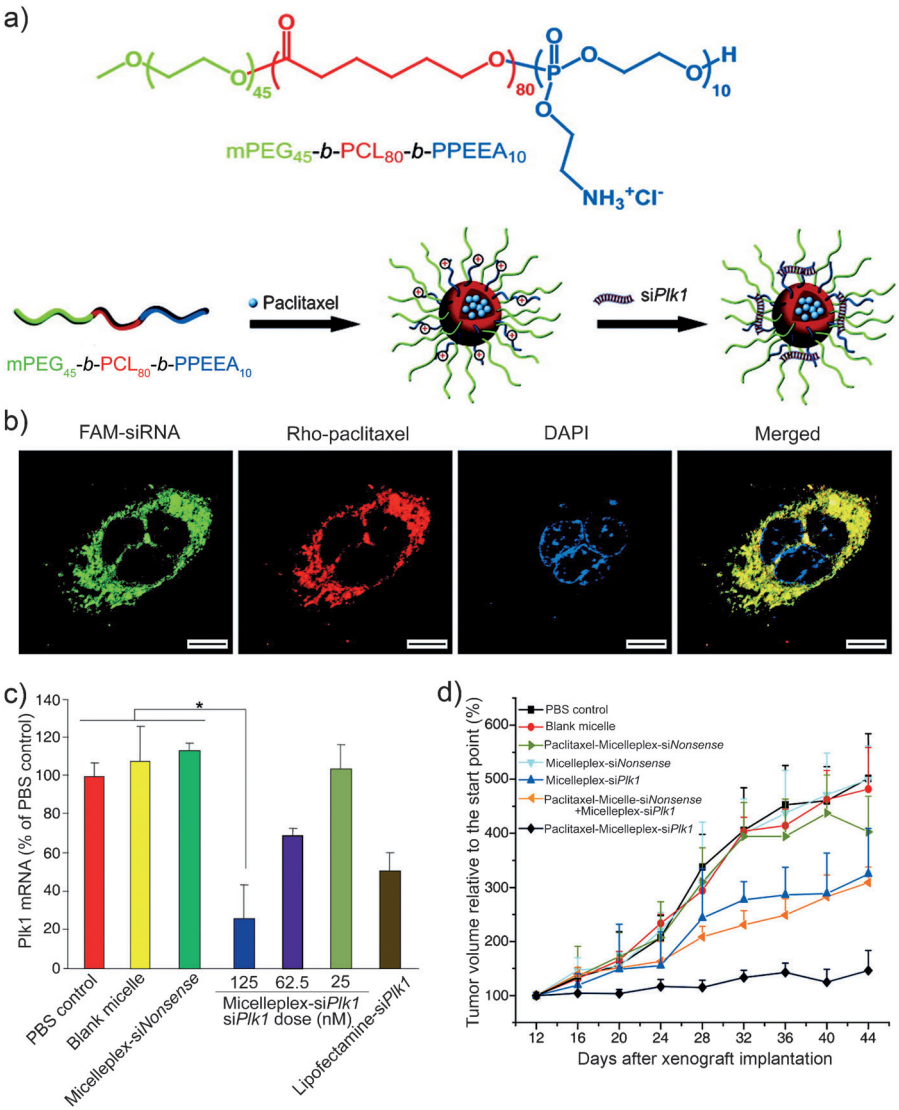

Figure 16. a) Chemical structure of $mPEG_{45}$-b-PCL_{80}-b-$PPEEA_{10}$ and schematic illustration of the formation of micellar nanoparticles and the loading with paclitaxel and siRNA. b) Confocal microscopy image of intracellular distribution of Rho-paclitaxel-micelleplex-FAM-siRNA in MDA-MB-435s cell after incubation for 2 h. c) Expression of Plk1 mRNA determined by quantitative real-time PCR. $*P < 0.007$. d) Inhibition of MDA-MB-435s xenograft tumor growth by paclitaxel-micelleplex-si*Plk1* in comparison with various other formulations. Reproduced with permission from Ref. [291], copyright 2011 American Chemical Society.

rates, 2) a hydrophilic stealth coating on the surface to improve circulation half-life, and 3) a lipid monolayer at the interface between the core and the shell to retard the escape of drugs from the polymer core as well as the influx of water, thus prolonging the total period of release. Specifically, PLGA and anti-cancer drug Dtxl were dissolved in acetonitrile, while lecithin and 1,2-distearoyl-snglycero-3-phosphoethanolamine-*N*-carboxy(PEG)2000 (DSPE-PEG) were codissolved in an aqueous solution of ethanol. By dropwise addition of the PLGA/Dtxl solution to the preheated lipid solution under gentle stirring, PLGA precipitated out as nanoparticles containing Dtxl, and the lipids self-assembled on the nanoparticles. The size and surface charge of the nanoparticles could be controlled by varying the ratio of lipid to polymer, thus the viscosity of the PLGA solution (Figure 17c). The PLGA-lipid-PEG nanoparticles showed a better sustained drug-release profile than the cases where the drugs were encapsulated in either PLGA-PEG or PLGA nanoparticles (Figure 17d), thus indicating a high efficacy of the lipid monolayer in reducing the diffusivity of the drug. These hybrid nanoparticles also showed significantly improved targeting efficiency toward prostate cancer cells that overexpress prostate-specific membrane antigen (PSMA) after the surface of the nanoparticles had been functionalized with an A10 RNA aptamer. At the current stage of development, it is still difficult to encapsulate large, hydrophilic protein drugs for this system based on hybrid nanoparticles.

