CHAPTER 3

Rational Design of Translational Nanocarriers

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3.1 The Three Key Elements for Translational Nanomedicine

Nanometer-sized drug carriers, including polymer–drug conjugates, dendrimers, liposomes, polymer micelles, and nanoparticles, have been extensively investigated in drug delivery for cancer chemotherapy.[1,2] Cancer drug delivery is a process using nanocarriers with appropriate sizes (usually between several nanometers and 200 nm) and stealth properties to preferentially carry drugs to tumor tissues *via* the enhanced permeability and retention (EPR) effect.[2] However, despite improved pharmacokinetic properties and reduced adverse effects,[1,3] currently cancer drug delivery has only achieved modest therapeutic benefits.[3,4] Thus, the design of nanocarriers with more efficient drug delivery and thus higher therapeutic efficacy is still a pressing need.

The cancer drug delivery process can be divided into three stages, shown in Figure 3.1. Initially, the drug-loaded nanocarrier circulates in the blood compartments, including the liver and the spleen. When passing through

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tumor blood vessels, the carrier may fall into the pores in the blood vessel wall and extravasate into the tumor tissue (EPR effect) (Figure 3.1A).[5,6] Next, it may further penetrate through the tumor tissue, which is nontrivial because of the high cell density and high interstitial fluid pressure (IFP) (Figure 3.1B).[7] Upon sticking to the surrounding cancer-cell membrane (Figure 3.1C), the carrier is expected to enter the cells *via* one or several possible pathways, and finally traverse the crowded intracellular structures and viscous cytosol to the targeted subcellular sites and release the carried drug cargo.

Thus, to achieve efficient drug delivery from the i.v. injection site to the target in the tumor cells, the nanocarrier must simultaneously meet two pairs of challenges (Figure 3.1): (a) the nanocarrier must retain the drug very tightly, ideally without any release, during the transport in the blood compartments and the tumor tissue, but must be able to efficiently release the drug once reaching the intracellular target to exert its pharmaceutical action; (b) the nanocarrier must be "slippery" or "stealthy" while in the blood compartments and in the tumor tissue until it reaches the targeted tumor cells. The stealth in the blood compartments enables it to effectively evade the reticuloendothelial system (RES) screening, particularly the capture by liver and spleen for a long blood circulation time. As the blood circulation time of the nanocarrier increases, so does its opportunity to pass the hyperpermeable tumor blood vessel and extravasation into the tumor tissue. After extravasating into the tumor, the nanocarrier must remain "stealthy" to penetrate deep into the center region to deliver the drug. This region lacks vascular perfusion

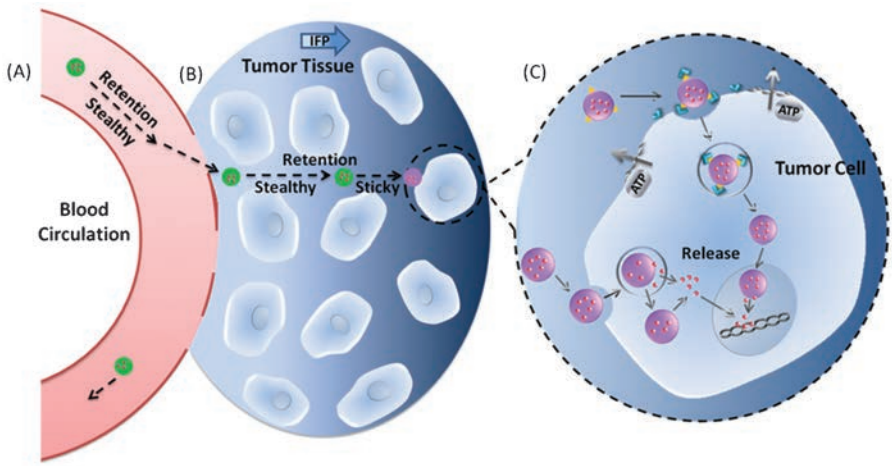

Figure 3.1 Cancer drug delivery process: (A) transport in the circulation, (B) transport through the tumor tissue, and (C) transport in the tumor cell. The nanocarrier must meet two pairs of challenges — For the drug: the nanocarrier must retain the drug very tightly during the transport in the blood compartments and the tumor tissue but efficiently release the drug once reaching the intracellular target; For the surface: the nanocarrier must be "very stealthy" during in the blood compartments for a long blood circulation time and remain "stealthy" in penetrating the tumor tissues but must become "sticky" or "cell binding" once interacting with tumor cells for efficient cellular uptake.

but harbors the most aggressive and resistant cells. On reaching the targeted cells the nanocarrier must become "sticky" or "cell binding" to interact with the tumor cell for efficient cellular uptake. A nanocarrier capable of simultaneously satisfying such opposite *2R2S* requirements at the right time and the right place, that is, "drug *R*etention in blood circulation *vs. R*elease in tumor cells (*2R*)" and "surface *S*tealthy in blood circulation and tumor tissues *vs. S*ticky to tumor cells (*2S*)", will deliver the drug specifically to the tumor cells, giving rise to high therapeutic efficacy and few side effects.

While the 2*R*2*S* capability of a nanocarrier may render the resulting nanomedicine efficacious and potentially safe for clinical translation, two other elements, namely the feasibility of the nanocarrier materials to be proved for use as excipients (referred to as material excipientability) and the ability to establish scaled-up production processes for good manufacturing practice (GMP) for the nanocarrier and its formulation with the drug (nanomedicine) (referred to as process scale-up ability) are also indispensible for the nanomedicine to be truly translational from the benchtop to the bedside (Figure 3.2).[8] Most of our current research is focused on using new material design and chemistry to improve the *2R2S* capability; however, research aimed at translational applications should comprehensively consider the other two elements at an early stage.

Herein, we briefly review the approaches addressing nanocarriers' *2R2S* capability and summarize the factors affecting material excipientability and process scale-up ability, aimed at promoting the developments of truly translational nanomedicine for cancer drug delivery.

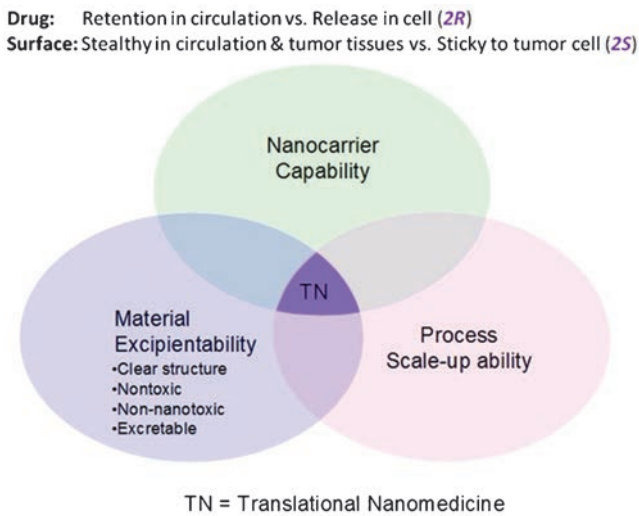

Figure 3.2 The three elements for translational nanomedicine: the nanocarrier should have the 2*R*2*S* capability and its material should be suitable for excipient use (referred to as material excipientability); the production of the nanocarrier and its formulation with drug (nanomedicine) should be able to scale up for good manufacture process (GMP) (scale-up ability). Reprinted with permission from ref. 8. Copyright 2012 Elsevier.

3.2 The *2R2S* Capability of Nanocarriers

3.2.1 *2R*: Drug Retention in Circulation *versus* Intracellular Release

3.2.1.1 Approaches to Minimize Premature Release from a Stable Carrier

Figure 3.3 illustrates two examples, an ideal one for the case when the carrier retains the drug during transport in the blood compartments and the tumor tissue, but releases it in the tumor cells, and another for a typical case of undesirable burst release when the carrier releases its drug cargo prematurely while still circulating in the blood. Such a burst release is generally observed for polymer particles[9,10] and liposomes.[11,12] As a result, the drug is dumped in the blood compartments, which causes not only local or systemic toxicity, but also lowers the drug availability to the tumor and thereby the therapeutic efficacy.

