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Challenges in design of translational nanocarriers

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ABSTRACT

Cancer drug delivery achieving high therapeutic efficacy and low side effects requires a nanocarrier to tightly retain the drug, efficiently reach the tumor, then quickly enter the tumor cells and release the drug. Furthermore, the nanocarrier intended for clinical applications should use materials safe as pharmaceutical excipients and its formulation (nanomedicine) should have good manufacture processes with scale-up ability. Thus, the challenge is to design safe, approvable, and easily scaled-up nanocarriers that simultaneously meet the two pairs of requirements of 'drug retention in circulation *versus* intracellular release' and 'stealthy in circulation *versus* sticky (cell-binding) in tumor' at the right places in order to deliver a cytosolic drug dose lethal to cancer cells with minimized side effects. Herein, we briefly review these elements aimed at promoting developments of translational nanocarriers.

1. The three key elements for translational nanomedicine: nanocarrier's 2R2S capability, material excipientability and process scale-up ability

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 the improved pharmacokinetic properties and the reduced adverse effects [1,3], currently cancer drug delivery has only achieved modest therapeutic benefits [3–5]. 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 Fig. 1: Initially, the drug-loaded nanocarriers circulate in the blood compartments, including the liver and the spleen. When passing through tumor blood vessels, some carriers may fall into the pores in the blood vessel wall and diffuse into the tumor tissue (EPR effect) (Fig. 1A) [6,7]. Next, they may further penetrate the tumor tissue, which is non-trivial because of the high cell density and high interstitial osmotic pressure (Fig. 1B) [8]. Upon sticking to the surrounding cancer-cell membrane (Fig. 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 *iv* injection site to the target in the tumor cells, the nanocarrier must simultaneously meet two pairs of challenges (Fig. 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 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 passing the hyperpermeable tumor blood vessel and extravasation into the tumor. But once in the tumor the nanocarrier must become "sticky" or "cell binding" to interact with tumor cells for efficient cellular uptake. A nanocarrier capable of simultaneously satisfying the opposite *2R2S* requirements at the right places, that is, "drug *R*etention in blood circulation *versus* *R*elease in tumor cells (2*R*)" and "*S*tealthy in blood *versus* *S*ticky in tumor (2 *S*)" will deliver the drug specifically to the tumor, giving rise to high therapeutic efficacy and few side effects.

While the 2*R*2*S* capability of a nanocarrier may render its resulting nanomedicine efficacy and safety potential for clinical translation, other two elements, the feasibility of the nanocarrier materials to be proved for use as excipients (referred to as material excipientability) and the ability to establish scaling up production processes for good manufacture processes (GMP) for the nanocarrier and its formulation with drug (nanomedicine) (referred to as process scale-up ability) are also indispensible for the nanomedicine truly translational from the benchtop to the bedside (Fig. 2). Most of our current research is

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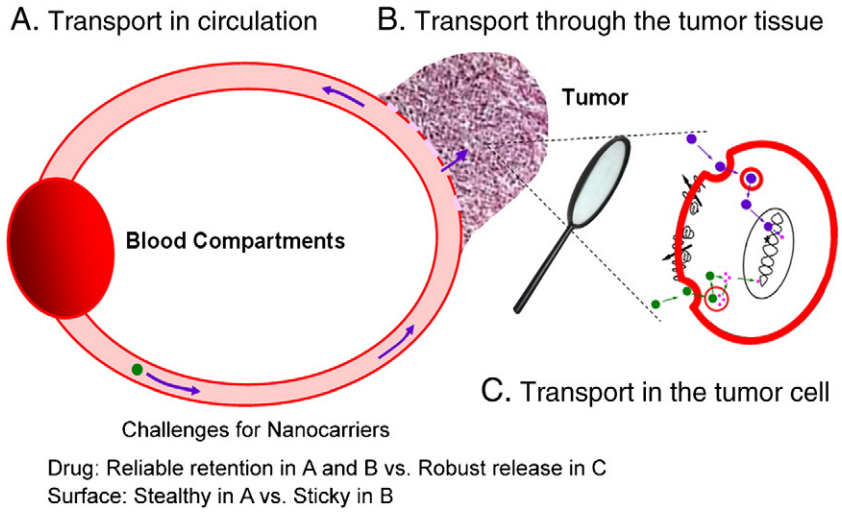


Fig. 1. Cancer drug delivery process: (A) transport in circulation, (B) transport through the tumor tissue, and (C) transport in the tumor cell. The nanocarrier must meet two pairs of challenges — for the drug: the nanocarrier must retain the drug very tightly during in the blood compartments and the tumor tissue but efficiently release the drug once in the intracellular target; For the surface: the nanocarrier must be "very stealthy" during in the blood compartments for a long blood circulation time but once in the tumor must become "sticky" or "cell binding" for efficient cellular uptake.

focusing on using new material design and chemistry to improve the 2*R*2*S* capability; however, those aimed at translational applications should comprehensively consider the other two elements at the early stage.

Herein, we briefly review approaches addressing nanocarrier 2*R*2*S* capability and summarize the factors affecting material excipientability and process scalability, aimed at promoting the developments of truly translational nanomedicine for cancer drug delivery.