6.5. Dendrimers

Dendrimers are tree-like spherical macromolecules with many branches emanating from a central core.[296] Dendrimers are synthesized in a shell-by-shell fashion starting either from initiator cores (the most commonly used divergence approach) or from the periphery (the less used convergence approach), resulting in narrow polydispersity, together with a high level of control over both size and degree of branching.[296b,297] The latter feature, the control over the degree of branching, distinguishes dendrimers from other polymers that display irregular, uncontrollable polymer networks. As a result of the unique step-by-step synthesis procedure, the choice over the central core and the repeated units is of tremendous importance because it determines the molecular weight, size, branch density, flexibility, water solubility, and versatility in the final functionalization of dendrimers.[298] Dendrimers were first reported in the 1980s,[299] but not until recently have they begun to show promise in the field of drug delivery.[296b,c] Host–guest interactions (e.g., drug loading) can take place either in the interior or on the periphery of dendrimers to result in different release profiles.

In one example, Minko and co-workers utilized poly(-propylenimine tetrahexacontaamine) dendrimer generation 5 (PPIG5) as starting materials to condense with siRNA against B-cell lymphoma (BCL, anti-apoptotic) mRNA.[300] The

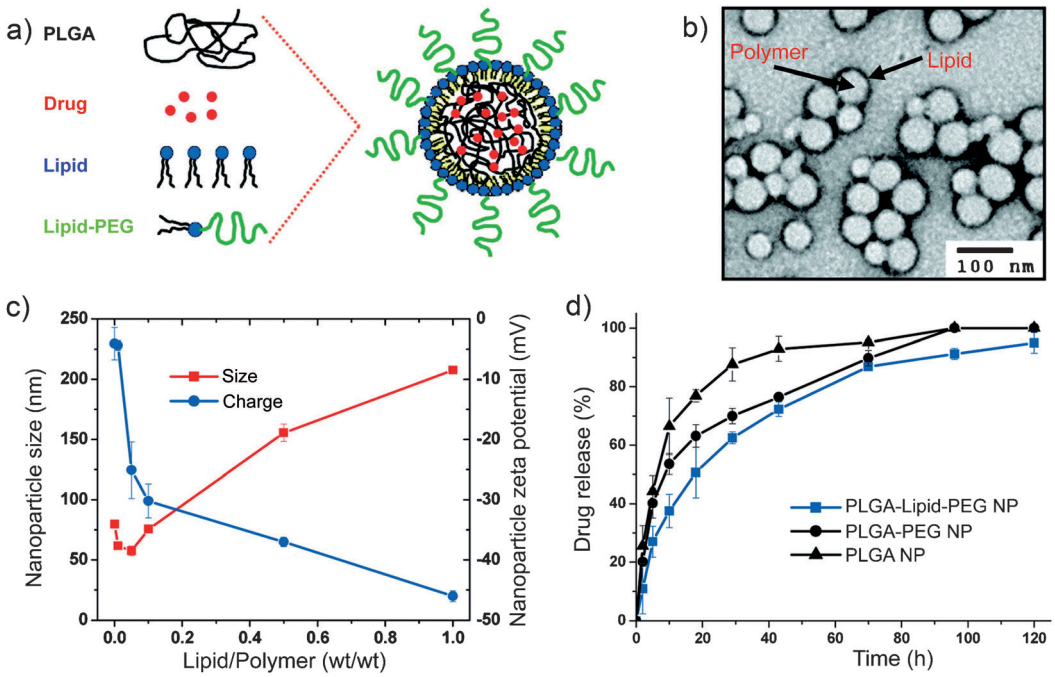

Figure 17. a) Schematic illustration of the formation of polymer–lipid hybrid nanoparticles. The nanoparticles comprised a hydrophobic poly(lactic-*co*-glycolic acid) (PLGA) core, a hydrophilic poly(ethylene glycol) (PEG) shell, and a lipid (lecithin) monolayer at the interface of the hydrophobic core and the hydrophilic shell. b) Transmission electron microscopy (TEM) image of the hybrid nanoparticles. c) Drug-encapsulation yield of PLGA-lipid-PEG hybrid nanoparticles in comparison with PLGA-PEG and PLGA nanoparticles. d) Drug-release profiles for the PLGA-lipid-PEG, PLGA-PEG, and PLGA nanoparticles, respectively. Reproduced with permission from Ref. [295], copyright 2008 American Chemical Society.

PPIG5-siRNA complexes were coated with PEG for steric stabilization and then a synthetic analogue of luteinizing hormone-releasing hormone (LHRH) peptide as the targeting motif. The PEG chains were linked to the surface of the dendrimers by dimethyl-3-3′-dithiobispropionimidate-HCl (DTBP), a redox-responsive disulfide linkage, to facilitate intracellular release of siRNA once the PEG chains underwent cleavage in the presence of reducing agents such as glutathione (Figure 18a). As shown in Figure 18b, targeted LHRH-PEG-DTBP-PPIG5-siRNA showed extensive cellular uptake for LHRH-positive A2780 human ovarian carcinoma cells, but not for LHRH-negative SKOV-3 cells. Nontargeted nanoparticles did not show significant intracellular accumulation in either of the two cell types. A similar trend for silencing efficiency toward BCL mRNA was observed (Figure 18c,d). In vivo investigations further demonstrated that LHRH-PEG-DTBP-PPIG5-siRNA had a higher tumor-targeting efficiency (for both dendrimers and siRNA) and lower clearance by the liver and kidneys (for siRNA) than nontargeted nanoparticles (Figure 18e,f).