Although the exact mechanism of burst release is still not fully understood, it is likely that drug-diffusion resistance can help explain and control it. A study on a model zero-order device indicated that the rate and extent of burst release from an otherwise stable carrier were affected by drug solubility and drug diffusion in an aqueous medium and by the drug loading content.[13] Such findings inspired more recent approaches to prevent burst release aimed at enhancing drug loading, inhibiting drug diffusion from the carrier, or both.

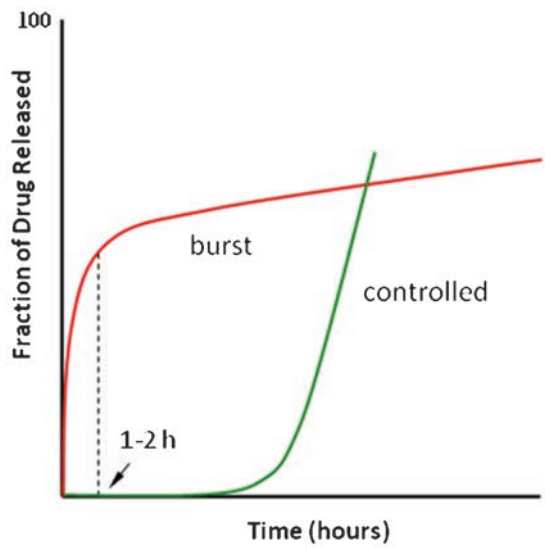

Figure 3.3 Sketch of ideal controlled release *vs.* premature burst release. Adapted with permission from ref. 8. Copyright 2012 Elsevier.

(1) Using new chemical processes to fabricate structured nanoparticles.

Polymeric micelles encapsulate drugs mostly *via* physical trapping based on hydrophobic interactions. They are generally fabricated by coprecipitation of the hydrophobic drugs with the hydrophobic blocks of amphiphilic copolymers by dialysis or the solvent-evaporation method,[14] assuming that the drugs and the hydrophobic blocks precipitate simultaneously and thus the drugs are completely embedded in the hydrophobic micelle core. However, in many cases this is not a very realistic assumption, as either the drugs can precipitate first or the core can form first, which prevents proper drug encapsulation in the core. For example, when the core forms first, most drug molecules may precipitate around the core, which are prone to burst release upon dispersion in an aqueous solution.[15]

Building on this finding, we proposed that coating the core with an additional hydrophobic layer would impose an extra diffusion barrier and thereby minimize burst drug release. Using a stepwise pH-controlled process, three-layer onion-structured nanoparticles (3LNPs) were synthesized that consisted of a poly(ε-caprolactone) (PCL) core, a pH-responsive poly[2-(*N*,*N*-diethylamino)ethyl methacrylate] (PDEA) middle layer, and a polyethylene glycol (PEG) outer coronal layer.[16] Compared to the conventional core-corona micelles, such 3LNPs were found to exhibit a significantly lower burst release of camptothecin (CPT) at physiological pH due to the effective barrier of the hydrophobic PDEA barrier.

The conventional method for preparing polymeric micelles through liquid solvent evaporation or dialysis offers little control of micellization *versus* drug precipitation. However, this can be accomplished with a near-critical fluid micellization (NCM) method to prepare drug-loaded polymeric micelles.[17] The solvating power of a near-critical fluid solvent is easily tunable with pressure. Thus, more selective and flexible micellization can be controlled by adjusting the pressure alone. At high pressures, drugs and polymers were molecularly homogenous in a near-critical solvent, whereas at moderate pressures micellization/drug encapsulation occurred (Figure 3.4A). With this process, PEG-PCL micelles, formed in a near-critical dimethyl ether/trifluoromethane, could be loaded with paclitaxel (PTX) as high as 12 wt% (Figure 3.4B). More recently, we prepared three-layered micelles formed by a stepwise NCM process that exhibited little, if any, burst release despite the high drug loading content (Figure 3.4C, D).[18] The biggest advantage of this NCM is that it uses the conventional Food and Drug Administration (FDA) approved materials to obtain high drug loading micelles with minimized burst or even burst-free. Such products are also free of contamination from organic solvents.

(2) Drug conjugation

The second approach to eliminate burst release is by conjugating drugs to the carriers *via* covalent bonds. Because the drug must be released once at the target, the covalent bonds or linkers must be cleavable in the tumor-cell environment. For instance, doxorubicin (DOX) was conjugated to a poly(L-aspartic acid) [P(Asp)] block in the block copolymer PEG-*b*-P(Asp) through amide[19,20] or hydrazone linkers.[21,22] Drugs can also be conjugated to the ends of hydrophobic blocks.[23,24] The resulting micelles formed from PEG-*block*-poly(L-amino acid)

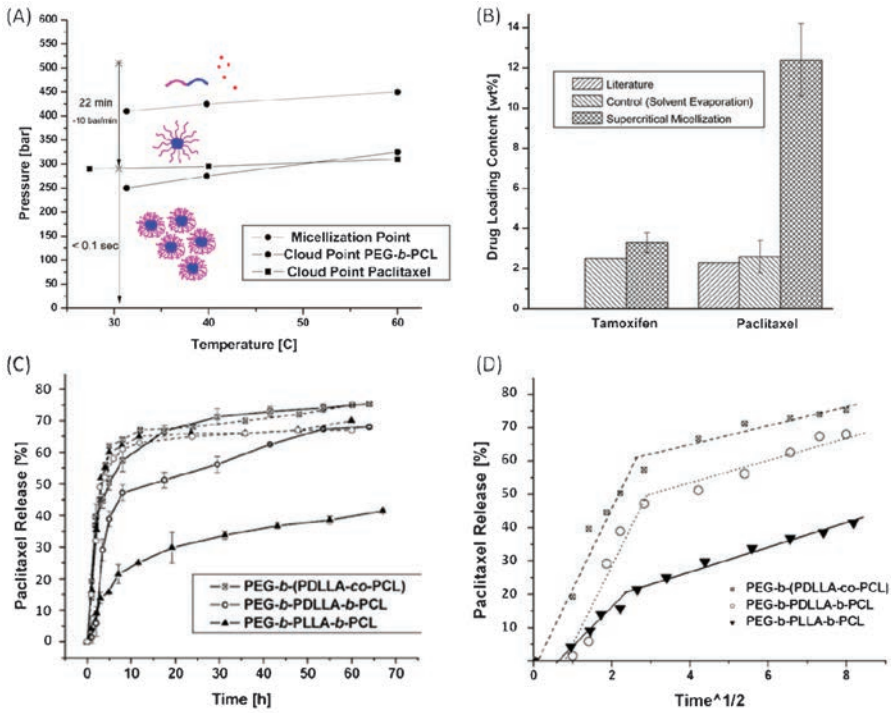

Figure 3.4 Nanoparticles prepared by a near-critical fluid micellization (NCM) method: (A) Micellization and cloud pressures of PEG-PCL and PTX in 70% dimethyl ether/30% trifluoromethane. (B) Drug loading contents of tamoxifen and PTX in PEG-PCL nanoparticles by the NCM process and conventional solution process. Adapted with permission from ref. 17. Copyright 2011 American Chemical Society. (C) Cumulative drug release as a function of time for a diblock and two triblocks copolymers. Solid lines represent micelles prepared by near-critical micelliazation while dashed lines represent micelles prepared conventionally by solvent evaporation. (D) Cumulative drug release plotted as a function of $t^{1/2}$ for experiments plotted in (C). Adapted with permission from ref. 18. Copyright 2012 American Chemical Society.

