Chapter 3

Construction and Biological Evaluation of Nanoparticle-Based Tumor Targeting Drug Delivery Systems

Hong Wu, Tiehong Yang and Li Fan

Additional information is available at the end of the chapter

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Abstract

Nanoparticle-based drug delivery systems have gained immense popularity due to their ability to overcome biological barriers, effectively deliver drugs, and preferentially target tissue. In this chapter, the current progresses and challenges, especially evaluation methods for nanodrugs in antitumor drug delivery systems, are summarized, citing our works targeted at cancer therapy. It includes four parts. First, the principle, advantages, and significance of nanoparticle-based tumor targeting drug delivery system are presented. Recent developments in nanoparticle-based tumor targeting drug delivery system including passive targeting, active targeting, and stimuli-responsive systems/triggered release are introduced. Second, current formulations of nanoparticle-based drug delivery systems are described, including lipid-based, polymeric and branched polymeric, metal-based, magnetic, and mesoporous silica. Third, analytical techniques used for evaluating nanodrugs *in vitro* and *in vivo* are emphatically described. Finally, disadvantages and challenges of nanodrug are also discussed.

Keywords: nanoparticle, nanomicelle, tumor targeting, biological evaluation, nanocarrier, nanodrug, controlled-release, drug delivery system

1. Introduction

The last decade has witnessed enormous advances in the development and application of nanotechnology in cancer detection, diagnosis, and therapy. A nanoparticle as per the National Institutes of Health (NIH) guidelines is any material that is used in the formulation of a drug resulting in a final product smaller than 1 micron in size. This chapter summarizes current progresses and challenges, especially evaluation method for nanodrug in antitumor drug

delivery systems, citing our works targeted at cancer therapy. This chapter mainly consists of four parts. The first part presents the principle, advantages, and significance of nanoparticle-based tumor targeting drug delivery system, including passive targeting, active targeting, and stimuli-responsive systems/triggered release. The second part introduces the formulations of nanocarriers, with emphases laid on lipid-based, polymeric and branched polymeric, metal-based, and mesoporous silica. Some nanodrug carriers designed by us are introduced in this part. They are active targeting and acid-responsible nanoparticles, novel copolymers, multifunctional acid-sensitive micelle, and tumor microenvironment multiple responsible nanodrug release system. The third part introduces analytical techniques used for the characterization of nanoparticles *in vitro* and *in vivo*, such as dynamic light scattering (DLS), transmission electron microscope (TEM), scanning electronic microscopy (SEM), NMR, FTIR, and UV-Vis were commonly used to characterize the nanodrugs. Techniques for cell biology, such as TEM, confocal microscopy, flow cytometry, Western blot, and immunohistochemistry (IHC), were employed to evaluate target ability of nanodrugs *in vitro. In vivo* imaging system, micro-CT, NMR, and drug biodistribution were used to assess the *in vivo* behavior and efficacy of nanodrugs. Finally, disadvantages and challenges of nanodrug are discussed. So far, there are so many papers but so few nanodrugs in cancer therapy. The uncertainty and limitation of nanodrugs in pharmacology, toxicology, immunology, large-scale manufacturing, quality standard setting, and regulatory issues make nanoparticle-based tumor targeting delivery system have a long way to go.

2. Construction of nanoparticle-based tumor targeting drug delivery systems and their targeting functionalities

2.1. Definition of nanoparticle-based drug delivery system and classification of targeting functionalities

Increasing demand for and awareness of the applications of nanotechnology in medicine has resulted in the emergence of a new fast-growing multidisciplinary area—nanomedicine. Nanoparticles (NPs) serve as promising delivery system for various cargos such as drugs. Drugs are incorporated in nanoparticles that have the ability to get through physiological barriers and access the whole systemic circulation and thus are cleared less rapidly than free drug.

Nanoparticle-based drug delivery system represents an opportunity to achieve sophisticated targeting strategies and multi-functionality. They can increase the antitumor efficacy of conventional chemo-therapeutics, decrease their systemic toxicity, prolong duration time in systemic circulation, also present the following advantages, (1) help to overcome problems of solubility and chemical stability of anti-cancer drugs; (2) protect anti-cancer drug from biodegradation or excretion; (3) help to improve distribution of chemo therapeutics; (4) designed to release their payload response to biological triggers; and (5) may decrease resistance of tumors against anti-cancer drugs.

Therefore, targeted delivery is of utmost importance in order to overcome current limitations in cancer therapy. Recent developments in nanoparticle-based tumor targeting drug delivery system could be concluded in four aspects, passive targeting, active targeting, and stimuli-responsive systems/triggered release.

2.2. Passive targeting

Passive targeting is realized by specific porous loose structure of tumor vessels, which is easier for nanoparticles to accumulate. This leaky cascularization is the so-called EPR effect (enhanced permeability and retention effect), which allows migration of macromolecules up to 400 nm into tumor site [1–5]. For example, pegylated liposomal doxorubicin (Doxil®/Caelyx®) and nab-paclitaxel (Abraxane®) are the first generation nanomedicine based on passive targeting [6]. Numerous macromolecules and nanocarriers have shown to accumulate in tumor via the passive targeting owing to the EPR effect [7, 8]. EPR-based chemotherapy is thus becoming an important strategy to improve the delivery of therapeutic agents to tumors for anticancer drug development, and macromolecular agents are potentially usefully for not only cancer therapy, but for cancer diagnosis and imaging [9]. Although passive targeting approaches form the basis of clinical therapy, they suffer from several limitations. Not all the tumors exhibit EPR effects, and the permeability of vessels may not be the same throughout a single tumor [10, 11]. For example, Kaposi sarcoma with fenestrated vasculature, nanomedicine therapeutics could passive target into tumors without any specific ligand attached to the surface of the nanocarrier. However, heterogeneity of the tumor, such as different hypoxic gradient, can severely impact on the efficacy of passive targeting delivery. Moreover, increased interstitial fluid pressure (IFP) is another limitation of passive targeting, which reduces convective transport, while the dense extracellular matrix hinders diffusion [12]. Finally, though passive targeting could be used for delivering nanomedicine to certain solid tumor, it does not prevent accumulation of nanocarriers in some organs with fenestrated endothelium, for example, the liver and spleen [13].

Therefore, the development of nanomedicine drugs with active targeting functionalities is certainly warranted. One way to increase the targeting efficacy of nanoparticle-based drug delivery systems is to attach affinity ligands, such as antibodies [14], peptides [15], aptamers [16] or small molecules such as folic acid and carbohydrates onto the surface.

2.3. Active targeting

Passive targeting allows for the efficient localization of nanoparticles within the tumor microenvironment. Active targeting facilitates the active uptake of nanoparticles by the tumor cells themselves. Nanoparticle-based drug delivery systems decorated with specific targeting ligands will recognize and bind to target cells and then enter the cells through receptor mediated endocytosis. In order to achieve high specificity, those receptors should be highly expressed on tumor cells, but not on normal cells. In our previous studies, folic acid [17], LHRH [18], HAb18 $F(ab')_2$ [19] and monoclonal antibody [14] have been conjugated on the nanoparticles surface to enhance their targeting efficacy. The active targeting nanoparticles first specific bind to the receptor on the cell surface, then get internalized in small concave formed on the

cell membrane. Small concave closed the opening to form endocytic vesicle, then early endosome. The newly formed endosome is transferred to specific organelles, and drugs could be released by acidic pH or enzymes [20–22]. This endocytosis procedure was also confirmed in our recent research [14], which was illustrated by the schematic below (**Figure 1**).

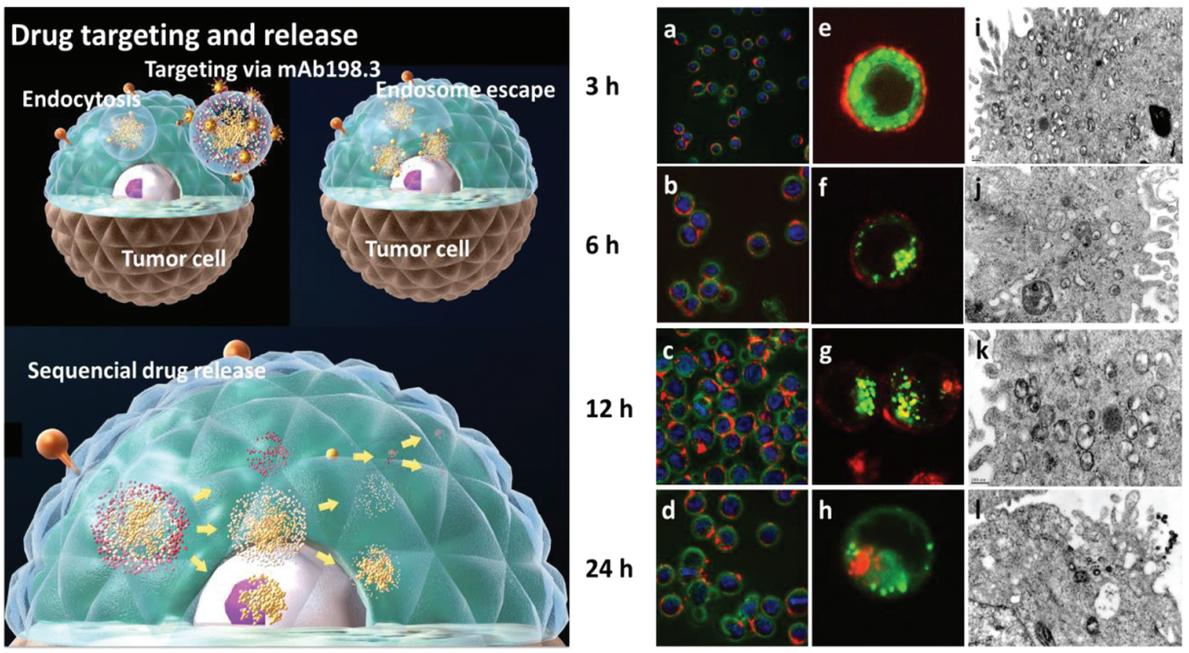

Figure 1. Illustrative schematic representing the endocytosis procedure of the SiO_2@AuNP delivery system after binding to cell surface targets. Followed by escaping from the endosomes/lysosomes, the drugs were sequentially released in cytoplasm to eliminate cancer cells. Confocal microscopy and TEM were introduced to testify the endocytosis and endosomal escape procedure of SiO_2@AuNP [14].

Among the potential targets for mAb-mediated nanoparticle delivery, human epidermal growth factor receptor 2 (HER2) [23], epidermal growth factor receptor (EGFR) [24], transferrin receptor (TfR) [25], and prostate-specific membrane antigen (PSMA) [26] have been extensively investigated. Over the last several years, aptamers have quickly become a new class of targeting ligands for drug delivery applications. Aptamer-based delivery systems of chemotherapy drugs (e.g., doxorubicin, docetaxel, daunorubicin, and cisplatin), toxins (e.g., gelonin and various photodynamic therapy agents), and a variety of small interfering RNAs were well established during past years [27]. Small molecules such as folic acid were also been widely used due to its inherent properties, which confer distinctive advantages and make it suitable ligand for nanoparticle targeting [28].