6.6. Hydrogels

Hydrogels are three-dimensional, cross-linked networks of hydrophilic (water-soluble) polymers, capable of retaining water or physiological fluids in large quantities.[301] Typical examples of naturally occurring polymers used for hydrogels include chitosan, hyaluronic acid, dextran, alginate, collagen, and gelatin; synthetic polymers include poly(2-hydroxyethyl methacrylate) (PHEMA), poly(2-hydroxypropyl methacrylate) (PHPMA), PAAm, poly(vinyl alcohol) (PVA), and poly(ethylene oxide) (PEO).[301b,c,302] These water-soluble polymers are rendered insoluble by physically or chemically induced cross-linking. For instance, while chitosan is soluble in acidic solutions, it becomes insoluble at neutral pH values;[303] alginate can be physically cross-linked in the presence of divalent cations such as Ca^{2+} and Ba^{2+};[304] most natural biopolymers are cross-linkable by carbodiimide reactions.[305] Compared with other formulations, hydrogels possess several unique advantages. The high water content of hydrogels makes them resemble biological tissues, thus reducing interfacial tension with biological fluids and promoting biocompatibility.[301b,c] The porosity of a hydrogel can be tuned by controlling the density of cross-linking in the gel matrix, which strongly affects drug loading and subsequent release rates. Although most synthetic hydrogels are not biodegradable in their original compositions, enzymatic, hydrolytic, and stimuli-responsive moieties can be incorporated into the networks to render them degradable under appropriate conditions.[306] Hydrogels can be prepared as nanoparticles, and are accordingly termed nanogels.[307]

Wang and co-workers have developed charge-converting nanogels that could be activated for drug-delivery applications by the acidity in tumors.[308] They first fabricated the parent nanogel based on poly(2-aminoethyl methacrylate hydrochloride) (PAMA) with PEG-diacrylate (PEGDA) as a cross-linker. The nanogel exhibited a uniform size of 100 nm in water and a positive zeta potential of + 30 mV. The positive charges on the surface of nanogels were advantageous for cell uptake because they interacted strongly with the negatively charged cell membranes. However, such charges also induce strong interactions with serum proteins, causing them to aggregate and become rapidly cleared from circulation. To solve this issue, the authors devised a similar method to the example we showed for liposomes: they added a layer of 2,3-