(PEG-*b*-PLAA) conjugated drugs eliminated any burst release.[25] Using labile linkers responsive to the tumor's extracellular or intracellular stimuli results in drug release triggered in a tumor extracellular environment.[26,27]

Our group demonstrated, using drugs as the hydrophobic part, directly making self-assembling amphiphilic prodrugs for fabricating burst-free carriers.[28] In this method, hydrophobic CPT molecules were conjugated to short oligomer chains of ethylene glycol (OEG) to form the amphiphilic phospholipid-mimicking prodrugs OEG-CPT or OEG-DiCPT (Figure 3.5). These prodrugs formed stable liposome-like nanocapsules with extremely high drug loading content but no burst release. Similar nanoparticles were prepared from an amphiphilic curcumin prodrug.[29]

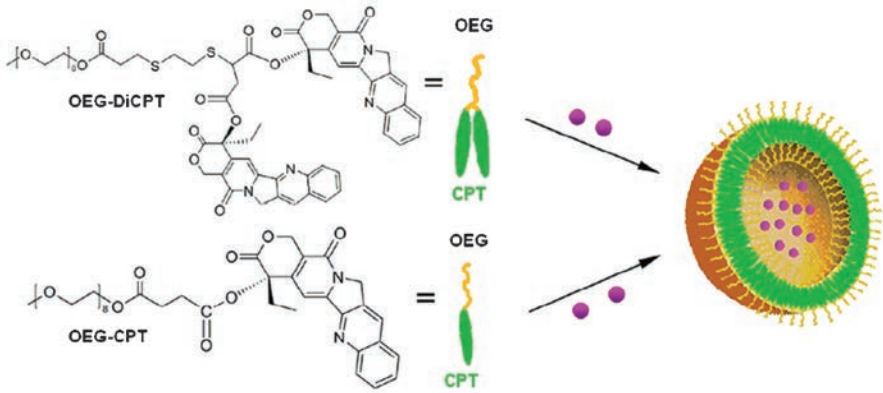

Figure 3.5 Amphiphilic CPT prodrugs (OEG-CPT and OEG-DiCPT) and their self-assembly into nanocapsules. Reprinted with permission from ref. 28. Copyright 2010 American Chemical Society.

The main disadvantage of such conjugation approaches is that they may change the drug chemical structure,[30] which in turn may reduce the pharmaceutical efficacy, not to mention the need for extensive preclinical tests and clinical trials before acquiring FDA approval.

(3) Core- or shell-crosslinked micelles

The third approach aimed at reducing the burst release is crosslinking the core or the corona shell of micelles. For example, Wooley *et al.* developed methods for fabricating shell-crosslinked micelles.[31] In order to de-crosslink the shell to allow drug release at the target site, a linker labile in the presence of intracellular glutathione (GSH) was used.[32] As intended, such crosslinked shells inhibited drug diffusion from the micelles, and hence reduced burst release. However, such a crosslinked shell becomes more rigid and hence loses its ability to repel serum proteins or other biomacromolecules,[33] and thus may not continue to be stealthy in circulation.

Covalent crosslinking of the micelle hydrophobic core can therefore be a preferable approach.[34,35] For instance, crosslinked micelles consisting of PEG-*b*-poly(acryloyl carbonate)-*b*-poly(D,L-lactide) (PEG-PAC-PDLLA) had high stability and significantly inhibited PTX release at low micelle concentrations compared to the non-crosslinked controls.[36] Lavasanifar *et al.* applied click chemistry and developed hydrolysable core-crosslinked PEG-*b*-poly(α-propargyl carboxylate-ε-caprolactone) (PEG-PPCL) micelles that exhibited a lower degree of PTX burst release than equivalent non-crosslinked micelles.[37] When the crosslinked core had disulfide linkers, it was shown to hold the drug tightly but release it quickly once in the tumor cell, due to the cleavage of the crosslinkages by intracellular GSH.[38] Similarly, thiolated Pluronic copolymer (Plu-SH) was demonstrated to form core-crosslinked micelles that were reversible *via* dithiothreitol (DTT)-breakable disulfide bonds, which inhibited the premature release in an aqueous solution.[39]

3.2.1.2 *Approaches to Increase Carrier Stability to Prevent Premature Release*

A thermodynamically unstable carrier (an unstable carrier for short) may dissociate before reaching its target and thus prematurely release the drug. Such an unstable carrier may dissociate fast or slowly, referred to as micelle dissociation kinetics (some authors[25] use "kinetic stability"). We always prefer carriers that are thermodynamically stable until they reach their target. At a given temperature, micelles form at the polymer concentrations above the critical micelle concentration (CMC):[40]

$$C_{\mathrm{CMC}} \approx \exp(-n\varepsilon_{\mathrm{h}}/k_{\mathrm{b}}T)$$

where $k_{\mathrm{b}}T$ is the thermal energy and ε_{h} is the monomer effective interaction energy with the bulk solution (related to χ in polymer physics). Polymers with a low CMC suggest a high thermodynamic stability, and *vice versa*. Usually, the longer the hydrophobic blocks, the more stable the micelles they form.[41] Thermodynamic stability is particularly important because locally, in circulation, micelles may dissociate if the block copolymer concentration falls below the CMC. It seems intuitive that a drug-loaded micelle may have a CMC that is different from its virgin drug-free analog, but, to a first approximation, it is common to neglect this difference.

Once the copolymer concentration falls below its CMC, the micelle dissociation rate can vary, depending on cohesive forces among the core-forming blocks. Chain insertion/expulsion and micellar fusion/splitting are two mechanisms that can explain the overall dynamic exchange between monomers and micelles.[41] Monte Carlo simulation indicated that chain insertion/expulsion played a major role when the polymer concentration was low.[42] Because chain mobility plays a crucial role, the hydrophobic blocks with a relatively high glass transition temperature (T_{g}) make the micelles dissociate much more slowly than those with a low T_{g}.[43] Furthermore, the size of the hydrophobic block and the hydrophilic-to-hydrophobic block mass ratio were found to affect the rate of micelle dissociation, from size-exclusion chromatography (SEC) experiments. For simple PEG-PCL copolymers, micelles formed from PEG-PCL (5000:4000 and 5000:2500) dissociated slowly; however, micelles formed from the PEG-PCL (5000:1000) dissociated quickly into monomers.[44]

Even though there is evidence that some polymeric micelles can be stable in serum even *in vivo*,[45] the stability of micelles in the blood is far from understood. Quite different from carriers tested in water or in buffer solutions, micelles in blood circulation can be extremely diluted and encounter various blood components which may promote micelle dissociation. Burt *et al.* prepared radiolabeled PTX-loaded PEG-PDLLA micelles and found that PTX was rapidly released from the micelles, and the diblock copolymer was cleaved into its two polymer components in the blood.[46,47] Maysinger *et al.* conjugated fluorescein-5-carbonyl azide diacetate to PEG-PCL micelles and noticed that they were stable in buffer solutions but unstable in serum-containing culture media with or without

cells.[48] Recently, Cheng *et al.* employed a fluorescence-resonance energy-transfer (FRET) technique to demonstrate that PEG-*b*-PDLLA micelles were not stable in the bloodstream due to the influence of α- and β-globulins rather than γ-globulin or serum albumin.[49] Based on those results, Cheng *et al.* summarized the possible mechanisms responsible for the micelle decomposition induced by serum proteins,[41] including protein adsorption,[48,49] protein penetration,[50,51] and drug extraction.[41] What exactly happens to the micelles after injection is poorly understood because it is hard to measure and estimate micelle concentration locally in the bloodstream.[52] Cheng *et al.* tracked unmodified copolymer micelles using the FRET imaging method, but unfortunately no direct evidence proved that the CMC was unchanged by incorporating a FRET pair.[53]

However, there is no doubt that, directionally, the lower the CMC, the higher the probability of micelle stability in the bloodstream. Therefore, the most common strategy to enhance the micelle stability is to reduce its CMC. Compared to liposomes, polymeric micelles usually have a much lower CMC, at a micromolar level, which imparts a higher stability. A further reduction of polymeric micelle CMC can be achieved by increasing the core-forming block hydrophobicity, molecular weight, or both.[40] One example is that of chemically modified Pluronics: Pluronic/PCL copolymeric nanospheres exhibited a lower CMC.[54,55] Another interesting finding is that stearic acid as side chains can keep micelles stable even in the presence of serum.[56] In the presence of serum albumin, α- and β-globulins, or γ globulins, the micelles from PEG-*b*-poly(*N*-hexyl stearate L-aspartamide) (PEG-*b*-PHSA) copolymers with nine stearic acid side chains still existed after two hours.