2. The 2R2S capability of nanocarriers

2.1. The 2R capability: drug retention in circulation versus intracellular release

2.1.1. Approaches to minimize premature drug release from a stable carrier

Fig. 3A illustrates two examples, ideal one for the case when the carrier retains the drug during the transport in the blood compartments and the tumor tissue, but releases it in the tumor cells, and the other 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 [9–11] and liposomes [12,13]. As a result, the drug is dumped in the blood compartments, which causes not only local or systemic toxicity, but also lowers drug availability to the tumor and thereby 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

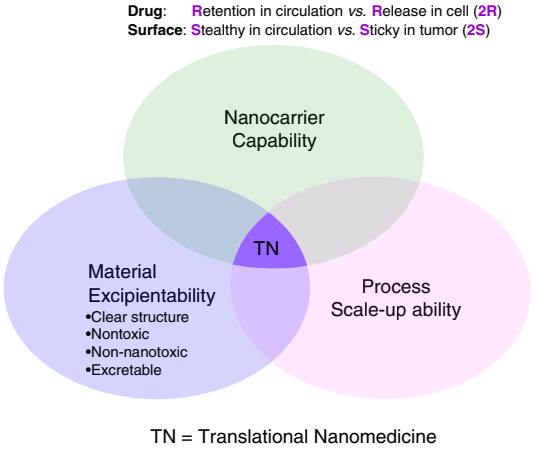


Fig. 2. The three elements for translational nanomedicine: the nanocarrier should have the 2*R*2*S* capability and its material should be suitable for excipient use (referred to as material excipientability); the production of the nanocarrier and its formulation with drug (nanomedicine) should be able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

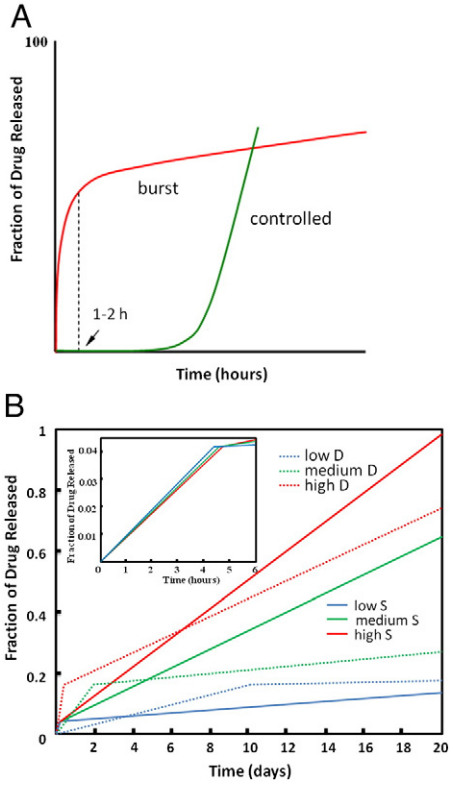


Fig. 3. (A) Sketch of ideal controlled release *versus* premature burst release; (B) Effects of drug solubility (S) and diffusion coefficient (D) on drug release kinetics in a zero-order drug-delivery system. Adapted with permission from reference [14]. Copyright 1997 Elsevier Science Ireland Ltd.

were affected by drug solubility and drug diffusion in an aqueous medium, as shown in Fig. 3B, and by the drug loading content [14]. Such findings inspired more recent approaches to prevent burst release aimed at enhancing drug loading, inhibiting drug diffusion from the carrier, or both.

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 [15], 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 drug can precipitate first or the core can form first, which prevents a 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 [16].
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 step-wise 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 [17]. Compared to the conventional core-corona micelles, such 3LNPs were found to exhibit a significantly lower burst release of camptothecin (CPT) at the 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 [18]. 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 the near-critical solvent, whereas at moderate pressures micellization/drug encapsulation occurred (Fig. 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% (Fig. 4B). Significantly, the burst release was suppressed despite the high drug loading content (Fig. 4C). More recently, we prepared 3-layered micelles formed by a stepwise NCM process that exhibited little if any burst release (Tyrrell, Shen, Radosz, unpublished work). 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 of organic solvents.

2). Drug conjugation
The second approach to eliminate the 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 of its block copolymer PEG-b-P(Asp) through amide [19,20] or hydrazone linkers [21–23]. Drugs can also be conjugated to the ends of hydrophobic blocks [24–28]. The resulting micelles formed from PEG-block-poly(L-amino acid) (PEG-b-PLAA)-conjugated drugs eliminated any burst release [29]. Using labile linkers responsive to the tumor's extracellular or intracellular stimuli results in drug release triggered in a tumor extracellular environment [30,31].
Our group demonstrated directly using drugs as the hydrophobic parts to make self-assembling amphiphilic prodrugs for fabricating