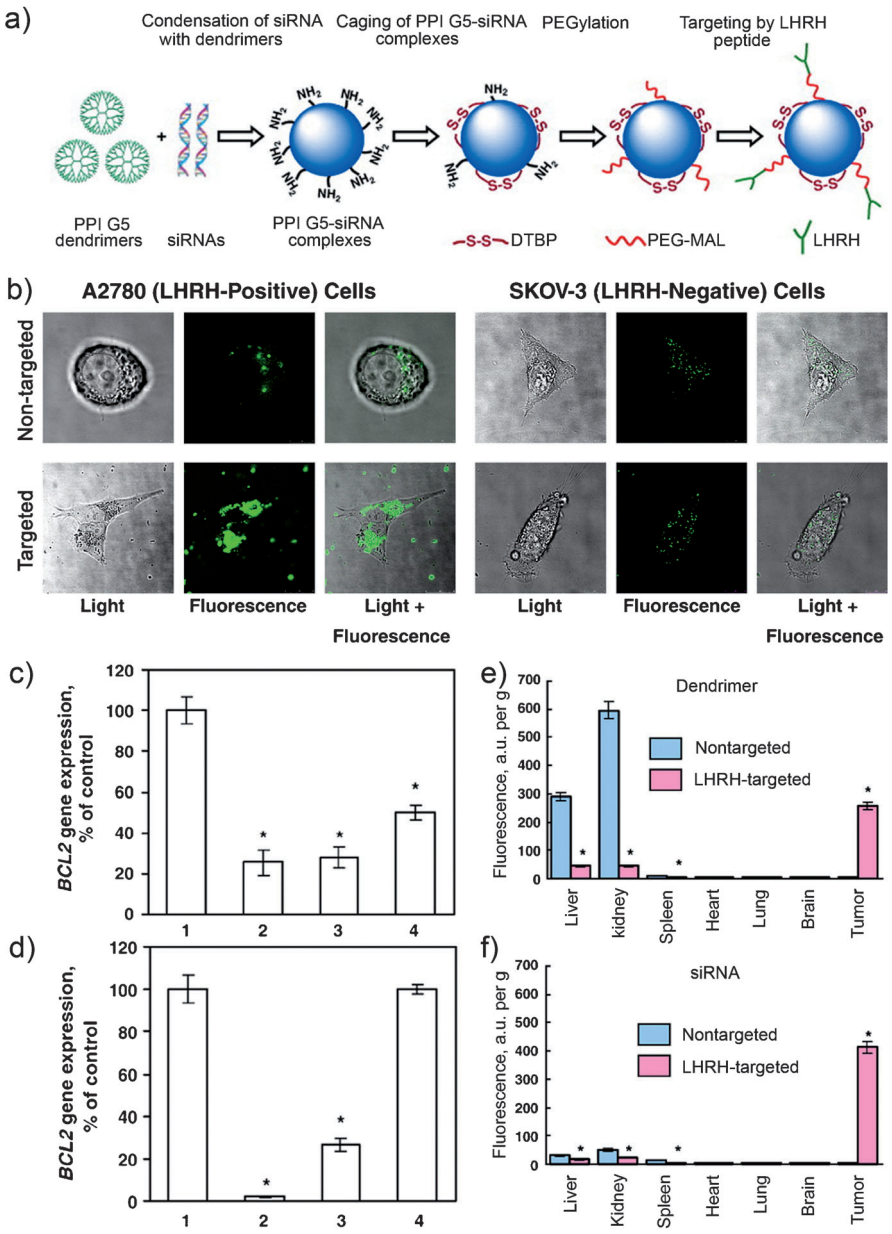

Figure 18. a) Preparation of stable, tumor-targeted nanoparticles for siRNA delivery. b) Confocal microscopy images of LHRH receptor-positive A2780 and LHRH receptor-negative SKOV-3 human ovarian cancer cells incubated with fluorescent labeled, nontargeted PEG-DTBP-PPI G5-siRNA-6-FAM Green particles and targeted LHRH-PEG-DTBP-PPI G5-siRNA-6-FAM Green particles. c,d) Suppression of BCL2 mRNA by different nanoparticles containing BCL2 targeted siRNA. c) 1. control (fresh media), 2. PPI G5-siRNA nanoparticles, 3. PEG-DTBP-PPI G5-siRNA nanoparticles, and 4. PEG-DTBP-siRNA-PPI G5 nanoparticles incubated with LHRH-positive A549 cancer cells. d) 1. control (fresh media), A549 cancer cells; 2.–4. targeted siRNA nanoparticles incubated with LHRH-positive 2. A2780 and 3. A549 cancer cells, and LHRH-negative 4. SKOV-3 cancer cells. $*P<0.05$. e,f) Average concentration per gram of organ weight of labeled e) dendrimers or f) siRNA. $*P<0.05$. Reproduced with permission from Ref. [300], copyright 2009 Elsevier.

dimethylmaleic anhydride (DMMA) to the surface of the nanogels, which changed the zeta potential of the gels to −17 mV. After incubation in an acidic environment (pH 6.8), the DMMA groups were gradually cleaved to convert the surface charge of the PAMA nanogels from negative to positive (Figure 19a). When the PAMA nanogels were coated with succinic anhydride (SA), they were not able to undergo effective charge conversion and the zeta potential remained negative. The charge-converting PSMA-DMMA nanogels elicited more accumulation in tumor cells (MDA-MB-435s) in vitro at pH 6.8 than at pH 7.4 (Figure 19b,c). When loaded with doxorubicin, PAMA-DMMA nanogels also caused higher mortality for MDA-MA-435s cells in vitro in a pH-dependent manner compared with the PAMA-SA nanogels (Figure 19d).

6.7. Phase-Change Materials

Stimuli-responsiveness is a promising strategy for realizing on-demand release of drugs or bioactive molecules. Among various types of stimuli, temperature variation has often been employed to initiate drug release because local body temperatures can vary in response to ambient conditions and in some cases, diseases.[309] One of the best-known systems is based on poly(N-isopropylacrylamide) (pNIPAAm) and its derivatives.[310] For nanoparticles (or bulk gels) comprising of cross-linked PNIPAAm chains, they shrink upon elevation of the temperature, forcing the encapsulated drugs to enter the surrounding medium. There are two intrinsic shortcomings associated with a pNIPAAm-based delivery system: noticeable cytotoxicity[310] and the existence in an "on" state because of their inability to completely inhibit drug diffusion.

To better use temperature variation as a stimulus for triggering drug release, Xia and co-workers introduced phase-change materials (PCMs), which are capable of undergoing reversible solid–liquid phase transitions in response to changes in temperature.[311] In the solid state, PCMs can effectively prevent any leakage of encapsulated drugs at temperatures below their melting points. However, when heated beyond their melting points, they exhibit a rapid phase change to the liquid state, thus releasing the payload. For PCMs based on fatty alcohols and fatty acids, they are particularly well-suited for drug-delivery applications in vivo because of their excellent biocompatibility. Notable examples include 1-tetradecanol (melting point: 38–39 °C), tridecanoic acid (41–42 °C), and