Furthermore, active targeting of nanocarriers has shown the potential to suppress multidrug resistance (MDR) via bypassing of P-glycoprotein-mediated drug efflux [29].

Although active targeting delivery systems looks promising, no one was currently approved for clinical use. Moreover, nanodrugs currently under clinical development lack specific targeting.

2.4. Stimuli-responsive systems/triggered release

Although passive and active targeting has been widely investigated, it still cannot guarantee sufficient high drug concentration in tumor site to achieve the complete eradication of tumors. Sufficient and sustained therapy is on the demand of controlled and sustained release of chemotherapeutics in tumor site. Therefore, it is highly desirable to design stimuli-responsive controlled drug delivery systems (CDDSs), which could release drugs by responding to tumor cell environmental changes, such as pH, temperature, glucose, adenosine-5′-triphosphate (ATP), glutathione (GSH), and H_2O_2 [30].

Among these stimuli, change in acidity as an internal signal is particularly crucial for the development of CDDSs that facilitate tumor targeting. Compared to the extracellular pH of normal tissues at pH 7.4, the measured tumor extracellular pH (pH_e) values of most solid tumors range from pH 6.5 to 7.2. Moreover, changes in pH are also encountered once the CDDSs enter cells via endocytosis where pH can drop as low as 5.0–6.0 in endosomes and 4.0–5.0 in lysosomes. The pH gradient is caused by hypoxia that upregulates glycolysis, followed by the production of lactate and protons in extracellular microenvironments [31]. pH-sensitive CDDSs can be used for delivering anti-cancer drugs to specific cancer cells, enhancing cellular internalization and rapid intracellular drug release. In order to increase the targeting activity, ligand-modified pH-sensitive CDDSs have been used for tumor targeting [32, 33]. In our previous study, many efforts have been made on several systems based on pH sensitive drug release characteristics. For instance, (1) pyrrolidinedithiocarbamate (PDTC) and doxorubicin

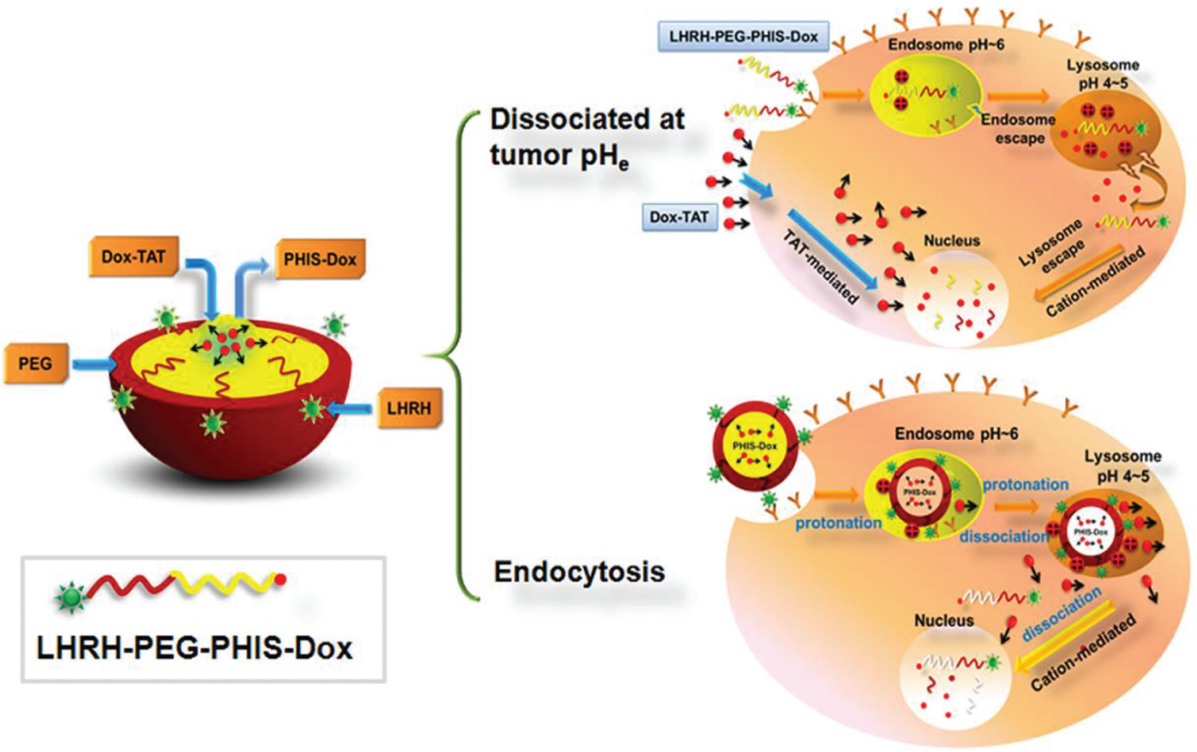

Figure 2. Schematic of targeting approaches and drug release procedures of LHRH-PEG-PHIS-Dox/Dox-TAT mixed micelles [18].

(DOX) was codelivered by copolymer folate-chitosan (FA-CS) nanoparticles to achieve targeted drug delivery, stimuli sensitive drug release, and to overcome multidrug resistance (MDR). (2) A novel delivery system based on LHRH-PEG-PHIS-Dox/Dox-TAT acid-sensitive micelles was developed, as shown in **Figure 2**. Such system could dissociate when responding tumor extracellular pHe and release Dox-TAT. This system showed remarkable antitumor efficacy and negligible systematic toxicity.

Higher concentration of GSH tripeptides is another important internal stimulus for rapid destabilization of CDDSs inside cells to accomplish rapid intracellular release [34]. The intracellular GSH concentration (1–10 mM) is substantially higher than extracellular levels (2 μM in plasma), providing a mechanism for selective intracellular release [35]. Gold nanoparticles were widely used for design GSH-triggered drug delivery systems. Its surface monolayer is stable under most physiological conditions, thus providing a reservoir of hydrophobic drugs, yet allowing controlled release by GSH though place exchange reactions of thiols on gold nanoparticle surfaces. These Au nanoparticles systems, which are under intensive study, display very intriguing properties, such as the precise control of intracellular drug release triggered by GSH. However, despite their great potential, additional investigations will be required to fully understand their pharmacokinetics, their interactions with the immune system, and the extent of cytotoxicity due to the surface and the geometry of the gold nanoparticles. Our research also focused on GSH-mediated drug release, such as siRNA [14] (**Figure 3**) and miR-218 mimics [36] (**Figure 4**) release from AuCOOH. After endocytosis, mediated by mAb198.3, the siRNA release process was illustrated by **Figure 3**. siRNA was released by the place exchange of glutathione (GSH) [37], and different band shifts on the denatured polyacrylamide gel page demonstrated the process of GSH-triggered siRNA release. In the research based on FA-CS@AuCOOH nanoparticles, temozolomide was released by diffusion due to FA-CS nanogel swelling, followed by miR-218 mimics was released by place exchange of GSH in tumor cells, which was illustrated in **Figure 4**. The sequential release of both chemo-drug and bio-drug exhibited significant synergistic effect against U87MG glioblastoma cells.

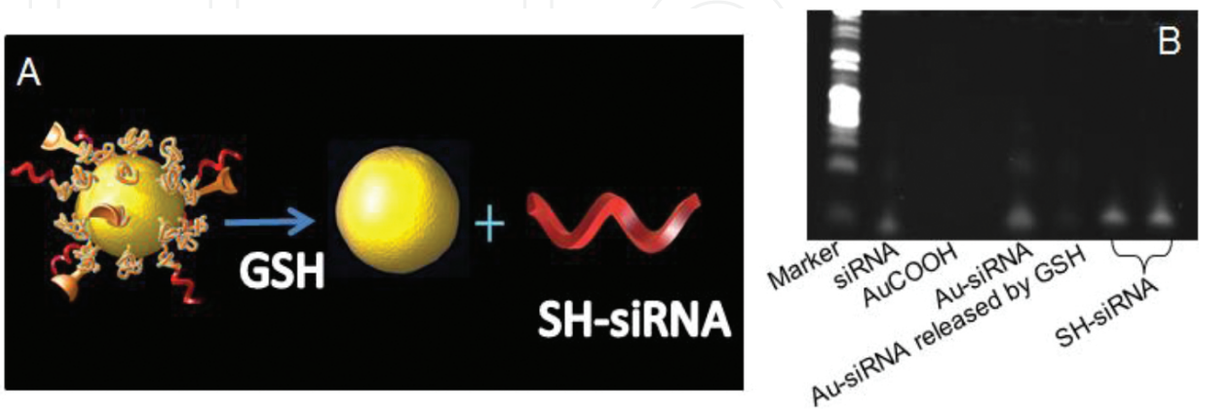

Figure 3. siRNA release procedure of outer AuNP layer. Schematic illustration of siRNA release procedures via GSH place exchange (A), confirmed by denatured SDS page (B) [14].

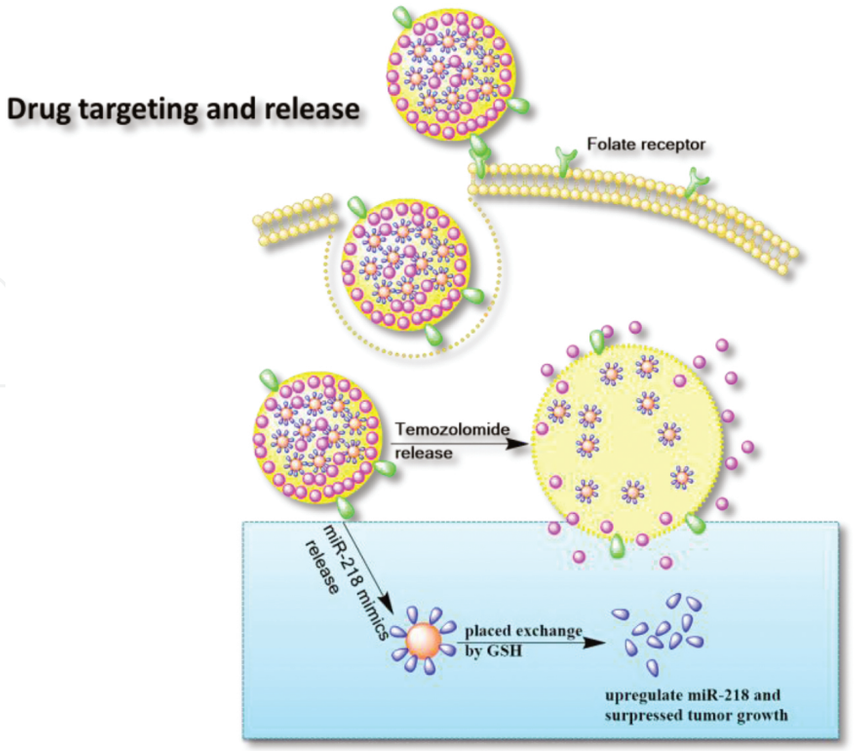

Figure 4. Schematic of drug design and drug release schedule. GSH mediated miR-218 mimics release from AuNP was emphasis by blue box [36].