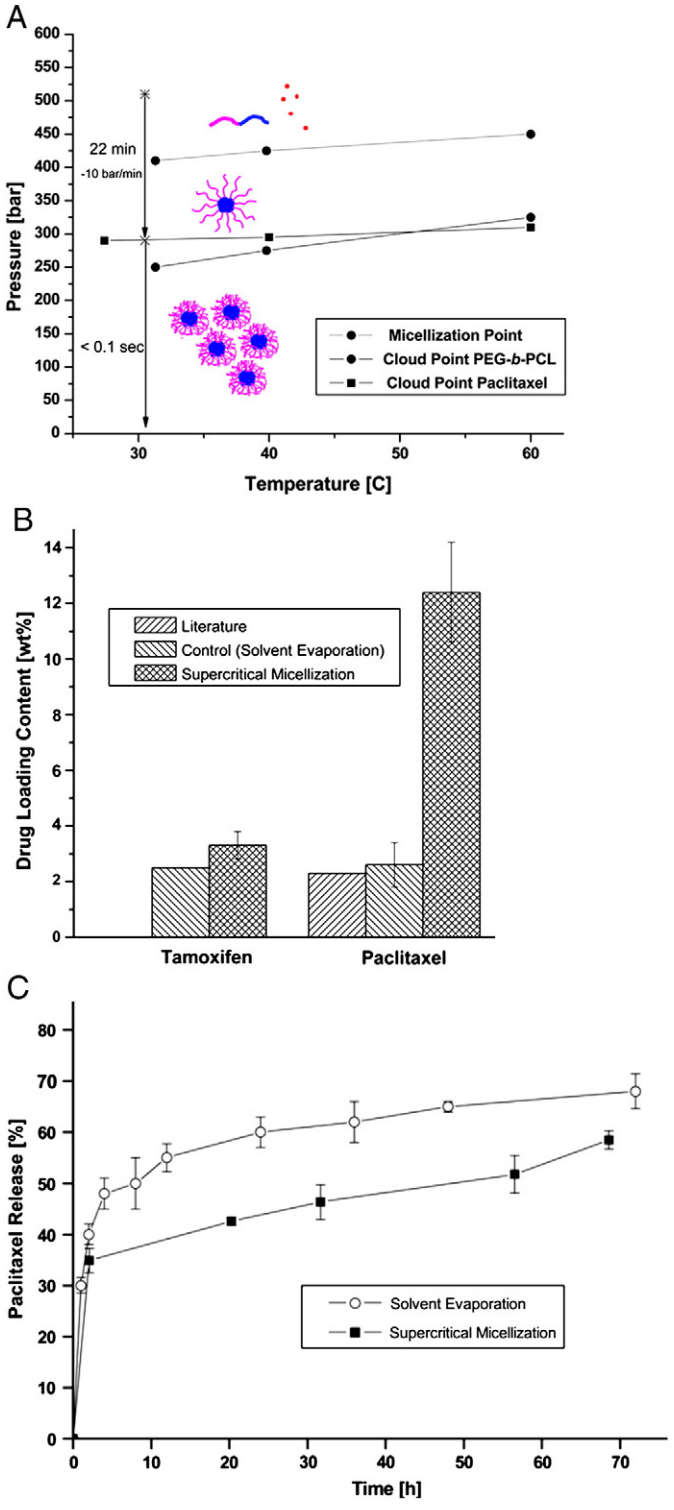


Fig. 4. Nanoparticles prepared by a near-critical fluid micellization (NCM) method: (A) Micellization and cloud pressures of PEG-PCL and PTX in 70% dimethyl ether/30% trifluoromethane; (B) Drug loading contents of tamoxifen and PTX in PEG-PCL nanoparticles by the NCM process and conventional solution process; (C) Drug release profile of PTX from PEG-PCL nanoparticles prepared by solvent evaporation (control) and the NCM. Adapted with permission from reference [18]. Copyright 2011 American Chemical Society.

burst-free carriers [32]. In this method, hydrophobic CPT molecules were conjugated to short oligomer chains of ethylene glycol (OEG) to form amphiphilic phospholipid-mimicking prodrugs OEG-CPT or OEG-DiCPT (Fig. 5). These prodrugs formed stable liposome-like nanocapsules with extremely high drug loading content but no burst release. Similar nanoparticles were prepared from amphiphilic curcumin prodrug [33].

The main disadvantage of such conjugation approaches is that they change the drug chemical structure [34], which in turn may reduce their 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 [35]. In order to de-crosslink the shell to allow drug release at the target site, the linker labile in the presence of intracellular glutathione (GSH) [36] was used. 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 [37], and thus may not continue to be stealthy in circulation.

Covalent crosslinking of the micelle hydrophobic core can therefore be a preferable approach [38–42]. 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 [41]. Lavasanifar et al. applied click chemistry and developed hydrolysable core-crosslinked PEG-b-poly(α-propargyl carboxylate-ε-caprolactone) (PEG-PPCL) micelles [42] that exhibited a lower degree of PTX burst release than equivalent non-cross-linked micelles. 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 [43]. Similarly, thiolated Pluronic (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 [44].

2.1.2. Approaches to increase carrier stability to prevent premature drug 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 [29] used "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), $C_{CMC} \sim \exp(-n\varepsilon_h/k_bT)$ [45], where k_bT 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 [46]. Thermodynamic stability is particularly important because locally, in circulation, micelles may dissociate if the block copolymer concentration falls below 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.

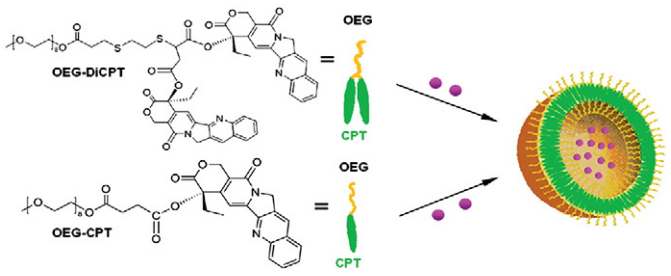


Fig. 5. Amphiphilic CPT prodrugs (OEG-CPT and OEG-DiCPT) and their self-assembly into nanocapsules. Reprinted with permission from reference [32]. Copyright 2010 American Chemical Society.

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 unimers and micelles [46]. Monte Carlo simulation indicated that chain insertion/expulsion played the major role when polymer concentration was low [47]. Because chain mobility plays a crucial role, the hydrophobic blocks with relatively high glass transition temperature (T_g) make the micelles dissociate much more slowly than those with low T_g [48]. 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 unimers [49].

Even though there is evidence that some polymeric micelles can be stable in serum [50] even *in vivo* [50], 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 radio-labeled 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 [51,52]. 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 [53]. 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 [54]. Based on those results, Cheng et al. [46] summarized the possible mechanisms responsible for the micelle decomposition induced by serum proteins, including protein adsorption [53–55], protein penetration [56–58] and drug extraction [46]. What exactly happens to the micelles after injection is poorly understood [59] because it is hard to measure and estimate micelle concentration locally in the bloodstream. Cheng et al. [60] 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.