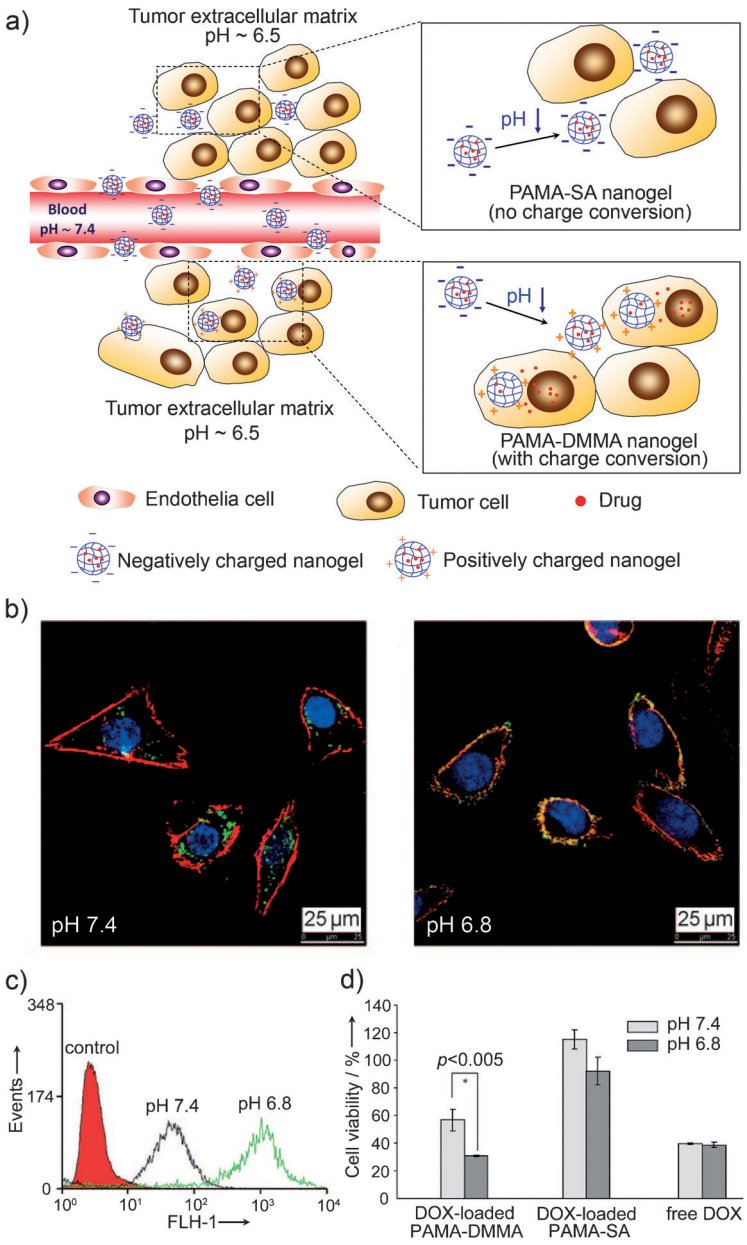

Figure 19. a) Comparison of drug release from pH-responsive PAMA-DMMA nanogels and PAMA-SA nanogels nonsensitive to pH change. b) Confocal microscopy images of MDA-MB-435s cells after incubation with PAMA-DMMA nanogels at pH 6.8 (left) and pH 7.4 (right) for 2 h. PAMA-DMMA was labeled with fluorescein isothiocyanate (FITC; green); the F-actin and nuclei of the cells were stained with rhodamine phalloidin (red) and 4′,6-diamidino-2-phenylindole (DAPI; blue), respectively. c) Comparison of cellular uptake of FITC-labeled PAMA-DMMA at pH 7.4 and 6.8 by flow cytometry. d) Cell viability of MDA-MB-435 s cells after incubation with doxorubicin (DOX)-loaded PAMA-DMMA, DOX-loaded PAMA-SA, and free DOX at the same DOX concentration of 16 mg mL^{-1}. *$P < 0.005$. Reproduced with permission from Ref. [308a], copyright 2010 Wiley-VCH.

dodecanoic acid (43–46 °C). In practice, the melting points of PCMs can be precisely tuned in the range of 38–46 °C by using binary or tertiary mixtures of these compounds at appropriate ratios.

As illustrated in Figure 20a, a model drug, fluorescein isothiocyanate (FITC)-dextran, was loaded into gelatin microbeads, which were then encapsulated in a 1-tetradecanol block (or beads). The block was then attached to the bottom of a container and immersed in phosphate-buffered saline (PBS). Upon increasing the temperature, the block started to melt and released gelatin beads within 60 s. The PCM completely disappeared within 120 s, releasing all of the gelatin microbeads. The release of FITC-dextran ensued, as continuous heating resulted in accelerated dissolution of gelatin microbeads. The rate of FITC-dextran release could be modulated by judicially selecting the materials used for PCM and microbeads. As shown in Figure 20b, gelatin microbeads could be used to achieve a fast rate of drug release as a result of the temperature responsiveness and hydrophilic nature of gelatin. In contrast, the release of FITC-dextran encapsulated in chitosan microbeads showed a more sustained profile because of the insolubility of chitosan in an aqueous medium at neutral pH values. When PLGA microbeads were used, the release rate was further reduced as a result of the strong hydrophobicity of the polymer. It was also demonstrated that dual temperature-regulated drug release could be achieved by incorporating an additional block of dodecanoic acid, which has a higher melting point than 1-tetradecanol. The release of FITC-dextran from gelatin microbeads within the diblock showed a stepwise profile, dependent on the specific temperature the block was exposed to (Figure 20c). It should be pointed out that although the examples given here are on the microscale, PCMs can be potentially scaled down to the nanoscale suited for various drug-delivery applications.[311]

6.8. Inorganic Nanoparticles

Inorganic nanoparticles comprise a very important category of drug-delivery systems because of their rich variety, precision in size/shape control, excellent physicochemical properties, and multifunctionality, although their inability to degrade has somewhat limited their scope of application. The most widely used inorganic nanoparticles include MSNs, carbon-based nanostructures (fullerenes, carbon nanotubes or CNTs, and graphene), noble-metal (typically Au) nanostructures, porous silicon, and hydroxyapatite. Here we only focus on the first three types of materials.

MSNs were developed in early 1990s[312] and have since become some of the most successful inorganic systems for drug delivery. MSNs are silica nanoparticles with sizes in the range of approximately 50–300 nm, containing hundreds of empty channels (i.e., mesopores) in a honeycomb-like arrangement. These nanoparticles possess a high surface area and pore volume, a stable mesostructure, a tunable pore diameter (ca. 2–10 nm), and easily functionalizable surfaces (including the channels).