Temperature is also a typical trigger at the tumor site, which could be exploited for drug delivery systems design [37, 38]. Thermo-responsive drug delivery is among the most investigated stimuli-responsive strategies. Usually, thermo-responsive nanocarriers were governed by a nonlinear sharp change with temperature, following by the release of the drug response to the temperature change. Ideally, thermo-responsive drug delivery systems should stay stable at body temperature (37°C) and rapidly release the payload within a locally heated tumor (40–42°C) to counteract rapid blood-passage time and washout from the tumor [38]. Poly(N-isopropyl acrylamide), PNIPAM was one of the most widely investigated thermo-sensitive materials, which exhibit a lower critical solution temperature. When surrounding temperature is above its LCST, the PNIPAM nanocarriers will shrink and push out the payload. For liposomes, thermos responsiveness usually arises from a phase transition of the constituent lipids and the associated conformational variations in the lipid bilayers [38, 39]. Thermo-responsive nanoparticle drug delivery systems typically present a lower critical solution temperature (LCST) at which they undergo coil-to-globule phase transitions. Thermo-sensitive liposomes usually composed of polymers with low LCST, which attached to lipid membranes due to hydrophobic interactions. The liposomes shrink to dehydrate and collapse, when the temperature achieve LCST, promoting drug release. By adjusting monomers types and ratio, polymer LCST can be tuned to different values, which could be used for controlling drug release at different environments [39].

ATP is a new member of physiological triggers to achieve “on-demand” therapeutic delivery with several merits, for example, high intracellular ATP concentration and sharp concentration

contrast between intracellular and extracellular environment make ATP a robust trigger signal to reduce premature drug release before cellular uptake and enhance intracellular accumulation of drugs [40]. ATP-triggered drug release system provides a more sophisticated drug delivery system, which can differentiate ATP levels to facilitate the selective release of drugs. Polymeric nanocarriers functionalized with an ATP-binding aptamer-incorporated DNA motif can selectively release the intercalating doxorubicin via a conformational switch when in an ATP-rich environment [41]. However, since the ATP binding modules are basically DNA or protein, potential concerns for immunogenicity from the components need to be addressed before clinical translations.

Glucose-responsive nanoparticles were widely investigated for insulin delivery [42]. Glucose nanosensors are being incorporated to precise and accurate tracking blood glucose levels. Also, they provide the guide for glucose-responsive nanoparticles which better mimic the body's demand for insulin. Besides, glucose-sensitive self-assembly is relevant for the application of anticancer therapeutic drug delivery. Since cancer cells metabolize differently than normal cells, glucose accumulate faster in tumor site than normal tissues and circulation [43–45]. Accumulation of glucose analogue ^{18}fluoro-2-deoxy-d-glucose (^{18}FDG) is 3.3–4.7 times greater for tumor than normal liver [46, 47]. A novel approach for glucose-triggered anticancer drug delivery from the self-assembly of neutral poly (vinyl alcohol) (PVA), and chitosan was been investigated by Satish Patil research group. This system could release glucose controllable by disintegration of layer by layer polymers. The capsules size and shape can be tuned because of physically cross-linked PVA hydrogel inside the multilayer. Because of the presence of borate in multilayer wall, the encapsulated drugs could be release programmable by different glucose concentration. The borate mediated self-assembly of PVA hydrogel and chitosan provide promising platform for intelligent anti-cancer drug delivery. The *in vivo* studies are under going in their laboratory [48].

Reactive oxygen species (ROS) play important roles in a variety of physiological and pathophysiological processes [49]. Moreover, many types of cancer cells exhibit high level of ROS stress [50]. An increase of H_2O_2 at cellular levels characteristic for cancer cells, which is a major component of ROS and a common marker for oxidative stress, plays a key role in carcinogenesis [51]. Thus, intracellular H_2O_2 in cancer cells was utilized as tumor site stimulus for drug delivery in cancer therapy. Synergistic release of anticancer drugs and O_2 can be achieved in an H_2O_2-responsive nanocarrier incorporated with catalase. Such a system demonstrated improved therapeutic efficacy against cisplatin resistant cell lines which often appear to be in hypoxia [52]. However, the most challenging problem for engineering ROS-controlled-release systems is to improve the responsive sensitivity to ROS species, because of low concentration and very short half-lives in most cellular. Although there are increasing number of ROS-controlled release systems have been reported, development of highly sensitive nanocarriers which are specifically responsive to physiological levels of ROS are highly desired.

Till now, no optimized targeting drug delivery platform has been announced. Each has its own advantages and flaws, even for those under preclinical or clinical testing. It might be possible that the combination sequential drug delivery system design could be more effective to precise drug delivery, paving the way for a more effective personalized therapy.

3. Nanoparticlated formulation-based drug delivery systems

What is such drug delivery systems composed of? Currently, many formulations of nanocarriers are utilized including lipid-based, polymeric and branched polymeric, metal-based, and mesoporous silica.

3.1. Lipid-based nanocarriers

The formulation of lipid-based nanomedicines against cancer has been hypothesized to improve drug localization into the tumor tissue and to increase the anticancer efficacy of conventional drugs, while minimizing their systemic adverse effects [53]. An ideal multifunctional lipid-based nanoparticle drug delivery system with targeting and triggering drug release functions should be composed of a matrix phospholipid, a destabilizing phospholipid, conjugation lipid, ligand attached, and a cell death marker. Chemotherapeutics and imaging agents were loaded in nanoparticles in aqueous phase [54]. Among various lipid-based formulations, classical examples are "liposomes," which primarily consist of phospholipids (major components of biological membranes) and have been extensively studied [55]. Prof. A.D. Bangham of the United Kingdom first published preparation of liposomes with entrapped solutes. Then, many scientists present a well-studied class of drug carriers generally characterized by the presence of a lipid bilayer that is primarily composed of amphipathic phospholipids [54].

3.2. Polymeric and branched polymeric nanocarriers

Polymer-based nanomedicine for improvement in efficacy of cancer therapeutics has been widely explored, including polymeric nanoparticles, polymer micelles, dendrimers, polymersomes, polyplexes, polymer-lipid hybrid systems, and polymer-drug/protein conjugates. Polymeric nanoparticles are defined by their morphology and composition. The therapeutic agent is either conjugated to the nanoparticles surface, or encapsulated and protected inside the polymeric core [56]. These polymeric nanoparticles are capable of loading wide range of drugs for a sustained or controlled release at tumor sites to provide enhanced antitumor efficacy with minimal systemic side effects. Also, these nanoparticles protect drugs from their rapid metabolism during systemic circulation and clearance by the liver, kidney, and reticuloendothelial system, which further improves drug's stability and target specificity [3, 57]. In recent years, major branch of our research was based on multifunctional poly(β-L-malic acid)-based nanoconjugates [18]. This nanoconjugate with a pH-dependent charge conversional characteristic was developed for tumor-specific drug delivery. As shown in **Figure 5**, nanoconjugates minimize nonspecific interactions with serum components and change the surface charge of nanoconjugates in response to the tumor acidity (pHe), leading to promoted cell internalization by the combination of electrostatic absorptive endocytosis and receptor-mediated endocytosis.

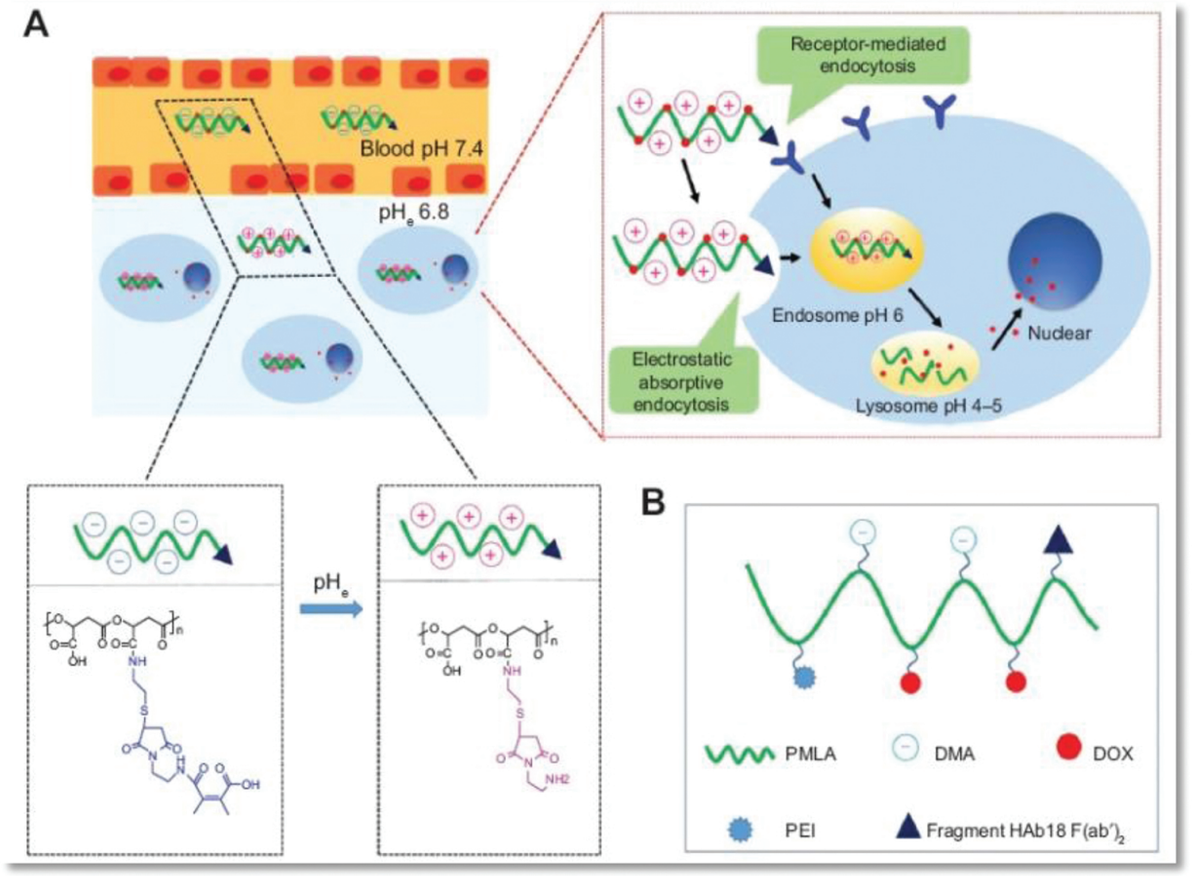

Figure 5. Schematic illustration of the stealth property and promoted tumor cell uptake of nanoconjugates (A) and DOX-loaded nanoconjugates (DOX/HDPEPM) (B). DMA, 2,3-dimethylmaleic anhydride; DOX, doxorubicin; HDPEPM, nanoconjugate formed by covalent attachment of fragment HAb18 F(ab')$_2$ and 2,3-dimethylmaleic anhydride to polyethylenimine-modified poly(β-L-malic acid); PEI, polyethylenimine; PMLA, poly(β-L-malic acid) [19].