Crosslinking is a straightforward method to stabilize micelles. While the covalent crosslinking of the micelle core or shell can inhibit burst release from a stable micelle, it can also inhibit or prevent micelle dissociation. For instance, PEG-PCL micelles with cores crosslinked by radical polymerization of the double bonds introduced to the PCL blocks turned out to be more stable.[57] Biodegradable thermosensitive micelles with crosslinked cores formed from PEG-*b*-[*N*-(2-hydroxyethyl methacrylamide)-oligolactates] [PEG-*b*-p(HEMAm-Lac_n)] kept their integrity upon dilution and only degraded after cleavage of the ester bonds in the crosslinkers.[58]

The caveat, however, is that crosslinking reactions usually occur after core formation, which can alter the structure and properties of the encapsulated drug. To overcome this potential problem, our group developed stable core-surface crosslinked micelles (SCNs), shown in Figure 3.6, made from

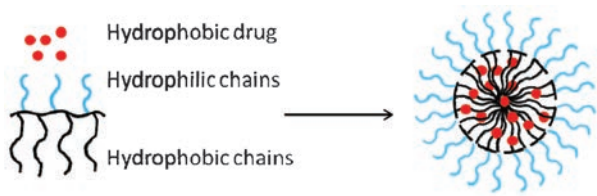

Figure 3.6 Formation of SCNs from amphiphilic brush polymers. Adapted with permission from ref. 59. Copyright 2004 American Chemical Society.

amphiphilic polymer brushes.[59] The key point is that the backbones of the polymer brushes acted as crosslinkages on the hydrophobic core surface, instead of chemical crosslinking, which substantially enhanced the micelle stability. Specifically, the resulting micelles had much lower CMCs than corresponding PEG-PCL block copolymers.

For the excretion of the nanocarriers from the body, crosslinked micelles must be able to break into small polymer chains. Toward this end, reversible crosslinking triggered by different stimuli like pH,[60] UV light,[61] or others[62] was later developed. Historically, pH-sensitivity was the first one used to trigger a desired carrier change because cancer or inflammation makes the extracellular pH at the disease site acidic.[63] For instance, micelles formed from the triblock copolymer PEG-*b*-poly[*N*-(3-aminopropyl)methacrylamide]-*b*-poly(*N*-isopropylacrylamide) (PEG-PAPMA-PNIPAM) were shell-crosslinked with terephthaldicarbaldehyde (TDA) at pH 9 *via* cleavable imine linkages.[60] However, at pH < 6 the hydrolytic cleavage of the imine crosslinkages occurred. Other examples[64,65] were inspired by crosslinking, using disulfide linkages that are sensitive to intracellular GSH ($\sim$0.5–10 mM as opposed to $\sim$20–40 μM in the bloodstream[66]). For example, micelles made of a PCL-*b*-poly[(2,4-dinitrophenyl)thioethyl ethylene phosphate]-*b*-PEG (PCL-*b*-PPE_{DNPT}-*b*-PEG) triblock copolymer, crosslinked with disulfide bonds, were found to be stable in circulation but quickly decomposed in intracellular fluid.[65]

Even if the micelle happens to be unstable, its decomposition rate can be reduced by choosing a stiff or bulky core. Toward this end, benzyl groups were introduced to increase the rigidity of hydrophobic cores.[67] Lavasanifar *et al.* synthesized benzyl carboxylate-substituted ε-CL monomers and prepared PEG-*b*-poly(α-benzyl carboxylate ε-caprolactone) (PEG-*b*-PBCL) copolymers.[67] For comparison, they also prepared PEG-*b*-poly(α-carboxyl-ε-caprolactone) (PEG-*b*-PCCL) by further catalytic debenzylation. Their results demonstrated that the stability of micelles with core structures containing aromatic groups (PEG-*b*-PBCL) was higher than that of the parent PEG-PCL micelles and of the PEG-*b*-PCCL micelles. The micelle decomposition rate can also be reduced by crystallizable hydrophobic blocks.[45,68] Another approach is to enhance ionic or hydrogen bonding interactions in the micelle core. For example, polyion complex (PIC) micelles with oppositely charged macromolecules, such as DNA or peptides, are resistant to enzymes in the bloodstream,[69] but they disassemble once the salt concentration rises above a certain threshold.[70] Hedrick *et al.* introduced urea functional groups,[71] while Zhu *et al.* introduced DNA base pairs[72] into block copolymers to show that hydrogen bonding can reduce micelle decomposition rates.

3.2.1.3 Approaches to Achieve Robust Intracellular Release

The chemical forces discussed above that make carriers retain drugs can conflict with the need for a rapid and complete release at the target site. Drugs become active only after liberation from their carriers.[73,74] DOX that was stably bonded to the nanoparticle core of poly(lactic-*co*-glycolic acid) (PLGA)[75] or P(Asp)[76]

showed low or even no anticancer activity.[77] The rate of drug release is also very important because tumor cells have intrinsic and acquired drug-resistance mechanisms to remove intracellular drugs,[78,79] *e.g.* as a result of cell-membrane-associated multidrug resistance to efflux drugs[79,80] and cell-specific drug metabolism or detoxification.[81] Tumor cells can also sequestrate some weakly basic drugs in their lysosomes and use biomacromolecules to bind drugs to limit their access to their targets. Thus, it is only the intracellular drug molecules free to bind to their targets that are useful therapeutically. Such free drug concentration in the cytosol, herein referred to as the effective cytosolic drug concentration [D] (effective [D] for short) determines the overall therapeutic efficacy.

Drug carriers that reach tumor cells are generally internalized by endocytosis[82,83] and routed to endosomes and then acidic lysosomes, as shown in Figure 3.7. The internalized carrier can release the drug in one of two possible ways or both: (1) within the lysosome, followed by drug diffusion, as illustrated with the upper path in Figure 3.7, and (2) in the cytosol, following the carrier escape from the lysosome, as illustrated in the lower path in Figure 3.7. For a specific tumor cell, [D] is a function not only of the cellular uptake of the carrier but also of its drug release rate (see Eq. 1 on the figure). If either ends up being "too little, too late," it can prevent reaching an effective [D].