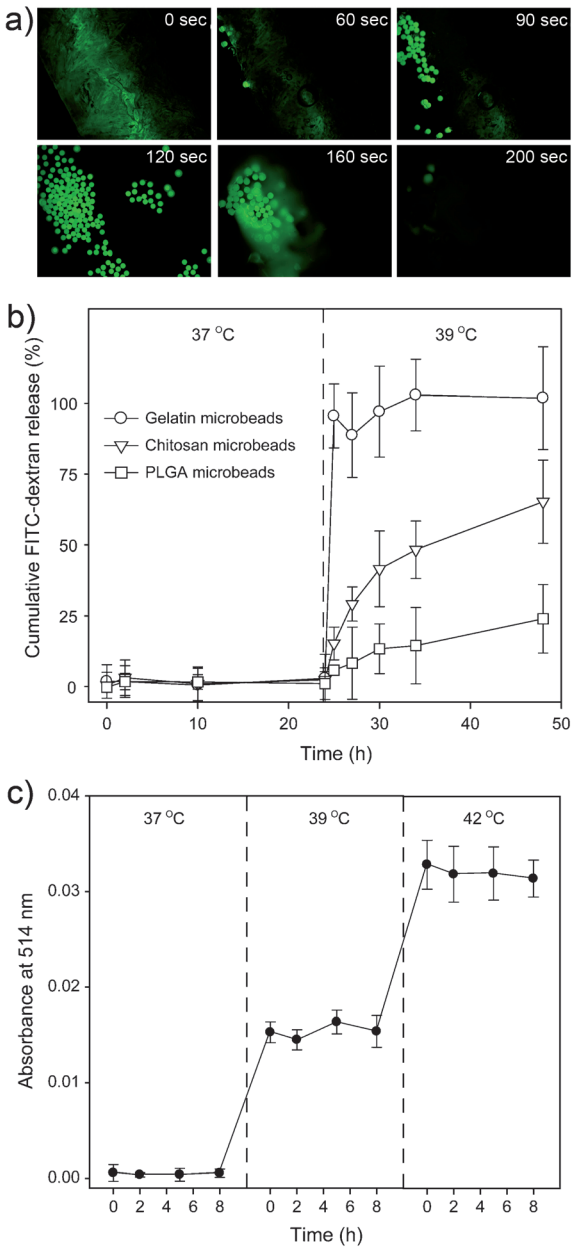

Figure 20. a) Time-lapse fluorescence micrographs showing the release of FITC-dextran from gelatin microbeads encapsulated in a 1-tetradecanol block. The temperature was gradually increased by adding warm water (60 °C) under gentle stirring. b) Release profiles at 37 and 39 °C for FITC-dextran from gelatin, chitosan, and PLGA microbeads encapsulated in the 1-tetradecanol blocks. c) Release profiles at 37, 39, and 42 °C for FITC-dextran from gelatin microbeads encapsulated in two blocks made of 1-tetradecanol and dodecanoic acid, respectively. Reproduced with permission from Ref. [309a], copyright 2010 Wiley-VCH.

Additionally, MSNs exhibit good biocompatibility and little immunogenicity.[313] MSNs have been successfully utilized in applications to deliver various theranostic agents, such as drugs, diagnostic probes, enzymes, and oligonucleotides.[313a,b]

CNTs have been used to deliver various types of therapeutic agents, including drugs,[314] peptides/proteins,[315] plasmid DNA,[315b,316] and RNA, into target cells through endocytosis.[317] The extremely high specific surface areas of CNTs allow for efficient drug loading. CNTs also possess excellent optical properties:[318] the absorption of NIR light by CNTs can be used for photothermal destruction of tumor cells,[319] while their NIR photoluminescence enables direct imaging of the target cells and tissues.[320] Furthermore, CNTs present distinctive resonance-enhanced Raman signatures, unique for Raman detection with large scattering cross-sections.[321] In one demonstration, Dai and co-workers conjugated paclitaxel, a widely used anticancer drug, to branched PEG chains anchored on single-walled CNTs (SWCNTs) (Figure 21 a).[314b] When tested in vivo with mice

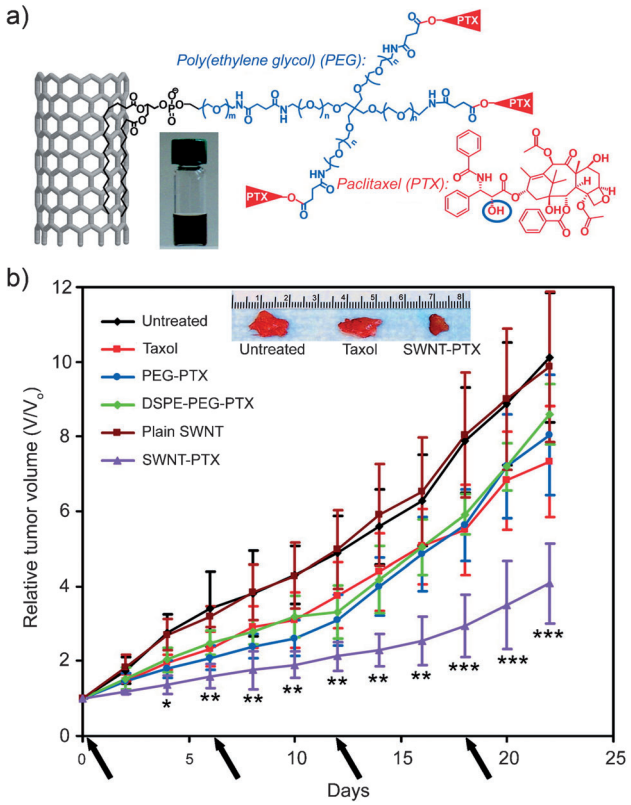

Figure 21. a) Schematic illustration of conjugation of paclitaxel (PTX) to single-walled carbon nanotubes (SWNT) functionalized by phospholipids with branched PEG chains. b) The administration of nanotube–PTX conjugates were shown to suppress tumor growth in a 4T1 breast cancer mice model. Reproduced with permission from Ref. [314b], copyright 2008 American Association for Cancer Research.

bearing 4T1 breast tumors (Figure 21 b), the SWCNTs-paclitaxel conjugate showed prolonged blood circulation, 10 times higher drug uptake by the tumor, and higher efficacy in suppressing tumor growth than free paclitaxel (clinical taxol).

Gold nanostructures offer a relatively new system for drug delivery. Very similar to CNTs, Au nanostructures also possess pronounced photothermal properties for direct cancer therapy without involving an anticancer drug, in addition to a variety of optical properties (e.g., fluorescence for Au nanoclusters,[322] multiphoton luminescence for Au nanorods and nanocages,[323] and strong optical absorptions

for all of them) for diagnostics. While drugs can be conjugated to the surface of solid Au nanostructures (e.g., nanospheres and nanorods), Au structures with hollow interiors (e.g., nanoshells[324] and nanocages)[217] allow for a much more efficient encapsulation of drugs within their cavities.