3.3. Metal-based nanocarriers

Metal-based inorganic nanoparticles with monodispersity have been extensively studied for imaging using magnetic resonance and high-resolution superconducting quantum interference devices for cancer therapy [58]. Among all inorganic nanoparticles, gold nanoparticles were mostly explored for anti-tumor therapeutics delivery, due to its surface properties, strong affinity to thiol and amine functionalities and relative non-toxic nature [59]. Gold nanoparticles have been used mostly as a probe for electron microscopy and as a delivery vehicle for biomolecules. Also, super paramagnetic iron oxide nanoparticles (SPION) and gadolinium chelates are gaining interest as MRI agents [60]. Magnetic nanoparticles (MNPs) are also gaining clinical importance as MRI contrast materials, such as ferumoxides and ferumoxtran; approved by the FDA for detecting solid tumors [61]. Gadolinium-conjugated TiO_2-DNA oligonucleotide nanoconjugates show prolonged intracellular retention period and T1-weighted contrast enhancement in magnetic resonance images. Moreover, the increased

retention time, Gd accumulation, and intracellular delivery may find its use in Gd neutron-capture cancer therapy [62]. Silver and platinum nanoparticles are also used for therapeutics delivery applications. Scientists at UC Santa Barbara presented a class of AgNPs that are exceptionally bright and photostable, carry peptides as model targeting ligands, can be etched rapidly and with minimal toxicity in mice, and that show tumor uptake *in vivo* [63]. These results illustrate how plasmonic nanoprobes based on etchable Ag cores will be a powerful tool in studies of targeted uptake and trafficking from a subcellular to tissue level. Nanoparticles built from platinum cross-linker present a novel platform for anti-tumor drug delivery. As novel cross-linker, platinum Pt (IV) diester derivative agglomerates PEG-based brush-arm star polymers (BASPs) with tunable structures was used for delivery several kinds of antitumor drug, such as doxorubicin, camptothecin, and cisplatin. The cross-linker disintegrates when reduced by glutathione, which is abundant inside cells, to release the drugs bound covalently to the star polymers. This process is well-controlled as the sizes and Pt-loading of the narrowly dispersed stars is tunable by variables such as brush length and cross-linker loading. Furthermore, *in vitro* and *in vivo* assays demonstrate an efficacy of anticancer activity and low off target toxicity [64].

3.4. Mesoporous silica–based nanocarriers

Multifunctional mesoporous silica nanoparticles (MSNs) are widely used as universal platform for drug delivery [65]. Highly attractive features, such as high internal surface area and pore volume, tunable pore sizes, colloidal stability, and the possibility to specifically functionalize the inner pore system and/or the external particle surface, make MSNs a promising and widely applicable platform for diverse biomedical applications including bioimaging for diagnostics [66], biosensing [67], biocatalysis [68], bone repair and scaffold engineering [69], and drug delivery [70]. For applications of multifunctional MSNs as drug delivery systems in future and further advanced in clinical trials, they should be designed with two different ways. One approach is to build up systems which could release drug response to stimuli already present in the organism, such as lower pH values and redox potential in endosomes (for triggered release functions). The other approach would rely on the use of external triggers (in combination with internal stimuli) to control the drug release behavior, for example, to release payloads in certain location of tissues or in certain time. Recent studies focus on the ultimate combination of diagnostic and therapeutic capabilities in the multifunctional mesoporous nanoparticles, such that the nanocarrier uses diagnostic information to control or tune its therapeutic actions [65]. Stimuli-free programmable drug release for combination chemo-therapy has been also investigated by Dr. Fan in our research group. In her previous work, she demonstrated programmed delivery of both chemotherapeutics and biodrug with tumor targeting efficacy by introducing SiO_2-based self-decomposable nanoparticles. The programmable drug delivery is realized by adjusting drug loading ratios and concentration with external stimuli-free characteristics [71]. The present system provides a simple and feasible system for design targeting and combination chemotherapy with programmed drug release (**Figure 6**).

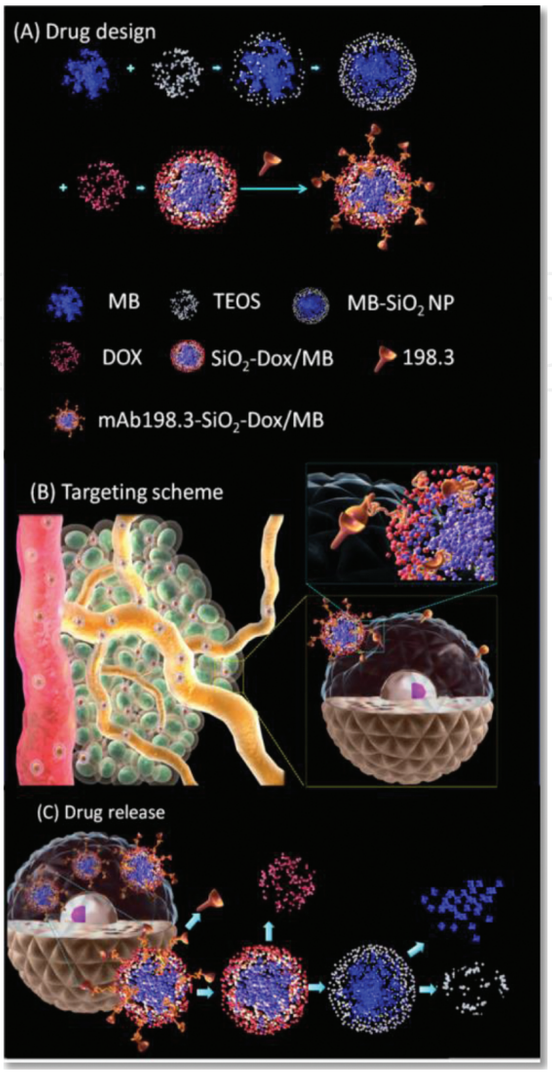

Figure 6. Schematic of the SiO_2 NP delivery system, its targeting scheme, and sequential drug release process. (a) Drug design of the mAb198.3-SiO_2-Dox/MB NP, (b) Targeting scheme of the NP drug (c) Multi-drug release process in a sequential manner [71].

4. Analytical techniques used for characterization of nanoparticles *in vitro* and *in vivo*

When materials are reduced at nanoscale dimensions, they show unique properties that are different from their massive counterparts. In order to characterize nanoparticles, their particle size, size distribution, morphology, composition, surface chemistry, and reactivity are important factors that need to be defined accurately. These properties make nanomaterials a suitable carrier for unique sensing applications and, at the same time, they may also create complications during the characterization process. Choosing the right method for the characterization of nanoparticles is a challenging task since one should be aware that each technique has its own limitations. The characterization of nanoparticles is carried out through various

techniques such as dynamic light scattering (DLS), scanning electron microscopy (SEM), transmission electron microscopy (TEM), NMR, FTIR, UV-Vis spectroscopy [72]. Techniques for cell biology, such as TEM, confocal laser scanning microscopy (CLSM), and flow cytometry, were employed to evaluate target ability of nanodrugs *in vitro*. *In vivo* imaging system and drug biodistribution were used to assess the *in vivo* behavior and efficacy of nanodrugs.

4.1. Dynamic light scattering (DLS) analysis

The size of nanoparticles is one of the key parameters that influence the interaction between nanoparticles and cells, which influenced cellular uptake [73, 74]. DLS is the most suitable technique to determine the particle size of nanoparticles (**Figure 7**).

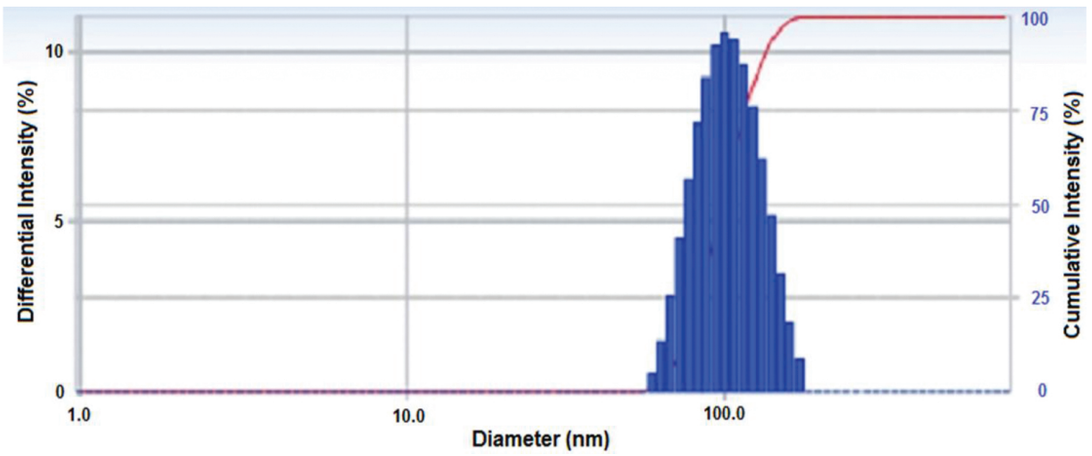

Figure 7. Particle size and size distribution of nanoparticles.

DLS is a technique in physics that can be used to determine the size distribution profile of small particles in suspension or polymers in solution, by measuring the random changes in the intensity of light scattered based on dynamic Brownian motion of the suspended particle. This technique is also called photon correlation spectroscopy (PCS) and quasi-elastic light scattering (QELS). The latter terms are more common in older literature. Typical applications are emulsions, micelles, polymers, proteins, nanoparticles or colloids. In general, the technique is best used for submicron particles and can be used to measure particle with sizes less than a nanometer. In this size regime (microns to nanometers) and for the size measurement (but not thermodynamics), the distinction between a molecule (such as a protein or macromolecule) and a particle and even a second liquid phase (such as in an emulsion) becomes blurred.