(1) Intra-lysosome release

The intra-lysosome release mechanism (upper path in Figure 3.7) works for most carriers that can be endocytosed into endosomes/lysosomes. The pH in endosomes decreases progressively, typically near 6 in early endosomes, near 5 in late endosomes, and about 4–5 in lysosomes.[84] This acidic pH and the

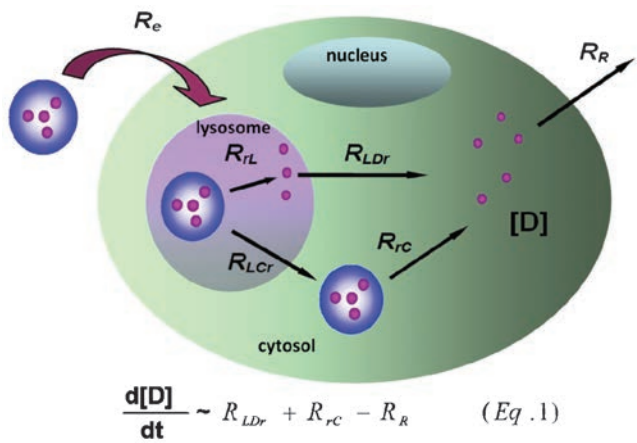

Figure 3.7 Cytosolic drug accumulation by drug delivery: [D], effective drug concentration in cytosol; R_e, endocytosis rate of the carrier; R_{rL}, drug release rate of the carrier in lysosomes; R_{rC}, drug release rate of the carrier in cytosol; R_{LDr}, lysosomal drug release rate; R_{LCr}, lysosomal-carrier escape rate; R_R, the overall rate of drug removal by P-gp pumps and drug consumption by other forms of drug resistance. Reprinted with permission from ref. 8. Copyright 2012 Elsevier.

special enzymes in lysosomes can trigger drug release from the carriers into lysosomes.[85] Because the harsh environment of lysosomes can easily degrade drugs sensitive to acid or these enzymes,[86,87] the drug must quickly diffuse out into the cytosol to avoid deactivation.

Polymer–drug conjugates, in which the drugs are conjugated to the polymer carriers *via* lysosomal pH-labile linkers, are the most popular design. Hydrazone and *cis*-aconityl are examples of such a linker.[88,89] Ulbrich *et al*. conjugated DOX to *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers *via* this hydrolytically labile spacer.[90] The results showed a fast DOX release from the polymer at intracellular pH 5, whereas at pH 7.4 the conjugates retained the drug. Recently, they synthesized new biodegradable star conjugates consisting of poly(amido amine) (PAMAM) dendrimer cores and HPMA grafts bearing DOX *via* hydrazone bonds.[89] The *in vitro* cytotoxicity and *in vivo* antitumor activity of all such conjugates were higher than those of classic conjugates. Another example is Wang *et al*.'s dual pH-responsive polymer–drug conjugate PPC-Hyd-DOX-DA, which could respond to the tumor extracellular pH gradients *via* amide bonds and the tumor intracellular pH gradients *via* hydrazone bonds.[91] Lysosomal degradable peptides [*e.g.* glycylphenylalanylleucylglycine (GFLG)], which are cleavable by lysosomal enzymes to release the drugs, are also used for drug conjugation.[74,92] For instance, DOX was conjugated to HPMA copolymers *via* GFLG peptides to form a cleavable HPMA-GFLG-DOX conjugate.[74]

Lysosomal pH has also been used to trigger drug release from pH-sensitive nanoparticles.[93,94] For example, pH-sensitive micelles composed of reducible poly(β-amino ester) (RPAE) cores dissociated rapidly in an acidic environment and at high levels of reducing reagents, inducing fast intracellular release.[94] Carriers with a core made from amine-containing hydrophobic polymers, such as polyhistidine (PHis), can be protonated and thus dissolve in acidic lysosomes, thereby releasing the drug.[95] Our group showed that a rapid cytoplasmic release from carriers could increase the anticancer activity of drugs.[96,97]

The additional advantage of such amine-containing polymers is that they may also have endosomal membrane-disruption activity induced by a "proton sponge" mechanism,[98] and thus disrupt the lysosomal membrane and further release the drug into the cytosol. Some specially designed polyacids, such as poly(propylacrylic acid) (PPAA),[99,100] were shown to disrupt endosomes at pH 6.5 or below, causing the cytosolic release of cargo molecules.

(2) Intra-cytosol release

An alternative to the carriers designed for intra-lysosome release discussed above is carriers designed for intra-cytosol release (lower path in Figure 3.7). Such intra-cytosol-release carriers retain the drug until escape from the endosome/lysosome[101] and then release the drugs into the cytosol, hence avoiding lysosomal drug retention and degradation. This is particularly important in small interfering RNA (siRNA) or gene delivery and thus various approaches have been explored to facilitate the endosomal release of DNA or RNA complexes.[102,103] In this approach the carriers must respond to the lysosomal environment for lysosomal escape and to the cytosolic environment for drug release.

Stealth carriers, such as HPMA[104] and pegylated particles, cannot diffuse through the lysosomal membrane and thus can be retained in the lysosomes for a long time. For instance, PEG-PCL particles were found confined in lysosomes.[105] Thus, they must be functionalized with lysosomal membrane-destabilizing polymers such as PPAA,[99,100] pH-dependent fusogenic peptides,[106,107] or cationic polymers such as polyethylenimine (PEI)[108] or histidine-rich peptides or polymers.[109] For cationic polymers or peptides, on the other hand, it is important first to mask their cationic charges (from primary and secondary amines) at physiological pH, so the carriers can be used for i.v. administration. However, once inside the tumor lysosome, the cationic charges are recovered to lyze the lysosomal membrane for escape. Such a ''negative-to-positive charge-reversal'' method makes the carrier stealthy in circulation, but enables endosomal lysis, once in lysosomes.[108,110]

Removal of a cleavable PEG layer can also allow lysosomal escape.[111] For instance, a PEG-cleavable lipid, *via* an acid-labile vinyl ether linker, was used for pegylation of (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine) (DOPE) liposomes. At acidic lysosomal pH the vinyl ether linker hydrolyzed and the PEG layer was removed from the DOPE liposomes, enabling DOPE, which has excellent fusogenic capacity, to fuse with the lysosomal membrane for escape.[112] Disulfide linkages were also used to detach PEG and make the drug-loaded carriers quickly escape from the endosomes.[113] After the particles were internalized by cells and trapped by endosomes, the PEG layer was removed. The exposed particles interacted with the endosomal membrane, increased the endosomal pressure, or both, resulting in destruction of the endosomal membrane to enable effective endosomal escape.[113]

Most carriers reaching the cytoplasm have already experienced an initial burst release and are in a slow, diffusion-controlled drug release process, *e.g.* nanoparticles with cores made of solid glassy polymers such as PCL or polylactide (PLLA).[114] According to Eq. 1 (see Figure 3.7), such a slow drug release profile may not be able to lead to a high [D] lethal to cancer cells. Thus, carriers responding to cytosolic signals have been developed for faster drug release. The most common is a cytosolic redox signal resulting from an elevated intracellular GSH concentration ($\sim$10 mM) compared to that in the bloodstream ($\sim$2 μM).[115] GSH can effectively cleave the disulfide bonds to release conjugated drugs.[66,116] It is thus used to trigger decomposition of micelles with hydrophobic parts linked by disulfide bonds[117] or other carriers crosslinked[118] or gated[119,120] with disulfide linkers. It has also been observed that removal of the PEG corona could increase the drug release rate.[121,122]

3.2.2 *2S*: Stealthy in Circulation and Tumor Penetration *versus* Sticky to Tumor Cells

The second major material challenge is how to impart nanocarriers' stealth ability to circulate in blood for a long time and after extravasation to penetrate

deep into the tumor, to the cells away from the blood vessels, but become effectively sticky upon interacting with tumor cells for fast cell internalization.

To be stealthy for a long circulation time in the blood compartments has been recognized as essential for a nanocarrier to achieve passive tumor targeting,[123,124] whereas transport in the tumor tissue after extravasation has been gradually realized in recent years.[125,126] Tumor resistance to anticancer drugs not only involves the cellular and genetic drug resistance mechanisms,[78,127] but also the physiological barriers of solid tumor tissues.[128,129] It is found that tumor drug distribution is not uniform. Drugs are rich in the areas surrounding the blood vessels and the concentration declines sharply away from the blood vessels, owing to the compact structure of tumor tissues.[130] Thus, the most aggressive tumor cells located in these hostile microenvironments (low pH and low pO_2) are actually exposed to few drugs.[7] Moreover, the exposure of those cancer cells to sublethal concentrations of anticancer drugs may facilitate the development of resistance.[7] Therefore, it is important for the nanocarrier to remain stealthy after extravasation for tumor penetration. It can be imagined that a nanocarrier strongly interacting with surrounding cells and matrix will be trapped there and cannot travel a long distance.