Gold nanocages represent a novel class of Au nanostructures with thin, porous walls, and hollow interiors (Figure 22a) and can be readily prepared using a galvanic replacement reaction between Ag templates and Au^{3+} in an aqueous solution. The edge length and wall thickness of Au nanocages can be separately adjusted to tune an array of optical properties, including multiphoton luminescence and optical scattering/absorption, to the desirable wavelengths. Owing to these properties, Au nanocages have been actively explored as contrast agents for imaging based on optical coherence tomography,[325] multiphoton microscopy,[323b,c,326] photoacoustic tomography,[326,327] and surface-enhanced Raman scattering.[328] Here we only focus on the applications of Au nanocages in drug delivery and cancer therapeutics. Figure 22b shows the capability to encapsulate a drug within the cavities of Au nanocages.[121c] To control the release of the drug, a thin layer of a thermoresponsive polymer based on PNIPAAm was attached to the surface of the nanocages through the Au–S linkage.[121c] Initial drug loading was accomplished by diffusion at an elevated temperature, at which the polymer chains shrank to expose the pores in the walls of the nanocages. Upon cooling to physiological temperature, the polymer chains relaxed back to the extended conformation and efficiently sealed the pores, preventing the drug molecules from escaping into the medium. When drug release was desired, a laser was applied to the nanocages to heat up the polymer coating by taking advantage of their strong photothermal effect. As a result, the pores on the walls of the nanocages were opened, allowing for faster diffusion of the encapsulated drugs (Figure 22c). It is worth noting that Au nanocages themselves can be directly used for cancer therapy without encapsulation of any drug. Figure 22d,e,f demonstrates the photothermal treatment effect of PEGylated Au nanocages in mice bearing U87MGwtEGFR tumors.[329] After 24 h exposure to a diode laser, at a power density of 0.7 W cm^{-2} for 10 min, the metabolic activity of the treated tumor dropped significantly (as measured by ^{18}FDG/PET imaging) in comparison to a saline control.

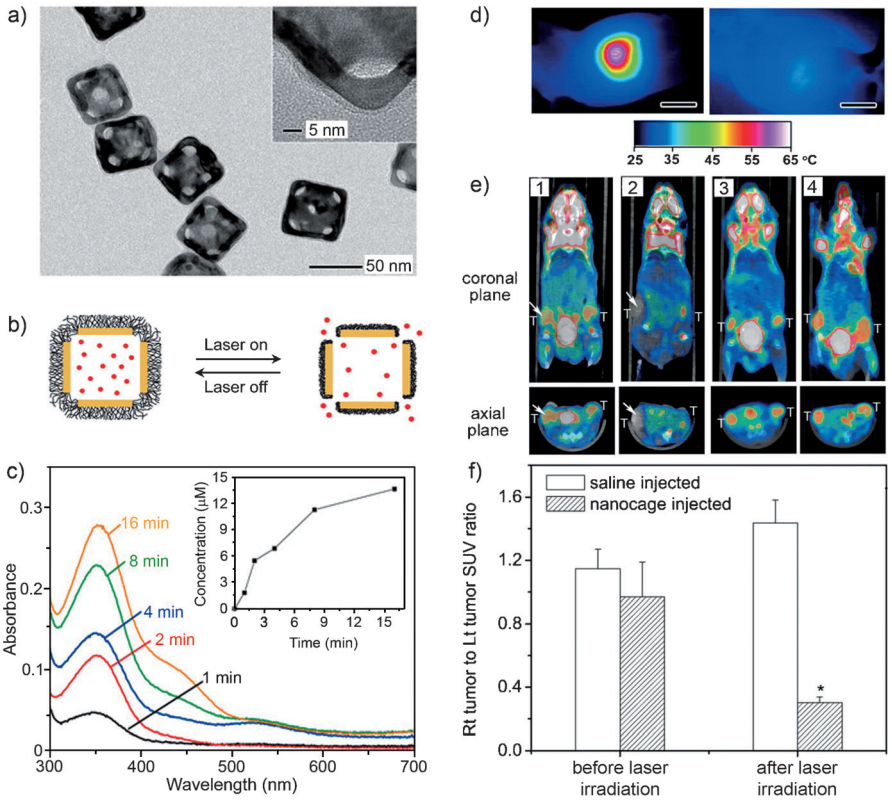

Figure 22. a) TEM images of Au nanocages whose surfaces were covered by a polymer brush of pNIPAAm-*co*-pAAm with a low critical solution temperature of 39 °C. The inset shows a magnified TEM image of the corner of such a nanocage. b) Schematic illustration showing the release of a drug controlled by light. c) Absorption spectra of alizarin-PEG released from the copolymer-covered Au nanocages when exposed to a pulsed NIR laser at a power density of 10 mW cm^{-2} for 1, 2, 4, 8, and 16 min. d) Thermographs of tumor-bearing mice after the injection of Au nanocages (left) and saline (right) for 10 min. e) ^{18}F-FDG PET/CT co-registered images of tumor-bearing mice intravenously administrated with either Au nanocages or saline, followed by exposure to a CW NIR laser: 1,2) nanocage-injected mouse before and after laser irradiation; and 3,4) saline-injected mouse before and after laser irradiation. The white arrows indicated the tumors exposed to the laser at a power density of 0.7 W cm^{-2} for 10 min. f) A plot showing the ratios of ^{18}F-FDG standardized uptake values (SUV, $*P < 0.001$) for laser-treated tumor (right tumor) and nontreated tumor (left tumor). Panels (a–c) are reproduced with permission from Ref. [121c], copyright 2009 Nature Publishing Group. Panels (d–f) are reproduced with permission from Ref. [329], copyright 2010 Wiley-VCH.