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 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 [45]. One of the examples is that chemically modified Pluronics, Pluronic/PCL copolymeric nanospheres, exhibited lower CMC [61,62]. Another interesting finding is that stearic acid as side chains can keep micelles stable even in the presence of serum [63]. 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 for 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 [64]. 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 [65].

The caveat, however, is that crosslinking reactions usually occur after the core formation, which can alter the structure and properties of the encapsulated drugs. To overcome this potential problem, our group developed stable core-surface crosslinked micelles (SCNs) shown in Fig. 6 made from amphiphilic polymer brushes [66]. The key point is that the backbones of the polymer brushes acted as cross-linkages on the hydrophobic core surface, instead of chemical cross-linking, which substantially enhanced micelle stability. Specifically, the resulting micelles had much lower CMC 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 [67], UV light [68], and others [69] 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 [70]. For instance, micelles formed from triblock copolymer PEG-b-poly(N-(3-aminopropyl)methacrylamide)-b-poly(N-isopropylacrylamide) (PEG-PAPMA-PNIPAM) were shell-crosslinked with terephthaldicarboxaldehyde (TDA) at pH 9 via cleavable imine linkages [67]. However, at pH<6 the hydrolytic cleavage of the imine cross-linkages occurred. Other examples [71–73] were inspired by crosslinking, using disulfide linkages that are sensitive to intracellular GSH (≈0.5–10 mM as opposed to ≈20–40 μM in the bloodstream [74]). 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 [73].

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 [75]. Lavasanifar et al. [75] synthesized benzyl carboxylate-substituted ε-CL monomers and prepared PEG-b-poly (α-benzylcarboxylate ε-caprolactone) (PEG-b-PBCL) copolymers. 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 [50,76]. 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 [77], but they disassemble once the salt concentration rises above a certain threshold [78]. Hedrick et al. introduced urea functional groups [79] while Zhu. et al. introduced DNA base pairs [80] into block copolymers to show that hydrogen bonding can reduce micelle decomposition rates.

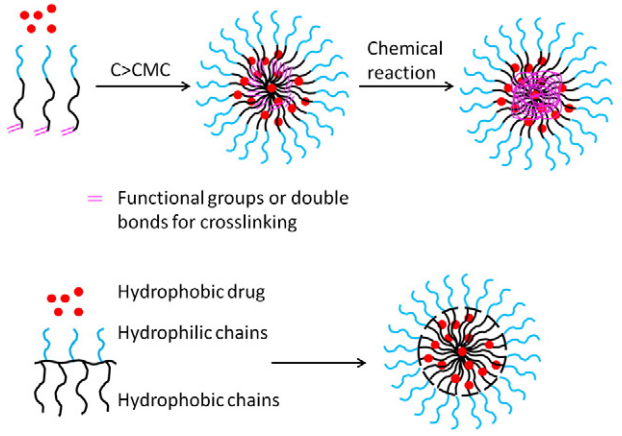


Fig. 6. Formation of core-crosslinked micelles by chemical reactions (a) and SCNs from amphiphilic brush polymers. Adapted with permission from reference [66]. Copyright 2004 American Chemical Society.

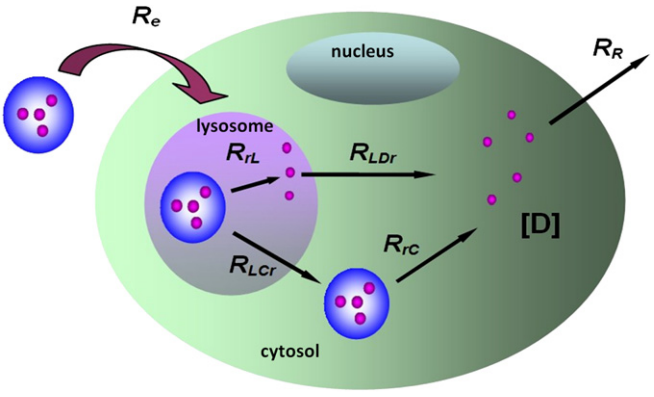
2.1.3. Approaches to achieve robust intracellular release

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 liberated from their carriers [81–83]. DOX that was stably bonded to the nanoparticle core of *poly(lactic-co-glycolic acid)* (PLGA) [84] or P(Asp) [85] showed low or even no anticancer activity [86]. The rate of drug release is also very important because tumor cells have intrinsic and acquired drug-resistance mechanisms to remove intracellular drugs [87–89], for example, as a result of cell-membrane-associated multidrug resistance to efflux drugs [88,90] and cell-specific drug metabolism or detoxification [91]. 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 [92,93] and routed to endosomes and then acidic lysosomes, as shown in Fig. 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 Fig. 7, and (2) in cytosol, following the carrier escape from the lysosome, as illustrated in the lower path in Fig. 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 (Eq. (1)). 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 Fig. 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 to 5 in lysosomes [94]. This acidic pH and special enzymes in lysosomes can trigger drug release from the carriers into lysosomes [95]. Because the harsh environment of lysosomes can easily degrade drugs sensitive to acid or these enzymes [96,97], the drug must quickly diffuse out into 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 [98–101]. Ulbrich et al. conjugated DOX to N-(2-hydroxypropyl) methacrylamide (HPMA) copolymers via this hydrolytically labile spacer [100]. Results showed a fast DOX release from the polymer at an 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 [101]. The *in vitro* cytotoxicity and *in vivo* anti-tumor activity of all such



$$\frac{d[D]}{dt} \sim R_{LDr} + R_{rC} - R_R \qquad (Eq.1)$$

Fig. 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.

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 [102]. Lysosomal degradable peptides (*e.g.* glycylphenylalanylleucylglycine (GFLG)), which are cleavable by lysosomal enzymes to release the drugs, are also used for drug conjugation [83,101,103,104]. For instance, DOX was conjugated to HPMA copolymers via GFLG peptides to form a cleavable HPMA-GFLG-DOX conjugate [83].