3.2.2.1 Approaches to Stealth Surfaces

(1) In circulation

The nanocarrier's stealth character hinges on many factors, including surface properties,[123] size,[131] and even shape.[132,133] In circulation, those with molecular weights below the renal threshold (*e.g.* 40 kDa for PEG) or sizes below 5 nm are rapidly cleared from the blood by glomerular filtration,[86] while those with diameters above 200 nm will be scavenged by the RES, mainly the liver and spleen.[131,134]

Most stealth carriers capable of avoiding opsonization[135] and interaction with the mononuclear phagocyte system (MPS)[131] are made from HPMA,[136,137] PEG, or polysaccharides[138] (*e.g.* heparin[139]). Nanoparticles coated with a layer of these polymers become stealthy by both hydration and steric hindrance.[140] For example, pegylation of particles or liposomes is well established,[135,141] and the DOX-loaded stealth liposome named Doxil® was approved by the FDA for cancer therapy.[142] Huang *et al.* reported that, on 100 nm liposomes pegylated with 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-PEG_{2000} (DSPE-PEG_{2000}), PEG chains were arranged in a mushroom configuration at a DSPE-PEG fraction less than 4 mol% but in a brush configuration at a DSPE-PEG content greater than 8 mol%.[124] The high density of PEG chains on the liposome surface with the brush configuration was the key to reduce liposome liver sequestration.[124] Discher *et al.* incorporated the PEG brushes onto polymersomes and obtained polymersomes having a blood circulation time two-fold longer than pegylated liposomes.[143] Dai *et al.* pegylated single-wall carbon nanotubes (SWNT) and found that, with the increase of linear PEG chain length from 2 kDa to 5 kDa, the blood circulation

time of pegylated SWNTs was significantly extended, but a further increase of the PEG chain length showed no significant effect.[144] Although pegylation reduces the recognition of the carriers by the MPS system and thereby extends their blood circulation time, the ''accelerated blood clearance (ABC)'' phenomenon was observed upon repeated injection of pegylated liposomes[145,146] due to IgM bound to pegylated liposomes secreted into the bloodstream after the first dose.[147] Such an immune reaction against the pegylated liposomes occurred in the spleen at least 2–3 days after the first administration.[145,146]

The carrier shape is also recognized as an important parameter that can substantially affect the blood circulation time. In fact, Mitragotri *et al.* reported that the particle shape, not size, played a dominant role in phagocytosis of polystyrene (PS) particles of various sizes and shapes: the rod-like particles entered the cells much faster.[148] Discher *et al.* found that flexible worm-like micelles efficiently evaded the RES and circulated in the blood for a week,[149,150] much longer than spherical micelles. Dai *et al.* found that carbon nanotubes pegylated with long PEG chains exhibited a long blood circulation time ($t_{1/2}$ = 22.1 h) upon intravenous injection into mice.[151] All these studies suggest that particle phagocytosis can be inhibited by minimizing its size-normalized curvature.[148,152] Thus, particle shape is an important variable to make it remain stealthy in circulation long enough for enhanced tumor accumulation.[149,150,153]

(2) In tumor tissue

Solid tumors are characteristic of poorly structured blood vessels,[154] a stiff extracellular matrix (ECM),[155–157] tightly packed cells,[158] high interstitial fluid pressure (IFP),[159,160] and drug metabolism and binding.[126] Together they impose strong diffusion barriers to nanocarriers, and even small molecules (Figure 3.8).[125,161]

For instance, it seems intuitive that as long as a nanocarrier extravasates from tumor blood capillaries and releases the carried small molecular drugs, the drug molecules will diffuse deep into the tumor tissue. Actually, free drugs, either hydrophobic or carrying positive charges, cannot migrate far from the nanocarrier due to their avid binding.[162] Diffusion of larger macromolecules, such as bovine serum albumin (BSA, 68 kDa, 9 nm in diameter) and immunoglobulin G (IgG, 150 kDa, 11 nm in diameter), in the tumor ECM is also hindered compared to that in buffered saline.[163] After extravasation, dextrans with a molecular weight between 40 and 70 kDa (and a diameter of 11.2–14.6 nm) were observed to be concentrated near the vascular surface.[130] Apparently, nanocarriers, which are larger in size than BSA and dextrans, are likely to face greater difficulties in tumor penetration.[161] Chan *et al.* systematically examined the effect of nanoparticle size on tumor penetration using sub-100 nm pegylated gold nanoparticles.[164] As expected, larger nanoparticles appeared to stay near the vasculature while smaller nanoparticles (20 nm in diameter) rapidly diffused into the tumor matrix (Figure 3.9). Similarly, Lee *et al.* demonstrated that PEG-PCL micelles with a mean diameter of 25 nm diffused further away from the blood vessels compared to those with diameters of 60 nm, which mainly remained in the perivascular

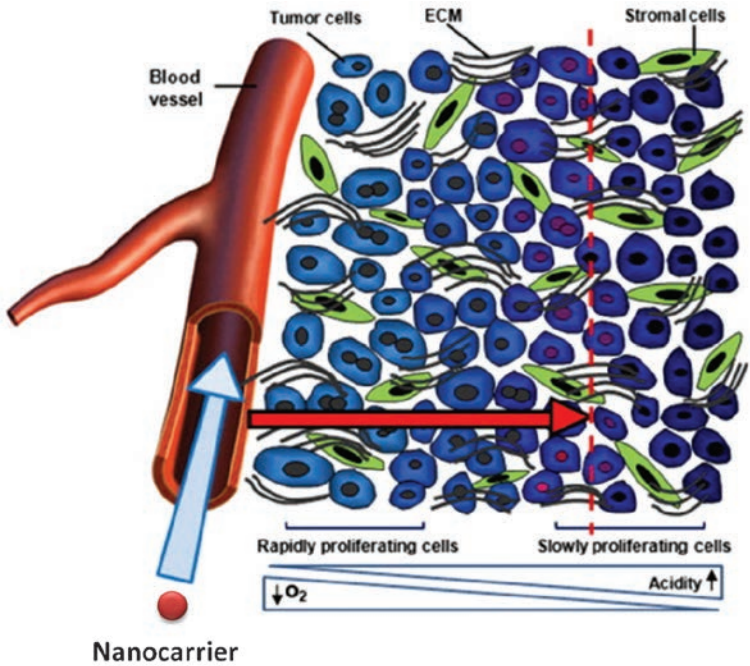

Figure 3.8 Scheme of solid tumor tissue which is characteristic of stiff ECM and compact tumor cells. Adapted with permission from ref.125. Copyright 2012 Elsevier.

regions.[165] Furthermore, Kataoka *et al.*[166] and Liang *et al.*[167] also proved that small-sized nanocarriers were essential for improved diffusion.[168] However, small-sized nanocarriers possess a high probability to be fast cleared during the circulation, as mentioned above.

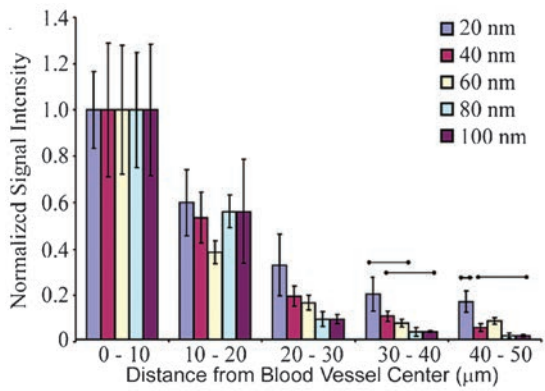

Figure 3.9 Size-dependent penetration of nanoparticles within tumor tissues. Reprinted with permission from ref. 164. Copyright 2009 American Chemical Society.