There are several advantages associated with DLS: simplicity; sensitivity and selectivity to NPs; short time of measurement; and the fact that calibration is not needed. Therefore, this technique is increasingly used for nanoparticle characterization in various science and industry fields [75, 76]. However, some problems are encountered when measuring samples with larger size distributions or multimodal distributions [77]. If the measured colloid is monodispersed, the mean diameter of the nanoparticles can be determined using the DLS technique. For polydispersed colloids, there is a risk during the DLS measurement, as small

particles can be screened by bigger particles, since bigger particles have more scattering property .

Some DLS instrument can measure not only particle size, but also Zeta potential at the same time [78]. Zeta potential is the surface charge of nanoparticles in solution (colloids). Nanoparticles have a surface charge that attracts a thin layer of ions of opposite charge to the nanoparticles surface. This double layer of ions travels with the nanoparticle as it diffuses throughout the solution. The electric potential at the boundary of the double layer is known as the Zeta potential of the particles and has values that typically range from +100 mV to −100 mV. Zeta potential is an important tool for understanding the state of the nanoparticle surface and predicting the long-term stability of the nanoparticle (**Figure 8**).

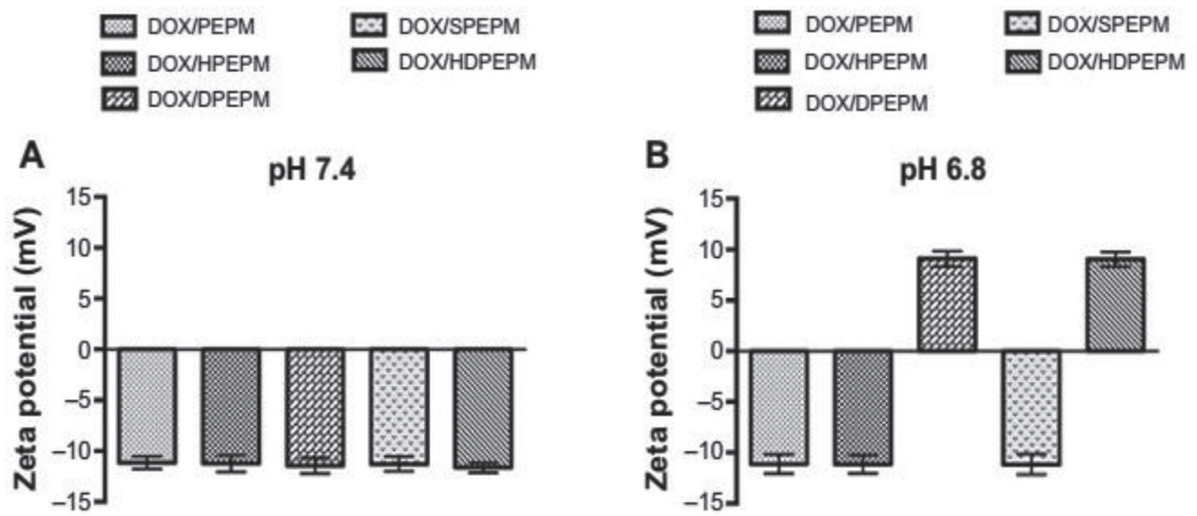

Figure 8. Zeta potential of different DOX-loaded nanoconjugates at (A) pH 7.4 and (B) pH 6.8 (n = 5). At pH 7.4, no charge-conversional behaviors were observed. When the pH was decreased from 7.4 to 6.8, both DOX/DPEPM and DOX/HDPEPM nanoconjugates showed a significant charge conversion [19].

4.2. Transmission electron microscope (TEM) and scanning electronic microscopy (SEM)

Particle morphology is another important parameter for the characterization of nanoparticles, and this is achieved with the help of microscopic techniques such as SEM and TEM. Both techniques produce a resolution that is a thousand times greater than the optical diffraction limit. SEM uses a beam of high-energy electrons to produce a variety of signals that contain information about the sample's surface composition, topography, and other properties such as electrical conductivity. We can analyze the sample at various times because X-rays generated by SEM do not lead to a loss of volume of the sample. However, electron microscopy creates a risk of radiation damage that is caused by the electron beam, which leads to the generation of free radicals. The diffusion of free radicals and the loss of mass may cause physical damage to the sample [78]. Also, TEM suffers from the limitations of poor contrast, especially in the event of peptide/protein nanoparticles and their conjugates. Besides particle morphology, TEM and SEM could also be used to study the physical size of nanoparticles (**Figure 9**).

However, there are some disadvantages associated with TEM and SEM: time consuming, high operator fatigue, few particles examined.

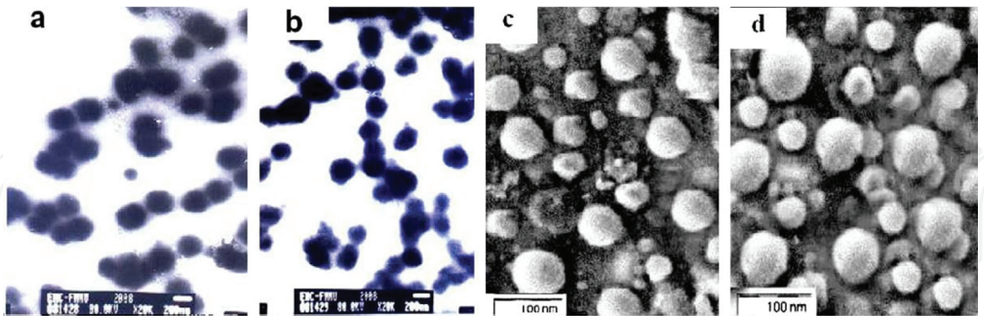

Figure 9. TEM and SEM micrographs of blank and drug-loaded nanoparticles (a) TEM of blank nanoparticles; (b) TEM of drug-loaded nanoparticles; (c) SEM of blank nanoparticles; (d) SEM of drug-loaded nanoparticles [79].

4.3. NMR, FTIR, and UV-Vis spectroscopy

NMR, FTIR, and UV-Vis spectroscopies are primary methods for determining the structure of compounds. They are also used in analyzing the structure of nanoparticles, especially to confirm the modification of polymer carriers. These are simply done and rapid. They can be combined to give overlapping information. NMR spectroscopy is one of the most nondestructive techniques in elucidating molecular structure as well as understanding the molecular dynamics of organic, organometallic, inorganic, polymeric, and biological molecules (**Figure 10**). It can be also used in nanoparticle size determination and nanoparticle surface study [80, 81]. IR spectra can be used to provide information on the functional groups as well as the structure of a molecule as a whole. UV-Vis spectra have broad features that could provide only limited information of structure but very useful for quantitative measurements.

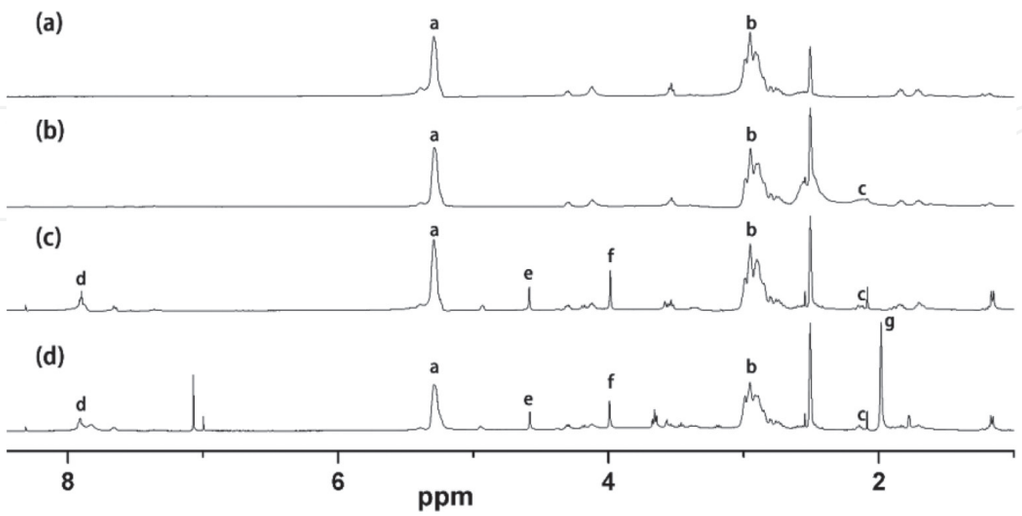

Figure 10. ^{1}H NMR spectra of (a) PMLA, (b) PEI-PMLA (PEPM), (c) DOX/PEPM, and (d)DOX/DPEPM. DMSO was used as the solvent [19].

The ability to enter target cell efficiently is a key character of nanoparticles. Techniques for cell biology, such as confocal microscopy, flow cytometry, were employed to evaluate target ability of nanodrugs *in vitro*.

4.4. Confocal laser scanning microscopy (CLSM)

CLSM is a technique for obtaining high-resolution optical images with depth selectivity. The key feature of CLSM is its ability to acquire the in-focus images from selected depths, a process known as optical sectioning. It could be used to observe the cellular uptake of fluorescence labeled nanoparticles, as well as nanoparticles-cell interaction (**Figure 11**).