Lysosomal pH has also been used to trigger drug release from pH-sensitive nanoparticles [105–107]. For example, pH-sensitive micelles composed of reducible poly(β-amino ester)s (RPAE) cores [107] dissociated rapidly in an acidic environment and at high levels of reducing reagents, inducing fast intracellular release. Carriers with a core made from amine-containing hydrophobic polymers such as polyhistidine (PHis) [106] can be protonated and thus dissolve in acidic lysosomes, thereby releasing the drug. Our group showed that a rapid cytoplasmic release from carriers could increase the anticancer activity of drugs [108,109].

The additional advantage of such amine-containing polymers is that they may also have endosomal membrane-disruption activity induced by a "proton sponge" mechanism [110], and thus disrupt the lysosomal membrane and further release the drug into the cytosol. Some specially designed polyacids, such as poly (propylacrylic acid) (PPAA) [111,112], 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 the intra-lysosome release discussed above is carriers designed for the intra-cytosol release (lower path in Fig. 7). Such intra-cytosol-release carriers retain the drug until escape from endosome/lysosome first [113] and then release drugs into cytosol, hence avoiding lysosomal drug retention and degradation. This is particularly important in siRNA or gene delivery and thus various approaches have been explored to facilitate the endosomal release of DNA or RNA complexes [114–116]. 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 [117] 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 [118]. Thus, they must be functionalized with lysosomal membrane-destabilizing polymers such as PPAA [111,112], pH-dependent fusogenic peptides [119–122], or cationic polymers such as polyethyleneimine (PEI) [123] and histidine-rich peptides and polymers [124]. For cationic polymers or peptides, on the other hand, it is important first to mask their cationic charges (from primary and secondary amines) at the physiological pH, so the carriers can be used for *iv* 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 [123,125].

Removal of a cleavable PEG layer can also allow lysosomal escape [126]. For instance, 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 [127]. Disulfide linkages were also used to detach PEG and make the drug-loaded carriers quickly escape from endosomes [128]. 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 [128].

Most carriers reaching the cytoplasm already experience an initial burst release and are in a slow, diffusion-controlled drug release process, as observed in a typical example shown in Fig. 4C for particles with cores made of solid glassy polymers such as PCL or polylactide (PLLA) [129]. According to Eq. (1), 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 cytosolic redox signal resulting from an elevated intracellular GSH concentration (~10 mM) compared to that in the bloodstream (~2 μM) [130]. GSH can effectively cleave the disulfide bonds to release conjugated drugs [74,125,131]. It is thus used to trigger decomposition of micelles with hydrophobic parts linked by disulfide bonds [132] or other carriers crosslinked [133] or gated [134,135] with disulfide linkers. It has also been observed that the removal of the PEG corona could increase the drug release rate [136,137].

2.2. The 2 S capability: stealthy in circulation versus sticky in tumor

2.2.1. Approaches to stealth surfaces

The second major material challenge is how to impart stealthy carriers for a long circulation time, which is needed to reach the tumor tissue, but become effectively sticky upon reaching tumor tissue, which is needed for the fast cellular uptake. The carrier's stealth character hinges on many factors, such as its surface properties [138], size [139] and even shape [140–142]. 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 [96], while those with diameters above 200 nm will be scavenged by RES, mainly the liver and spleen [139,143].

Most stealth carriers capable of avoiding opsonization [144] and interaction with the mononuclear phagocyte system (MPS) [139] are made from HPMA [145,146], PEG, or polysaccharides [147] (e.g. heparin

[148]). Nanoparticles coated with a layer of these polymers become stealthy by both hydration and steric hindrance [149]. For example, pegylation of particles or liposomes is well-established [144,150–155], and the DOX-loaded stealth liposomes named Doxil® were approved by the FDA for cancer therapy [156]. Huang et al. reported that, on the 100 nm liposomes pegylated with 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-PEG_{2000} ($DSPE\text{-}PEG_{2000}$), PEG chains were arranged in a mushroom configuration at the DSPE-PEG fraction less than 4 mol% but in a brush configuration at the DSPE-PEG content greater than 8 mol% (Fig. 8) [157]. The high density of PEG chains on the liposome surface with the brush configuration was the key to reduce liposome liver sequestration [157]. Discher et al. incorporated the PEG brush onto polymersomes and obtained polymersomes having a blood circulation time two-fold longer than pegylated liposomes [158]. 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 further increase of the PEG chain length showed no significant effect [159]. 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 [160–163] due to IgM bound to pegylated liposomes secreted into the bloodstream after the first dose [161]. Such immune reaction against the pegylated liposomes occurred in the spleen at least 2–3 days after the first administration [162,163].

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 cell much faster [164]. Discher et al. found that flexible worm-like micelles efficiently evaded RES and circulated in the blood for a week [165,166], much longer than spherical micelles. Dai et al. found that carbon nanotubes pegylated with long PEG chains exhibited a long blood circulation ($t_{1/2} = 22.1$ h) upon intravenous injection into mice [152]. All these studies suggest that particle phagocytosis can be inhibited by minimizing its size-normalized curvature [164,167]. Thus, particle shape is an important variable to make it remain stealth in circulation long enough for enhanced tumor accumulation [165,166,168–170].