Besides size, the surface charge of nanocarriers also influences their penetration into tumor tissues. Cationic liposomes (150 nm) were observed not able to travel far into the tumor interstitium.[169] Recently, Forbes *et al.* compared the penetration of oppositely charged gold nanoparticles (+30 *vs.* −36 mV, 6 nm) into cylindroidal cell aggregates.[170] Cationic nanoparticles were taken up by the proliferating cells on the periphery of the cylindroids, whereas anionic nanoparticles were better at penetrating the extracellular matrix and entered hypoxic necrotic cells in the core of the mass. As a matter of fact, the extracellular matrix presents as an effective electrostatic bandpass, suppressing the diffusive motion of both positively and negatively charged objects, which allows uncharged particles to easily diffuse through while effectively trapping charged particles (Figure 3.10).[171] Jain *et al.* demonstrated that the optimal particles for delivery to tumors should be neutral after exiting the blood vessels.[172]

Another issue that needs addressing is affinity.[173] The affinity plays an important role in antibody-based tumor targeting nanocarriers. It was visualized that the antibody distributed mostly in perivascular regions rather than homogeneously in tumor cells.[174] Reports revealed there was an inverse relationship between affinity and penetration, *i.e.* the antigen–antibody interaction in the tumor tissue imposed a binding-site barrier that retarded antibody penetration and caused a heterogeneous distribution.[175–177] The higher the affinity of binding and the higher antigen density caused fewer free molecules to be able to penetrate farther into the tumor interstitium.[175,176] Increasing the antibody dose gave better penetration and more uniform distribution.[175]

Therefore, it is clear that to deliver a sufficient drug concentration to the tumor center region lacking vascular perfusion, where the most aggressive and resistant cells reside, the nanocarrier should not release the carried drug after

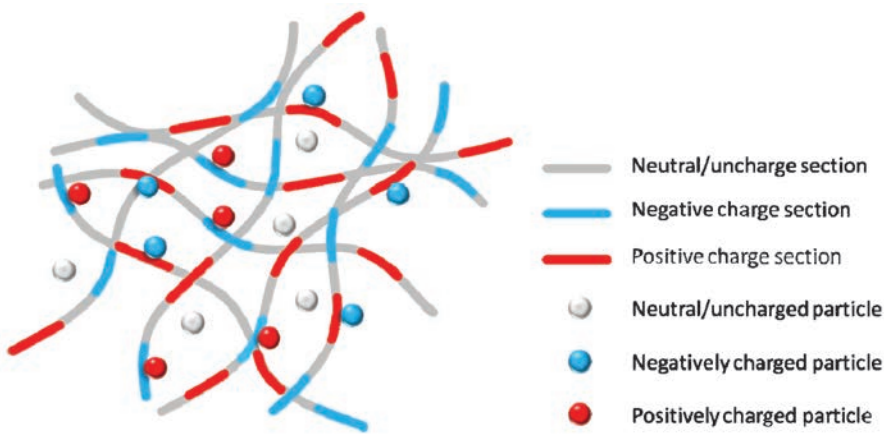

Figure 3.10 Scheme of the ECM exerts the filtering function in tumor tissue. Charged particles (red, blue) are trapped in the respective region of opposite charge (blue, red), while neutral particles (gray) can diffuse nearly unhindered.

extravasation but should further diffuse deep into the tumor. This requires the nanocarrier to remain slippery and have as small a size as possible. Thus, it is better for the nanocarrier to be neutral and not to present any binding groups (including targeting groups) until reaching the center of the tumor.

3.2.2.2 *Approaches to Becoming Sticky to Tumor Cells for Cellular Uptake*

After reaching the targeted region the nanocarrier should efficiently enter the cells for drug release. Now the same properties that impart stealth to the nanocarrier prevent it from cellular uptake by tumor cells. Nanocarriers that are negatively charged will be repelled from the cell membrane due to the electrostatic repulsion. The PEG corona of pegylated polymeric micelles or liposomes retards their interaction with cell membranes due to steric hindrance. Thus, once in the tumor, the carrier must become cell-binding or sticky to targeting tumor cells for fast cellular uptake.[178] The challenge is how to reconcile these two opposite requirements, stealth for circulation and diffusion *versus* sticky for targeting. For instance, it is well known that positively charged carriers reliably stick to cell membranes due to electrostatic adsorption triggering fast cellular uptake, but positively charged carriers are not suitable for *in vivo* applications because they are systemically toxic[179] and have short circulation times.[180]

One strategy to convert a carrier from stealth circulation to sticky targeting is to equip it with PEG groups that are cleavable upon encountering a tumor-specific stimulus. Once the PEG chains are removed, the bare particle can be adsorbed onto the cell membrane. Toward this end, Thompson *et al.* prepared acid-labile PEG-conjugated vinyl ether lipids to stabilize fusogenic DOPE liposomes.[181] At lower pH, the PEG layer was removed by the acid-catalyzed hydrolysis of the vinyl ether bond, triggering membrane fusion. Similarly, Harashima *et al.* connected PEG to the lipid through a matrix of metalloproteinase (MMP)-cleavable peptide.[182,183] MMP is overexpressed in tumor-tissue angiogenesis, invasion, and metastasis[184] and thus the peptide can be degraded quickly in tumors. They prepared a multifunctional envelope-type nano device (MEND) using the PEG-peptide lipid and found that pDNA expression was dependent on the MMP expression level in the host cell.

Positive charges can promote carrier adsorption on the negatively charged membrane and hence trigger adsorption-mediated endocytosis. Thus, an alternative is to use tumor extracellular acidity to impart positive charges to the carrier by a "charge-reversal" technique (illustrated in Figure 3.11). Amine-containing carriers, such as PCL-*b*-PEI,[108] poly(L-lysine) (PLL),[185] and PAMAM dendrimers,[110] were amidized to acid-labile β-carboxylic acid amides to make them negatively charged at physiological pH. Once in weakly acidic tumor extracellular fluid, the amides hydrolyzed and regenerated the amines with cationic charges, which led to fast cellular uptake (Figure 3.11A). In yet another example, a pH-responsive layer becomes positively charged at tumor extracellular acidity but collapses, forming a middle layer, at neutral pH (Figure 3.11B).[16] Bae

et al. reported tumor extracellular pH-triggered TAT-presenting micelles. The TAT moieties were anchored to a PEG micelle corona and shielded at pH >7.0 by their electrostatic complexation with poly(methacryloyl sulfadimethoxine) (anionic PSD)-PEG (PSD-*b*-PEG) diblock copolymer. At pH 6.6, however, PSD turned to a nonionized form and fell off the TAT, exposing it and enabling the micelle a fast cellular uptake.[186] Another design was to anchor TAT onto the PEG corona through a pH-sensitive PHis spacer. At pH 7.4, the PHis was water insoluble, which kept the TAT moieties buried in the PEG corona. At pH lower than 7.2, however, ionization of the PHis spacer made it water soluble, which stretched it, exposing the TAT on the corona surface.[187]

The most common approach enabling a carrier to become sticky to the cell membrane is to decorate it with a ligand whose receptors are overexpressed on the cancer-cell membrane. The ligand–receptor binding enables receptor-mediated endocytosis, promoting cellular uptake.[11,188] Only a few ligands are needed for rapid internalization.[189] More ligand groups can theoretically increase uptake, but a high surface ligand density may make the carrier less stealthy as a result of opsonization-mediated clearance.[190] Many examples of targeting ligands include folic acid,[191] peptides,[192–194] antibodies,[195–197] transferrin,[198] aptamers,[199,200] and other moieties[201] that have been tested and subsequently reviewed.[2,202]