7. Summary and Outlook

The utilization of nanoparticles as drug carriers promises a significant improvement in cancer treatment. Targeted delivery can reduce the systemic side effects that patients must endure under traditional chemotherapy by ensuring that pronounced cytotoxic levels of the drugs are only present at the tumor sites. Besides targeting, nanoparticles have been designed to release their payloads in response to a variety of different stimuli, either those specific to the tumor microenvironment, such as acidic pH values and elevated secretion of certain enzymes (e.g., matrix metalloproteinases, MMPs),[330] or external ones, such as light exposure and heating, among others. Nanoparticles also offer multifunctionality, combining both diagnostic (i.e., image contrast enhancement or molecular recognition capability)

and therapeutic features, a combination known as theranostics.

In recent years, nanoparticle-based delivery systems have also started to show promise for cancer immunotherapy because they allow the (co)delivery of tumor-associated immunomodulatory agents and/or antigens to dendritic cells. The activated dendritic cells would relay the "danger" information to T cells, which then initiate the continuous and long-term elimination of targeted tumor cells.[331] This intriguing research theme is expected to be actively and intensively explored to greatly expand the versatility and potency of nanoparticles as cancer therapeutics.

Despite the impressive progress, a number of challenges still remain to be addressed on the route toward widespread applications of nanomedicine. For example, a fundamental hurdle lies in the development of methods for optimal drug loading into and release from nanoparticles. Precise engineering of the physiochemical properties of a carrier must be performed to ensure that it can stabilize the encapsulated drug molecules during inert periods (e.g., shelf storage, blood circulation), but become activated to release the drug once they have entered the tumor site. In principle, a nanoparticle-based delivery system should seamlessly integrate high drug-loading capacity, long circulation half-life, effective targeting capability, releasing programmability, stimuli responsiveness, and diagnostic features.

Oftentimes the targeting and therapeutic capabilities of nanoparticles are rather limited because of the highly heterogeneous and complex tumor microenvironment that contains a mixture of several subpopulations, including primary cancer cells, cancer stem cells, mutated variants, and tumor-associated stromal cells.[332] On the other hand, many tumors share some characteristics of normal tissues, such as secretion of MMPs and expression of folate receptors that also occur at many sites other than the tumors, albeit at lower concentrations.[333] To tackle this issue, two schemes have been devised, which involve multiple delivery/targeting mechanisms. Instead of aiming at only one target in the tumor, nanoparticles can be designed to simultaneously carry two or more drugs with programmed release profiles, or the drug itself can be modified in such a way that it targets multiple antitumor moieties at the same time.[334] Similarly, multiple antibodies specific to a tumor type (e.g., HER-2 and MMP antibodies for some breast tumors)[335] can be conjugated to the surface of nanoparticle carriers to maximize the probability and accuracy of their tumor recognition.

The in vivo clearance of nanoparticles is another critical issue for consideration during their design. While most polymer-based nanoparticles can be designed to degrade (at least to a certain degree) once they have accomplished their tasks as drug-delivery vehicles, many other systems (e.g., inorganic nanoparticles) cannot be readily degraded, and yet they are too potent to be left aside. In these cases, it is necessary to incorporate a higher level of complexity into the design of the delivery system based on its physiological relevance to both cancerous and normal tissues. One potential solution resides in multistep programmed systems in which large particles (ca. 50 nm) are assembled from a number of small particles (< 6 nm in size) through the use of degradable linkers.[336] The initially administered large particles can improve the targeting efficiency that is facilitated by the EPR effect of the tumor vasculature, but as soon as they enter the tumor regions, the linkers will be degraded by tumor-specific enzymes that disassemble them into small particles with a size of less than 6 nm, which can be subsequently eliminated from the body by kidney filtration.

Besides the efforts on nanoparticles themselves, it is of tremendous importance to develop realistic in vitro testing platforms that can effectively evaluate the performance of nanoparticle-based drug-delivery systems. To date, the majority of the delivery systems work well in vitro, but fail when they are tested in the much more complicated in vivo microenvironment. Pronounced differences lie between in vitro tumor models and preclinical models (i.e., small or large laboratory animals), and between animals and human bodies. Over the past decade, three-dimensional (3D) culture systems based on porous scaffolds or hydrogels have gradually replaced the conventional two-dimensional (2D) cultures on plastic tissue culture plates (TCPs) in an effort to better mimic the in vivo organization of tissues. More comfortingly, a novel and exciting concept termed "organ-on-a-chip" was proposed a couple of years ago by Ingber and co-workers based on the pioneering work conducted by their own and Schuler's group.[337] In such an approach, 3D miniaturized in vitro human tissues/organs (e.g., liver, lung, heart, kidney, and blood vessels) are created from perfusion cultures on microfluidic chips, and connected to each other to form a multi-organ, human-mimicry platform that can be used for testing drugs and nanoparticles. Using this "organ-on-a-chip" platform, one can conduct a more effective evaluation of the nanoparticles as drug-delivery systems and thus predict their in vivo behaviors. Importantly, because of the "humanized" feature of the platform, it is expected that preclinical models might be eventually eliminated.

Given recent technical advancements along with knowledge accumulated over the past decades, we believe that smart, targeted nanoparticles as drug carriers will revolutionize the field of cancer therapy by significantly improving both the quality and duration of a patient's life. We hope to see, in the near future, the development of personalized cancer therapeutics based on nanoparticles with increasingly sophisticated designs and integrations.

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