2.2.2. Approaches to becoming sticky in tumor for cellular uptake

However, the same properties that impart stealth in circulation can make the carrier slow in cellular uptake by tumor cells. Carriers that are negatively charged will be repelled from the cell membrane due to the electrostatic repulsion (Fig. 9B). The PEG corona of pegylated polymeric micelles or liposomes retards their interaction with cell membranes due to steric hindrance (Fig. 9C). Thus, once in the tumor, the carrier must become sticky to targeting tumor cells for fast cellular uptake [171]. The challenge is how to reconcile these two opposite requirements, stealth circulation and sticky targeting. For instance, it is well known that positively charged carriers reliably stick to cell membranes due to electrostatic adsorption triggering fast cellular uptake (Fig. 9A); but positively charged carriers are not suitable for *in vivo* applications because they are systemically toxic [172] and have a short circulation time [173].

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 on and fused with the cell membrane. Toward this end, Thompson et al. prepared acid-labile PEG conjugated vinyl ether lipids to stabilize fusogenic DOPE liposomes [174]. 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 [175,176]. MMP is overexpressed in tumor-tissue angiogenesis, invasion, and metastasis [177] 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.

Alternatively, positive charges can promote carrier adsorption on the negatively charged membrane and hence trigger adsorption-mediated endocytosis. A practical approach is to use tumor extracellular acidity to impart positive charges to the carrier by a "charge-reversal" technique (illustrated in Fig. 10). Amine-containing carriers, such as PCL-b-PEI [123], poly(L-lysine) (PLL) [178] and PAMAM dendrimers [125], were amidized to acid-labile β-carboxylic acid amides to make them negatively charged at the physiological pH. However, in weakly acidic tumor extracellular fluid, the amides hydrolyzed and regenerated the amines with cationic charges, which led to fast cellular uptake (Fig. 10A). In yet another example, a pH-responsive layer becomes positively charged at tumor extracellular acidity but collapses, forming a middle layer at neutral pH (Fig. 10B) [17]. 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 [179]. 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 TAT on the corona surface [180].

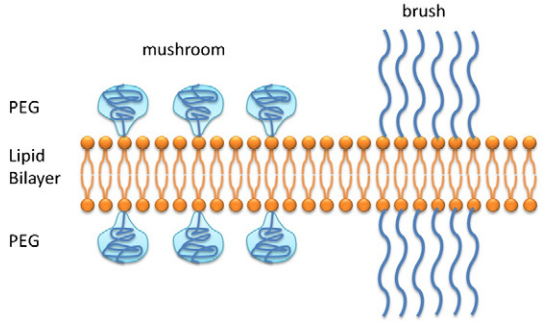


Fig. 8. The mushroom and brush configurations of PEG chains arranged on the lipid bilayer.

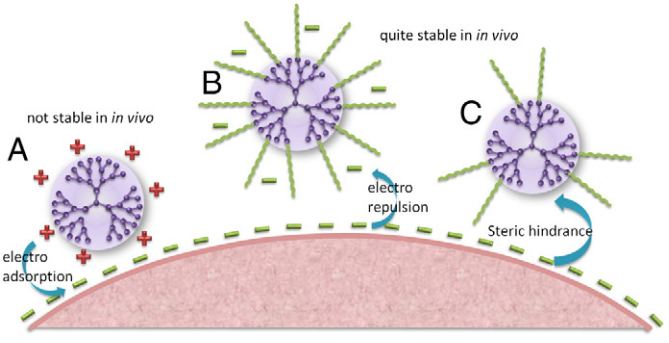


Fig. 9. Paradox of "stealthy" and "sticky". (A) positively-charged carriers are internalized quickly due to electrostatic adsorption, but are not suitable *in vivo*; (B) negatively-charged carriers are quite stable *in vivo*, but are internalized quite slowly because of electrostatic repulsion; (C) carriers with PEG corona are internalized slowly because of steric hindrance.

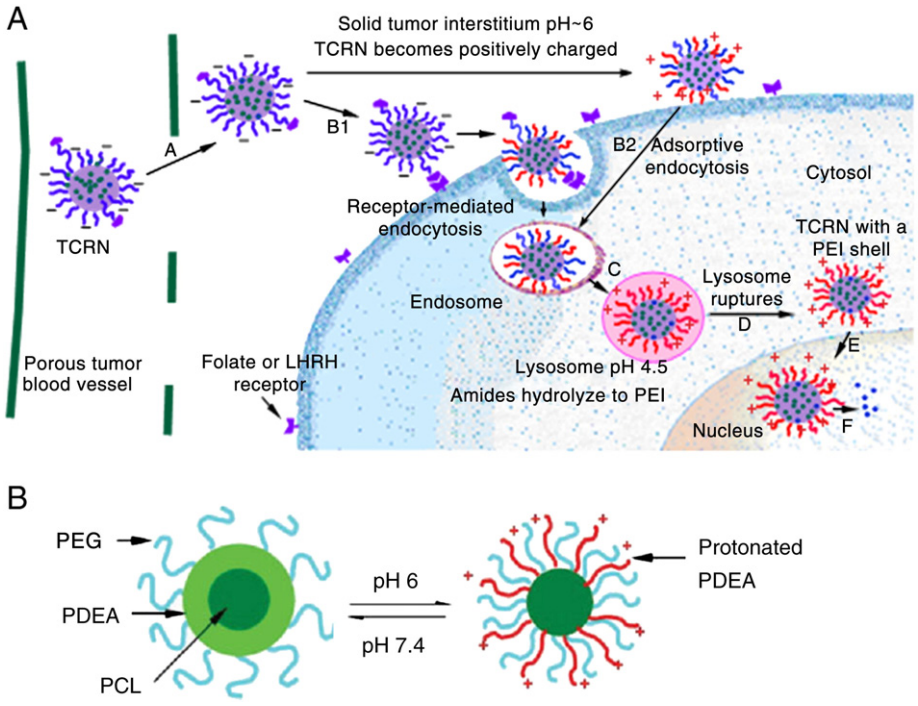


Fig. 10. (A) The charge-reversal concept for drug delivery. Reprinted with permission of reference [15]. Copyright 2010 Elsevier Ltd; (B) The pH-responsive three-layered nanoparticles (3LNPs). Reprinted with permission from reference [17]. Copyright 2008 Wiley InterScience.