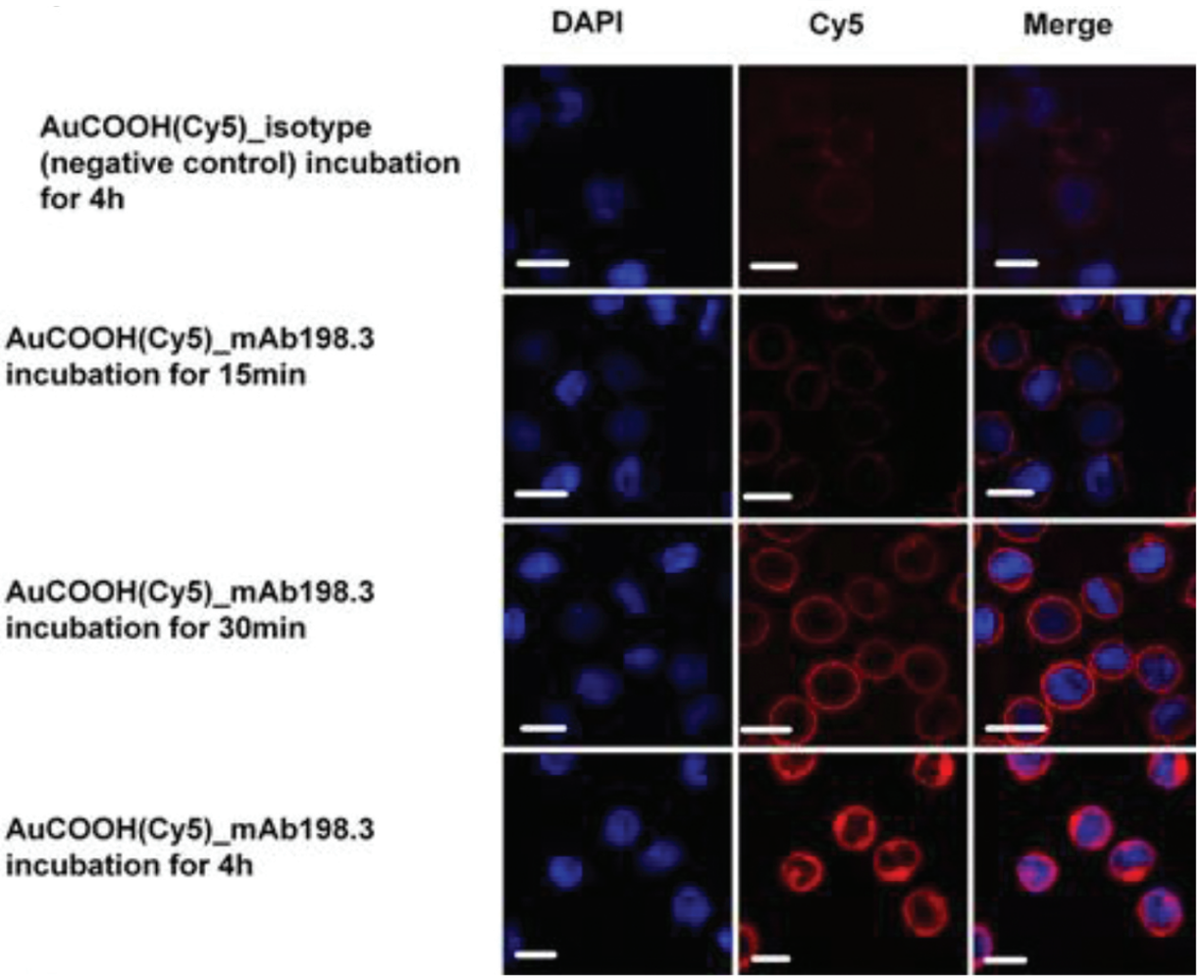

Figure 11. Confocal images of Colo 205 cells incubated with AuCOOH(Cy5)_isotype (negative control) and AuCOOH(Cy5)_mAb198.3 and nucleus stained with DAPI. Incubated time: 15 min, 30 min and 4 h. (Blue fluorescence is associated with DAPI, and red fluorescence is associated with Cy5). Scale bar at 20 μm [36].

4.5. Flow cytometry

Flow cytometry is a laser-based, biophysical technology employed in cell counting, cell sorting, biomarker detection, and protein engineering, by suspending cells in a stream of fluid and

passing them by an electronic detection apparatus. It is extensively used in research for the cell apoptosis and fluorescence quantitative analysis of nanoparticles to evaluate its targeting efficacy (**Figure 12**).

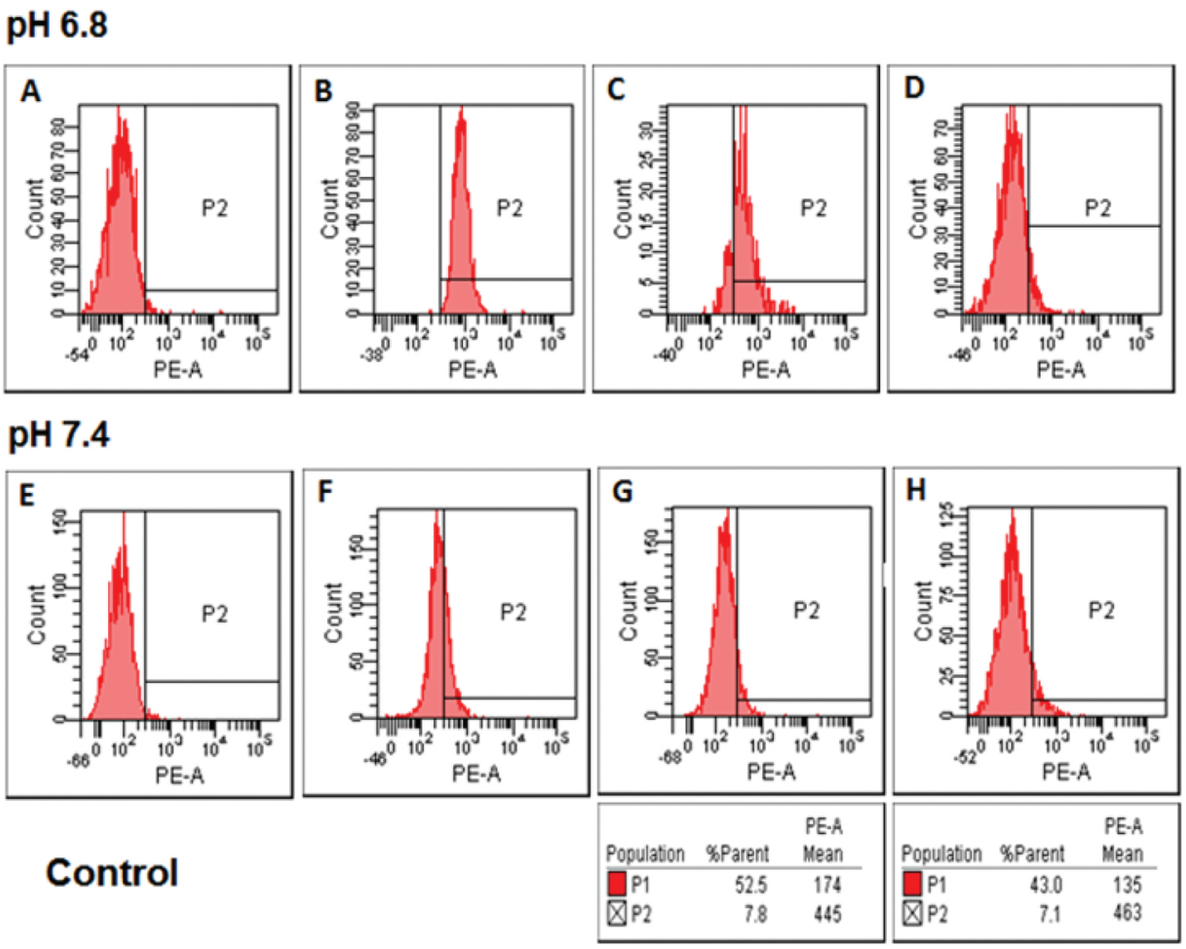

Figure 12. FACS analysis of A2780/DoxR cells incubated for 1 h at 37°C with untreated cell as control (A, E), LHRH-PEG-PHIS-Dox/Dox-TAT (B, F), LHRH-PEG-PHIS-Dox/Dox (C, G) and LHRH-PEG-PHIS-Dox (D, H) at pH 7.4 or pH 6.8, respectively [18].

4.6. *In vivo* imaging system

The ability of nanoparticles to achieve high, local concentrations of drugs at a target site provides the opportunity for improved system performance and patient outcomes along with reduced systemic dosing. Current technologies for tumor imaging, such as *in vivo* imaging system, are able to yield high-resolution images for the assessment of nanoparticles uptake in tumors at the microscopic level; a microscopic visual representation of a biological component inside the body [82]. The imaging procedure often utilizes a variety of diagnostic tools to provide insight regarding disease states, molecular characterization, and biological processes (**Figure 13**).

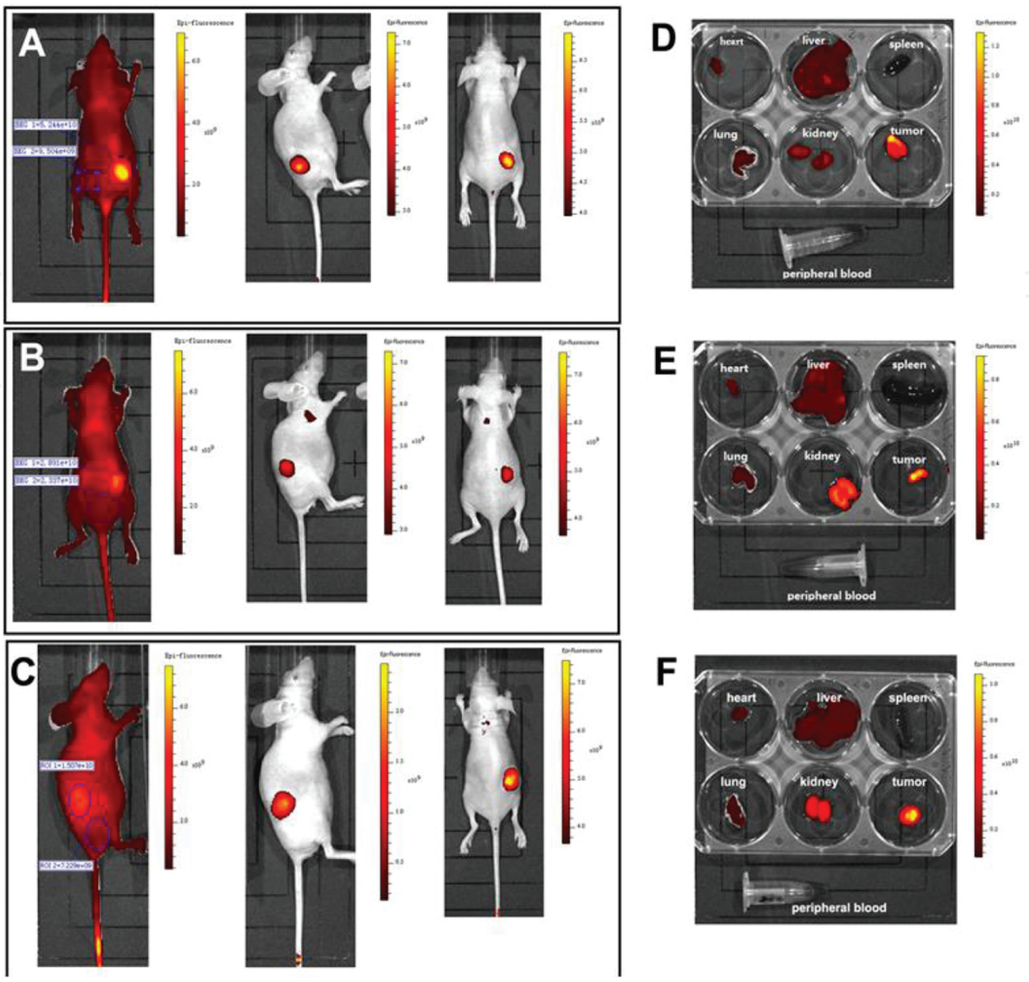

Figure 13. In vivo imaging of Colo 205 tumor bearing mice. Fluorescent signal captured by IVIS Lumina Imaging System in tumor bearing mice after injection with AuCOOH(Cy5)_ mAb198.3 (a), AuCOOH(Cy5)_ isotype (b), and mAb198.3_Cy5 (c) for 24 h. Luminescent image of resected organs from Colo 205 tumor-bearing mouse injected with AuCOOH(Cy5)_ mAb198.3 (d), AuCOOH(Cy5)_ isotype (e), and mAb198.3_Cy5 (f) for 24 h [36].