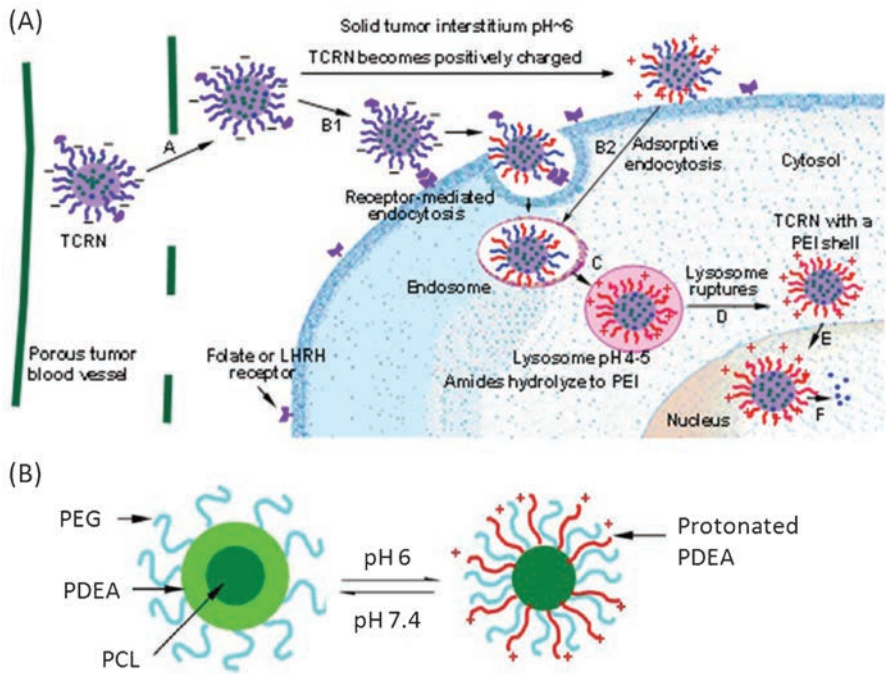

Figure 3.11 (A) The charge-reversal concept for drug delivery. Reprinted with permission from ref. 14. Copyright 2010 Elsevier; (B) The pH-responsive three-layered nanoparticles (3LNPs). Reprinted with permission from ref. 16. Copyright 2008 American Institute of Chemical Engineers.

3.3 The Material Excipientability and Production Process Scale-Up Ability

The *2R2S* capability for nanocarriers discussed in the previous sections determines the adsorption, distribution, metabolism, and excretion (ADME) of the carried drug. Such a nanocarrier simultaneously having *2R2S* capability can deliver a high cytosolic drug concentration and give rise to high therapeutic efficacy. However, this is not sufficient for it to be translational.[203–205] The nanocarrier itself should also have proper ADME. According to Choi and Frangioni, safety and clearance (renal or hepatic) and a proper stealth surface should be included among the basic criteria for clinical translation of formulation/materials administered to humans,[205] "from the benchtop to the bedside" translation. Thus, a nanocarrier must meet the requirements for the pharmaceutical excipient for i.v. uses. For simplicity, this ability of the nanocarrier material(s) to be used or approved to be an excipient, herein denoted as excipientability, is the second element for a nanocarrier to be translational (see Figure 3.2). It goes without saying that the production of the nanocarrier and the resulting nanomedicine should be able to be scaled-up and establish the required GMP, or scale-up ability, for short. Some of these important points of the two key elements are summarized as follows.

1) Safety. The nanocarrier itself should have proper ADME and no nanotoxicity, and should be nontoxic and easy to excrete completely from the body *via* the liver (into bile) or the kidneys (into urine) or both. This is because retention of polymers or nanosized materials in the body, even inert polymers like polyvinylpyrrolidone (PVP),[206–208] can cause health problems. The threshold for rapid renal excretion is about 5.5 nm in hydrodynamic diameter. This corresponds to a molecular weight of about $\sim$ 45 kDa for HPMA[209] and 40 kDa for PEG.[86]

2) Approval. In order to expedite and increase the probability of the approval success, the carrier should have a clear and simple structure with known degradation products. An even better case would be that it is made of FDA-approved building blocks.

3) Production scale-up. This involves the feasibility of making large volumes of consistently reproducible quality to establish GMP. For instance, because the molecular weight of a polymer–drug conjugate strongly affects its pharmacokinetics, the polymer itself must have consistently low polydispersity and reproducible average molecular weight from batch to batch. The same applies to drug-loaded micelles made of block copolymers, such as PEG-PCL, in addition to reproducible particle size, particle-size distribution, and drug-loading efficiency and content. As the micelle structure becomes more and more complicated, the number of quality control parameters drastically increases,[14,210] which makes it more and more difficult to produce an acceptably consistent formulation. Also, although not crucial to clinical success, it is also worth considering a high, ideally close to 100%, drug-loading

efficiency to simplify the manufacturing process and minimize losses of these very expensive anticancer drugs.

4) High drug-loading content. In current commercial formulations, the drug-loading content tends to be on the low side.[211–213] High drug-loading contents are needed to minimize the body's exposure to excipient carrier matter, even if it is biocompatible and relatively benign. For instance, PEG-containing liposomal carriers may induce acute immune toxicity, manifested in hypersensitivity reactions (HSRs).[214,215]

3.4 Challenges of Rational Design for Translational Nanomedicine

With the above analysis in mind, it is clear that the key to translational nanomedicine is to develop nanocarriers with optimal *2R2S* capability, excipientability, and scale-up ability.

As for the nanocarrier *2R2S* capability, we still do not have ones that can *fully and simultaneously* achieve the *2R2S* capability, despite a large volume of the scientific literature on each topic separately, or on various subsets of them, giving rise to unsatisfied therapeutic efficacy and side effects. As a consequence, a particular problem of those systems is that a large majority doses of the drugs are still sequestrated in the liver or spleen, even though the tumor drug accumulations are indeed enhanced compared to free drugs.[133,216] For instance, the PF-PTX micelles[217] and IT-101 CPT conjugates[218] give drug accumulation in tumors much better than Taxol® and CPT, respectively, but the total amounts of drugs accumulated in the liver were still about 4.5 and 3.5 times of those in tumors. In many cases, only a few percent of the injected drugs were in the tumors. Thus, for many nanomedicine systems, liver toxicity is the killer for further developments. Other necessities are how to achieve effective cellular uptake of the nanocarriers once in the tumor and robust intracellular release. Delayed or insufficient intracellular release directly leads to lower cytotoxicity than the free drugs.[219,220]

The material excipientability of nanocarriers and the production scale-up ability of the nanocarriers and their nanomedicine systems are equally important. For instance, a large variety of inorganic nanomaterials and sophisticated polymeric nanostructures have been proposed and investigated as nanocarriers for cancer drug delivery. These studies provide useful proof-of-concepts and rich insights into various aspects of cancer drug delivery essential to the design of nanocarriers towards *2R2S* capability, but those aimed at clinical applications must comprehensively design and characterize their materials, nanosize effects, and scale-up ability. Of the three, the material is the basic concern for a translational nanocarrier. If the material used for the nanocarrier is not proper for *in vivo* clinical uses (for instance, inherently toxic or non-clearable from the body), the resulting nanocarrier, even with perfect nanosize effects and *2R2S* capability, would not be able, or take an impractically long time, to be translated into clinics. Thus, except for proof-

of-concepts, it is better to look into these issues early at the bench in order for a successful nanocarrier to move forward quickly.

3.5 Conclusion

The challenge to develop truly translational nanocarriers and nanomedicine is to use excipientable materials and processes of scale-up ability to produce nanocarriers with optimal *2R2S* capability. While research aimed at proof-of-concepts remains important, it is important to increasingly focus on comprehensive approaches or systems that include *all* the three key elements as early as possible in the innovation chain, to speed up developments of translational nanomedicine.

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