The most common approach to make the carrier stick to the cell membrane is to decorate it with a ligand with receptors that are over-expressed on the cancer-cell membrane, which enables receptor-mediated endocytosis [12,181] promoting their cellular uptake. Only a few ligands are needed for rapid internalization [182]. More ligand groups can theoretically increase uptake, but their increasing surface density may make the carrier less stealthy as a result of opsonization-mediated clearance [183]. Many examples of targeting ligands include folic acid [184], peptides [185–187], antibodies [188–190], transferring [121], aptamers [191,192] and other moieties [193], that have been tested and subsequently reviewed [2,149,194,195].

2.3. The necessity to simultaneously have 2R2S capability: our own experience

To achieve a sufficiently high effective [D] in the cancer cell, the nanocarrier must meet all the four requirements as discussed above: stealth and drug retention during the circulation *versus* sticky and robust intracellular release in the cancer cell once in tumor. If any of these conditions is not met, such a carrier will fail in its mission.

We shall illustrate it with an example of our own work showing that missing a single requirement can ruin the whole effort. This example concerns a new type of polyester dendrimers that enable high-loading efficiency of hydrophobic drugs at low temperatures without using organic solvents [196,197]. Such dendrimer carriers easily met the reliable retention and robust release requirements, namely, that it slowly released DOX without a burst release at 37 °C and pH 7, but quickly released it at pH 4–5 in PBS solution (Fig. 11A) suggesting a perfect intracellular DOX release. In fact, DOX encapsulated in the dendrimer was efficiently delivered to drug-resistant cells (Fig. 11D), while free DOX hardly entered such cells (Fig. 11C). Surprisingly, however, the (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) (MTT) result (Fig. 11B, unpublished data) showed that the large amount of the carried DOX barely exerted any pharmaceutical actions, as it had a very similar cytotoxicity to free DOX. Adding chloroquine to break endosomes/lysosomes didn't increase the cytotoxicity (unpublished data) suggesting there was no lysosomal sequestration. It is very possible that DOX could not diffuse out the dendrimer efficiently in the cell, causing the low therapeutic efficacy.

3. The material excipientability and production process scale-up ability - elements indispensible for translational nanomedicine

The *2R2S* capability for nanocarriers discussed in the previous sections is necessary and determines the adsorption, distribution, metabolism and excretion (ADME) of the carried drug. Such a nanocarrier simultaneously having *2R2S* capability can deliver a high cytosolic concentration of the drug and give rise to high therapeutic efficacy. However, this is not sufficient for it to be translational [198–200]. The nanocarrier itself should also have proper ADME. According to Choi and Frangioni [200], 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, "from the benchtop to the bedside" translation. Thus, a nanocarrier must meet the requirements for the pharmaceutical excipient for iv uses. For simplicity, this ability of the nanocarrier material(s) to be used or approved to be an excipient, or herein denoted as excipientability, is the second element for a nanocarrier to be translational (Fig. 2). It goes without saying that the production of the nanocarrier and the resulting nanomedicine should be able to scale up and establish 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) [201–203], can cause health problems. The threshold for rapid renal excretion is about 5.5 nm in hydrodynamic diameter. This corresponds to the molecular weight of about ~45 kDa for HPMA [204] and 40 kDa for PEG [96].