4.7. Drug biodistribution analysis

Another method to assess the *in vivo* behavior and efficacy of nanodrugs is drug biodistribution analysis. This is a method of tracking where drugs of interest travel in an experimental animal or human subject by the determination of drug concentration in targeted site and other organs.

5. Disadvantages and challenges of nanodrug

Nanodrug since its emergence has proved to be promising novel drug delivery system. In recent years, great progress was achieved in making drugs owning the characteristics of targeted and controlled release via nanotechnologies. However, there are some challenges in the use of large size materials in drug delivery. Some of these challenges are poor targeting and therapeutic effects, sustained and targeted delivery to site of action, poor bioavailability,

generalized side effects, *in vivo* stability, intestinal absorption, and plasma fluctuations of drugs [83]. Taking the active targeting strategy as an example, it is not always as effective as expected. The main mechanism behind active targeting is the recognition of the ligand by its target substrate. But because of the heterogeneity of tumor cells, receptors on the surface of tumor cells are different from cell to cell. Therefore, the interaction between cell receptors and ligands linked to nanoparticles becomes unreliable, which the nanoparticles was relied on to enter into the cell. This results in poor targeting and therapeutic effects in some cases [84]. Besides, distribution through the tumor is severely limited by its relatively large size which slows diffusion and may become trapped in the ECM. Other obstacles with nanocarriers that must be concerned include complicated synthesis, in vivo aggregation and recognition by the reticuloendothelial system leading to high clearance. This is further complicated when the therapeutic is covalently attached to the drug carrier as in the case of many polymers. Finally, most studies are at the basic research stage at present. Since it was unknown about environmental influence and genetic effect of novel nanomaterials, much works and a long process for acceptance by public were needed for more nanodrugs to be used in clinic.

To reach the promise of nanodrugs, it is necessary to take a step back and look at the problems facing drug delivery as a whole rather than designing around only one or two obstacles. Incremental designs may not be sufficient to accomplish the task of treating cancer effectively. Instead, a revolution in concept is needed. Nanodrug delivery system with simple synthesis routes and high targeting/therapeutic efficacy may point the way out.

So far, there are so many publications but so few nanodrugs in cancer therapy [85]. The uncertainty and limitation of nanodrugs in pharmacology, toxicology, immunology, large-scale manufacturing, and regulatory issues make it become an important research field in nanoparticle-based tumor targeting delivery system. And how we can overcome these difficulties, it is a long way to go.

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Author details

Hong Wu*, Tiehong Yang and Li Fan

*Address all correspondence to: wuhong@fmmu.edu.cn

School of Pharmacy, Fourth Military Medical University, Xi'an, China

References

[1] Bamrungsap S, Zhao Z, Chen T, Wang L, Li C, Fu T, Tan W. Nanotechnology in therapeutics: a focus on nanoparticles as a drug delivery system. *Nanomedicine*, 2012, 7: 1253–71.

[2] Matsumura Y, Maeda H. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. *Cancer Res*, 1986, 46: 6387–92.

[3] Sinha R, Kim GJ, Nie S, Shin DM. Nanotechnology in cancer therapeutics: bioconjugated nanoparticles for drug delivery. *Mol Cancer Ther*, 2006, 5: 1909–17.

[4] Smith AM, Duan H, Mohs AM, Nie S. Bioconjugated quantum dots for in vivo molecular and cellular imaging. *Adv Drug Deliver Rev*, 2008, 60: 1226–40.

[5] Srikanth M, Kessler JA. Nanotechnology-novel therapeutics for CNS disorders. *Nat Rev Neurol*, 2012, 8: 307–18.

[6] Wicki A, Witzigmann D, Balasubramanian V, Huwyler J. Nanomedicine in cancer therapy: challenges, opportunities, and clinical applications. *J Control Release*, 2015, 200: 138–57.

[7] Maeda H. Tumor-selective delivery of macromolecular drugs via the EPR effect: background and future prospects. *Bioconjugate Chem*, 2010, 21: 797–802.

[8] Torchilin V. Tumor delivery of macromolecular drugs based on the EPR effect. *Adv Drug Deliver Rev*, 2011, 63: 131–5.

[9] Tanaka T, Shiramoto S, Miyashita M, Fujishima Y, Kaneo Y. Tumor targeting based on the effect of enhanced permeability and retention (EPR) and the mechanism of receptor-mediated endocytosis (RME). *Int J Pharm*, 2004, 277: 39–61.

[10] Jain RK. Barriers to drug delivery in solid tumors. *Sci Am*, 1994, 271: 58–65.

[11] Sriraman SK, Aryasomayajula B, Torchilin VP. Barriers to drug delivery in solid tumors. *Tissue Barriers*, 2014, 2: e29528.

[12] Jain RK, Stylianopoulos T. Delivering nanomedicine to solid tumors. *Nat Rev Clin Oncol*, 2010, 7: 653–64.

[13] Gaumet M, Vargas A, Gurny R, Delie F. Nanoparticles for drug delivery: the need for precision in reporting particle size parameters. *Eur J Pharm Biopharm*, 2008, 69: 1–9.

[14] Fan L, Zhang Y, Wang F, Yang Q, Tan J, Renata G, Wu H, Song C, Jin B. Multifunctional all-in-one drug delivery systems for tumor targeting and sequential release of three different anti-tumor drugs. *Biomaterials*, 2016, 76: 399–407.

[15] Jhaveri A, Torchilin V. Intracellular delivery of nanocarriers and targeting to subcellular organelles. *Expert Opin Drug Del*, 2016, 13: 49–70.

[16] Wu Q, Wu L, Wang Y, Zhu Z, Song Y, Tan Y, Wang XF, Li J, Kang D, Yang CJ. Evolution of DNA aptamers for malignant brain tumor gliosarcoma cell recognition and clinical tissue imaging. *Biosens Bioelectron*, 2016, 80, 1–8.

[17] Fan L, Li F, Zhang H, Wang Y, Cheng C, Li X, Gu CH, Yang Q, Wu H, Zhang S. Co-delivery of PDTC and doxorubicin by multifunctional micellar nanoparticles to achieve active targeted drug delivery and overcome multidrug resistance. *Biomaterials*, 2010, 31: 5634–42.

[18] Yang T, Li F, Zhang H, Fan L, Qiao Y, Tan G, Zhang H, Wu H. Multifunctional pH-sensitive micelles for tumor-specific uptake and cellular delivery. *Polym Chem*, 2015, 6(8): 1373–82.

[19] Zhou Q, Yang T, Qiao Y, Guo S, Zhu L, Wu H. Preparation of poly(beta-L-malic acid)-based charge-conversional nanoconjugates for tumor-specific uptake and cellular delivery. *Int J Nanomed*, 2015, 10: 1941–52.

[20] Farokhzad OC, Langer R. Impact of nanotechnology on drug delivery. *ACS Nano*, 2009, 3: 16–20.

[21] Crommelin DJ, Park K, Florence A. Pharmaceutical nanotechnology: unmet needs in drug delivery. *J Contro Release*, 2010, 141: 263–4.

[22] Yang L, Webster TJ. Nanotechnology controlled drug delivery for treating bone diseases. *Expert Opin Drug Del*, 2009, 6: 851–64.

[23] Shirshahi V, Shamsipour F, Zarnani AH, Verdi J, Saber R. Active targeting of HER2-positive breast cancer cells by Herceptin-functionalized organically modified silica nanoparticles. *Cancer Nanotechnol*, 2013, 4: 27–37.

[24] Master AM, Sen Gupta A. EGF receptor-targeted nanocarriers for enhanced cancer treatment. *Nanomed*, 2012, 7: 1895–906.

[25] Deshpande PP, Biswas S, Torchilin VP. Current trends in the use of liposomes for tumor targeting. *Nanomed*, 2013, 8: 1509–28.

[26] Huang BN, Abraham WD, Zheng YR, Lopez SCB, Luo SS, Irvine DJ. Active targeting of chemotherapy to disseminated tumors using nanoparticle-carrying T cells. *Sci Transl Med*, 2015, 7(291): 291ra94.

[27] Zhang Y, Hong H, Cai W. Tumor-targeted drug delivery with aptamers. *Curr Med Chem*, 2011, 18: 4185–94.

[28] Zwicke GL, Mansoori GA, Jeffery CJ. Utilizing the folate receptor for active targeting of cancer nanotherapeutics. *Nano Rev*, 2012, 3: 18496-http://dx.doi.org/10.3402/nano.v3i0.18496.

[29] Kapse-Mistry S, Govender T, Srivastava R, Yergeri M. Nanodrug delivery in reversing multidrug resistance in cancer cells. *Front Pharmaco*, 2014, 5: 159.

[30] Zhu CL, Wang XW, Lin ZZ, Xie ZH, Wang XR. Cell microenvironment stimuli-responsive controlled-release delivery systems based on mesoporous silica nanoparticles. *J Food Drug Anal*, 2014, 22: 18–28.

[31] Lee ES, Gao Z, Bae YH. Recent progress in tumor pH targeting nanotechnology. *J Control Release*, 2008, 132: 164–70.

[32] Yang ZZ, Li JQ, Wang ZZ, Dong DW, Qi XR. Tumor-targeting dual peptides-modified cationic liposomes for delivery of siRNA and docetaxel to gliomas. *Biomaterials*, 2014, 35: 5226–39.

[33] Zhang W, Dong D, Li P, Wang D, Mu H, Niu H, Duan J. Novel pH-sensitive polysialic acid based polymeric micelles for triggered intracellular release of hydrophobic drug. *Carbohyd Polym*, 2016, 139: 75–81.

[34] Cheng R, Feng F, Meng F, Deng C, Feijen J, Zhong Z. Glutathione-responsive nanovehicles as a promising platform for targeted intracellular drug and gene delivery. *J Control Release*, 2011, 152: 2–12.

[35] Hong R, Han G, Fernandez JM, Kim BJ, Forbes NS, Rotello VM. Glutathione-mediated delivery and release using monolayer protected nanoparticle carriers. *J Am Chem Soc*, 2006, 128: 1078–9.

[36] Fan L, Yang Q, Tan J, Qiao Y, Wang Q, He J, Wu H, Zhang Y. Dual loading miR-218 mimics and Temozolomide using AuCOOH@FA-CS drug delivery system: promising targeted anti-tumor drug delivery system with sequential release functions. *J Exp Clin Canc Res: CR* 2015, 34: 106.

[37] Shao PY, Wang BC, Wang YZ, Li J, Zhang YQ. The application of thermosensitive nanocarriers in controlled drug delivery. *J Nanomater*, 2012, 23(50): 505706-18.

[38] Mura S, Nicolas J, Couvreur P. Stimuli-responsive nanocarriers for drug delivery. *Nat Mater*, 2013, 12: 991–1003.