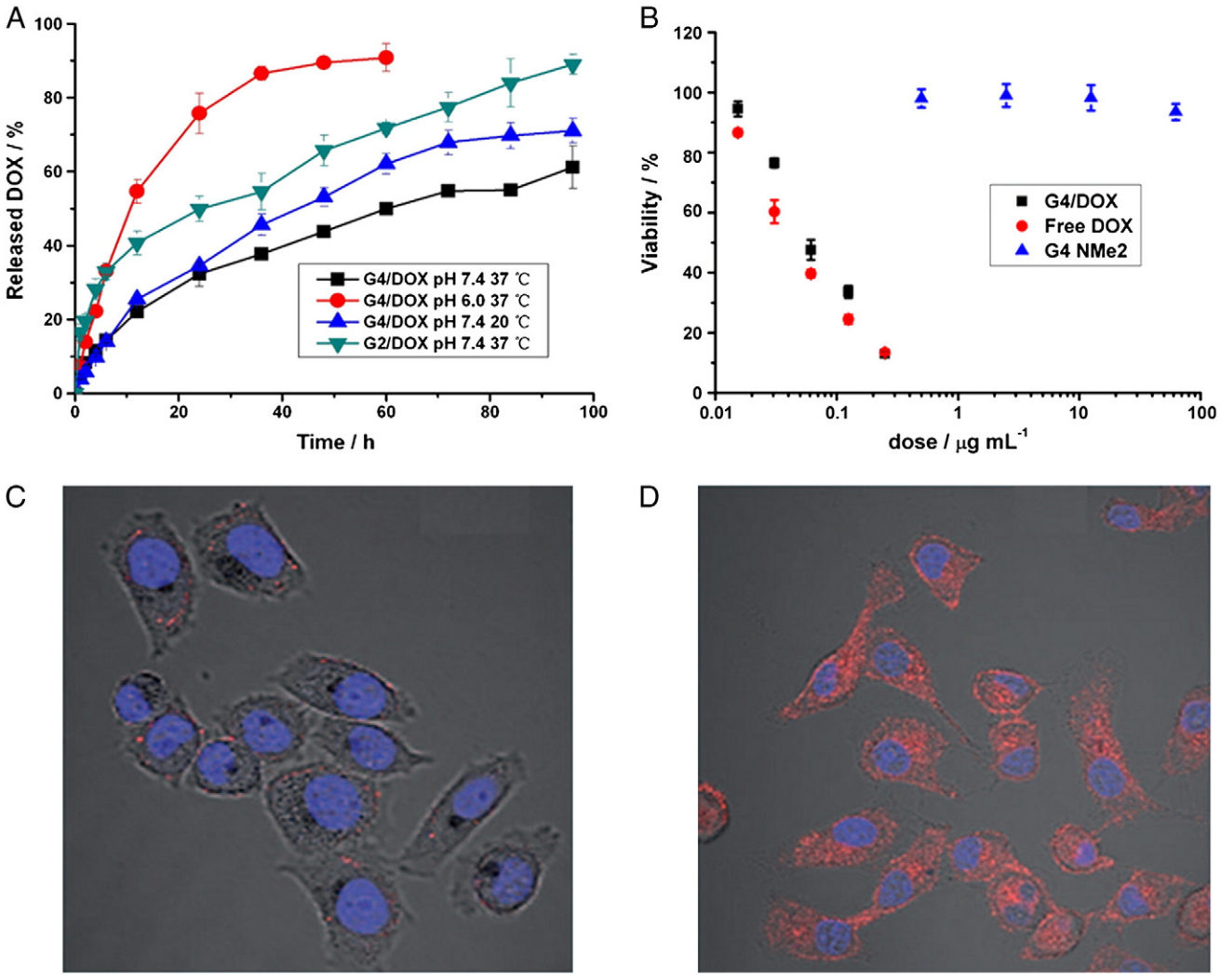


Fig. 11. (A) The effects of dendrimer generation, pH and temperature on drug release profiles from DOX-loaded second (G2) and fourth generation (G4) dendrimers; (B) MTT assay of G4, G4/DOX, and free DOX; Cellular uptake of free DOX(C) or G4/DOX(D) observed by confocal scanning laser fluorescence microscopy. MCF-7/ADR cells were incubated with DOX or G4 /DOX for 4 h at 37 °C. Adapted with permission from reference [197]. Copyright 2011 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

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 [15,205], 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 manufacture process and minimize losses of these very expensive anti-cancer drugs.
4) *High drug loading content*: In current commercial formulations, the drug loading content tends to be on the low side [206–208]. 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) [209,210].

4. Challenges for developments of translational nanomedicine for cancer chemotherapy

With 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 large majority doses of the drugs were still sequestrated in liver or spleen, even though the tumor drug accumulations were indeed enhanced compared to free drugs [142,211]. For instance, the PF-PTX micelles [212] and IT-101 CPT-conjugates [213] gave drug accumulation in tumors much better than Taxol® and

CPT, respectively, but the total amounts of drugs accumulated in liver were still about 4.5 and 3.5 times of those in tumors. In many cases only several percents 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 free drugs [214,215].

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 design of nanocarriers towards the *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 to, or take an impractically long time, to be translated into clinics. Thus, except for proof of concepts, it's better to look into these issues early at the bench in order for a successful nanocarrier to move forward quickly.

Nomenclature

ABC	accelerated blood clearance
ADME	adsorption, distribution, metabolism, excretion
CMC	critical micelle concentration
CPT	camptothecin
DOPE	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
DOX	doxorubicin
DSPE	1,2-distearoyl-sn-glycero-3-phosphoethanolamine
DTT	Dithiothreitol
EPR	enhanced permeability and retention
FDA	Food and Drug Administration
FRET	fluorescence resonance energy transfer
GFLG	glycylphenylalanylleucylglycine
GMP	good manufacturing practices
GSH	glutathione
HPMA	N-(2-hydroxypropyl)methacrylamide
HSRs	hypersensitivity reactions
MEND	multifunctional envelope-type nano device
MMP	matrix metalloproteinase
MPS	mononuclear phagocyte system
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCM	near-critical fluid micellization
OEG	oligomer chain of ethylene glycol
P(Asp)	poly(L-aspartic acid)
p(HEMAm-Lac_n)	N-(2-hydroxyethyl methacrylamide)-oligolactates
PAC	poly(acryloyl carbonate)
PAMAM	Poly(amido amine)
PAPMA	poly(N-(3-aminopropyl)methacrylamide)
PBCL	poly(α-benzylcarboxylate ε-caprolactone)
PCCL	poly(α-carboxyl-ε-caprolactone)
PCL	poly(ε-caprolactone)
PDEA	poly[2-(*N,N*-diethylamino)ethyl methacrylate]
PDLLA	poly(D,L-lactide)
PEG	polyethylene glycol
PEI	polyethyleneimine
PHis	polyhistidine
PHSA	poly(N-hexyl stearate L-aspartamide)
PIC	polyion complex
PLAA	poly(L-amino acid)
PLGA	poly(lactic-co-glycolic acid)
PLL	poly(L-lysine)
PLLA	poly(L-lactide)
PNIPAM	poly(N-isopropylacrylamide)
PPAA	poly(propylacrylic acid)
PPCL	poly(α-propargyl carboxylate-ε-caprolactone)
PPE_{DNPT}	poly((2,4-dinitrophenyl)thioethyl ethylene phosphate)
PS	polystyrene
PSD	poly(methacryloyl sulfadimethoxine)
PTX	paclitaxel
PVP	polyvinylpyrrolidone
RES	reticuloendothelial system
RPAE	reducible poly(β-amino ester)s
SCNs	core-surface corsslinked micelles
SEC	size-exclusion chromatography
SWNT	single-wall carbon nanotube
TDA	terephthaldicarboxaldehyde

Take-home message

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 the 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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