[39] Ta T, Porter TM. Thermosensitive liposomes for localized delivery and triggered release of chemotherapy. *J Control Release*, 2013, 169: 112–25.

[40] Sun W, Gu Z. ATP-responsive drug delivery systems. *Expert Opin Drug Del*, 2016, 1–4.

[41] Mo R, Jiang T, DiSanto R, Tai W, Gu Z. ATP-triggered anticancer drug delivery. *Nat Commun*, 2014, 5: 3364.

[42] Veiseh O, Tang BC, Whitehead KA, Anderson DG, Langer R. Managing diabetes with nanomedicine: challenges and opportunities. *Nat Rev Drug Discov*, 2015, 14: 45–57.

[43] Hahn T, Barth S, Hofmann W, Reich O, Lang I, Desoye G. Hyperglycemia regulates the glucose-transport system of clonal choriocarcinoma cells in vitro. A potential molecular mechanism contributing to the adjunct effect of glucose in tumor therapy. *Int J Cancer*, 1998, 78: 353–60.

[44] Okumura M, Yamamoto M, Sakuma H, Kojima T, Maruyama T, Jamali M, Cooper DR, Yasuda K. Leptin and high glucose stimulate cell proliferation in MCF-7 human breast cancer cells: reciprocal involvement of PKC-alpha and PPAR expression. *Biochim Biophys Acta*, 2002, 1592(2): 107–16.

[45] Ganapathy V, Thangaraju M, Prasad PD. Nutrient transporters in cancer: relevance to Warburg hypothesis and beyond. *Pharmacol Therapeut*, 2009, 121: 29–40.

[46] Nolop KB, Rhodes CG, Brudin LH, Beaney RP, Krausz T, Jones T, Hughes JM. Glucose utilization in vivo by human pulmonary neoplasms. *Cancer*, 1987, 60: 2682–9.

[47] Yonekura Y, Benua RS, Brill AB, Som P, Yeh SDJ, Kemeny NE, Fowler JS, Acgregor RR, Stamm R, Christman DR, Wolf AP. Increased accumulation of 2-deoxy-2-[F-18]fluoro-D-glucose in liver metastases from colon-carcinoma. *J Nucl Med*, 1982, 23: 1133–7.

[48] Manna U, Patil S. Glucose-triggered drug delivery from borate mediated layer-by-layer self-assembly. *ACS Appl Mater Inter*, 2010, 2: 1521–7.

[49] Li JJ, Han Y, Chen QX, Shi HD, Rehman SU, Siddiq M, Ge ZS, Liu SY. Dual endogenous stimuli-responsive polyplex micelles as smart two-step delivery nanocarriers for deep tumor tissue penetration and combating drug resistance of cisplatin. *J Mater Chem B*, 2014, 2: 1813–1824.

[50] Trachootham D, Alexandre J, Huang P. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? *Nat Rev Drug Dis*, 2009, 8: 579–91.

[51] Lopez-Lazaro M. Dual role of hydrogen peroxide in cancer: possible relevance to cancer chemoprevention and therapy. *Cancer Lett*, 2007, 252: 1–8.

[52] Chen H, He W, Guo Z. An H(2)O(2)-responsive nanocarrier for dual-release of platinum anticancer drugs and O(2): controlled release and enhanced cytotoxicity against cisplatin resistant cancer cells. *Chem Commun*, 2014, 50: 9714–7.

[53] Arias JL, Clares B, Morales ME, Gallardo V, Ruiz MA. Lipid-based drug delivery systems for cancer treatment. *Currt Drug Targets*, 2011, 12, 1151–65.

[54] Puri A, Loomis K, Smith B, Lee JH, Yavlovich A, Heldman E, Blumenthal R. Lipid-based nanoparticles as pharmaceutical drug carriers: from concepts to clinic. *Crit Rev Ther Drug*, 2009, 26: 523–80.

[55] Fenske DB, Chonn A, Cullis PR. Liposomal nanomedicines: an emerging field. *Toxicol Pathol*, 2008, 36: 21–9.

[56] Prabhu RH, Patravale VB, Joshi MD. Polymeric nanoparticles for targeted treatment in oncology: current insights. *Int J Nanomed*, 2015, 10: 1001–18.

[57] Chan JM, Valencia PM, Zhang L, Langer R, Farokhzad OC. Polymeric nanoparticles for drug delivery. *Meth Mol Biology*, 2010, 624, 163–75.

[58] Alexis F, Pridgen EM, Langer R, Farokhzad OC. Nanoparticle technologies for cancer therapy. *Handbook of Experimental Pharmacology*, Rosenthal, W., Ed. Springer Science +Business Media: Heidelberg, 2010, 55-86.

[59] Patra CR, Bhattacharya R, Mukhopadhyay D, Mukherjee P. Application of gold nanoparticles for targeted therapy in cancer. *J Biomed Nanotechnol*, 2008, 4: 99–132.

[60] Liu Y, Miyoshi H, Nakamura M. Nanomedicine for drug delivery and imaging: a promising avenue for cancer therapy and diagnosis using targeted functional nanoparticles. *Int J Cancer*, 2007, 120: 2527–37.

[61] Shubayev VI, Pisanic TR. 2nd; Jin S. Magnetic nanoparticles for theragnostics. *Adv Drug Del Rev*, 2009, 61: 467–77.

[62] Paunesku T, Ke T, Dharmakumar R, Mascheri N, Wu AG, Lai B, Vogt S, Maser J, Thurn K, Szolc-Kowalska B, Larson A, Bergan RC, Omary R, Li DB, Lu ZR, Woloschak GE. Gadolinium-conjugated TiO2-DNA oligonucleotide nanoconjugates show prolonged intracellular retention period and T1-weighted contrast enhancement in magnetic resonance images. *Nanomed-Nanotechnol*, 2008, 4: 201–207.

[63] Braun GB, Friman T, Pang HB, Pallaoro A, Hurtado de Mendoza T, Willmore AM, Kotamraju VR, Mann AP, She ZG, Sugahara KN, Reich NO, Teesalu T, Ruoslahti E. Etchable plasmonic nanoparticle probes to image and quantify cellular internalization. *Nature Mater*, 2014, 13: 904–11.

[64] Liao L, Liu J, Dreaden EC, Morton SW, Shopsowitz KE, Hammond PT, Johnson JA. A convergent synthetic platform for single-nanoparticle combination cancer therapy: ratiometric loading and controlled release of cisplatin, doxorubicin, and camptothecin. *J Am Chem Soc*, 2014, 136: 5896–9.

[65] Argyo C, Weiss V, Brauchle C, Bein T. Multifunctional mesoporous silica nanoparticles as a universal platform for drug delivery. *Chem Mater*, 2014, 26: 435–451.

[66] Lee JE, Lee N, Kim T, Kim J, Hyeon T. Multifunctional mesoporous silica nanocomposite nanoparticles for theranostic applications. *Account of Chem Res*, 2011, 44: 893–902.

[67] Liu JL, Li CY, Li FY. Fluorescence turn-on chemodosimeter-functionalized mesoporous silica nanoparticles and their application in cell imaging. *J Mater Chem*, 2011, 21: 7175–81.

[68] Ariga K, Ji Q, Mori T, Naito M, Yamauchi Y, Abe H, Hill JP. Enzyme nanoarchitectonics: organization and device application. *Chem Soc Rev*, 2013, 42: 6322–45.

[69] Salinas AJ, Esbrit P, Vallet-Regi M. A tissue engineering approach based on the use of bioceramics for bone repair. *Biomater Sci-Uk*, 2013, 1: 40–51.

[70] Li Z, Barnes JC, Bosoy A, Stoddart JF, Zink JI. Mesoporous silica nanoparticles in biomedical applications. *Chem Soc Rev*, 2012, 41: 2590–605.

[71] Fan L, Jin B, Zhang S, Song C, Li Q. Stimuli-free programmable drug release for combination chemo-therapy. *Nanoscale*, 2016.

[72] Zaman M, Ahmad E, Qadeer A, Rabbani G, Khan RH. Nanoparticles in relation to peptide and protein aggregation. *Int J Nanomedicine*, 2014, 9: 899–912.

[73] Desai MP, Labhasetwar V, Walter E, Levy RJ, Amidon GL. The mechanism of uptake of biodegradable microparticles in Caco-2 cells is size dependent. *Pharm Res*, 1997, 14(11): 1568–1573.

[74] Zauner W, Farrow NA, Haines AM. In vitro uptake of polystyrene microspheres: effect of particle size, cell line and cell density. *J Control Release*, 2001;71(1): 39–51.

[75] Brar SK, Verma M. Measurement of nanoparticles by light-scattering techniques. *TrAC – Trend Anal Chem*, 2011, 30(1): 4–17.

[76] Khlebtsov BN, Khlebtsov NG. On the measurement of gold nanoparticle sizes by the dynamic light scattering method. *Colloid J*, 2011, 73(1): 118–127.

[77] Powers KW, Brown SC, Krishna VB, Wasdo SC, Moudgil BM, Roberts SM. Research strategies for safety evaluation of nanomaterials. Part VI. Characterization of nanoscale particles for toxicological evaluation. *Toxicol Sci*, 2006, 90(2): 296–303.

[78] Klang V, Valenta C, Matsko NB. Electron microscopy of pharmaceutical systems. *Micron*, 2013, 44: 45–74.

[79] Fan L, Wu H, Zhang H, Li F, Gu CH, Yang Q. Antitumor drug Paclitaxel-loaded pH-sensitive nanoparticles targeting tumor extracellular pH. *Carbohydr Polym*, 2009, 77(4): 773–8.

[80] Babu PK, Oldfield E, Wieckowski A. Nanoparticle surfaces studied by electrochemical NMR. *Modern Asp Electrochem*, Springer, 2004, 36: 1–50.

[81] Gomez MV, Guerra J, Myers VS, Crooks RM, Velders AH. Nanoparticle size determination by (1)H NMR spectroscopy. *J Am Chem Soc*, 2009, 131(41): 14634–5.

[82] Cho CF, Ablack A, Leong HS, Zijlstra A, Lewis J. Evaluation of nanoparticle uptake in tumors in real time using intravital imaging. *J Vis Exp*, 2011, (52): pii: 2808.

[83] Jiang W, Kim BY, Rutka JT, Chan WC. Advances and challenges of nanotechnology-based drug delivery systems. *Expert Opin Drug Deliv*, 2007, 4(6): 621–33.

[84] Bertrand N, Wu J, Xu X, Kamaly N, Farokhzad OC. Cancer nanotechnology: the impact of passive and active targeting in the era of modern cancer biology. *Adv Drug Deliv Rev*, 2014, 66: 2–25.

[85] Venditto VJ, Szoka Jr FC. Cancer nanomedicines: so many papers and so few drugs. *Adv Drug Deliv Rev*, 2013, 65(1): 80–8.