



Stimuli-responsive cross-linked micelles for on-demand drug delivery against cancers[☆]



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ABSTRACT

Stimuli-responsive cross-linked micelles (SCMs) represent an ideal nanocarrier system for drug delivery against cancers. SCMs exhibit superior structural stability compared to their non-cross-linked counterpart. Therefore, these nanocarriers are able to minimize the premature drug release during blood circulation. The introduction of environmentally sensitive cross-linkers or assembly units makes SCMs responsive to single or multiple stimuli present in tumor local microenvironment or exogenously applied stimuli. In these instances, the payload drug is released almost exclusively in cancerous tissue or cancer cells upon accumulation via enhanced permeability and retention effect or receptor mediated endocytosis. In this review, we highlight recent advances in the development of SCMs for cancer therapy. We also introduce the latest biophysical techniques, such as electron paramagnetic resonance (EPR) spectroscopy and fluorescence resonance energy transfer (FRET), for the characterization of the interactions between SCMs and blood proteins.

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1. Introduction

Nanotechnology offers new opportunities for diagnosis and treatment of a variety of cancers [1–5]. Multifunctional nanoparticles possessing functions including tumor targeting [6–10], imaging [11–14] and therapy [10,15–17] are under intensive investigation aiming to overcome limitations associated with conventional cancer diagnosis and therapy [18–20]. Over the past decade, polymeric micelles

have been extensively investigated as nanocarriers to deliver conventional anticancer drugs. These nanoparticles provide several distinct advantages for the drugs, such as improved solubility, prolonged *in vivo* circulation time and preferential accumulation at tumor site via the enhanced permeability and retention effect [21–24]. Despite the recent progress in the research of micellar nanoparticles, some shortcomings are gradually revealed which may limit their application in clinic. In blood circulation, blood proteins and lipoproteins such as high density lipoprotein (HDL), low density lipoprotein (LDL), very low density lipoprotein (VLDL) and chylomicron may interact with the polymeric micellar nanoparticles [25]. This process can result in the early disintegration or aggregation of micelles and premature drug release [26]. Besides, polymeric micelles are thermo-dynamic self-assemble system which has a well-known equilibrium existed between micelles and unimers (assembly unit) in aqueous condition. After being injected into the blood stream, conventional self-assembled polymeric micelles are susceptible to dilution below the critical micelle concentration (CMC). This may lead to the dissociation of micelles into unimers.

Cross-linking strategy has been utilized to solve the above mentioned stability problems following the pioneer work by Wooley's group [27]. Since then, this strategy has been exploited by a number of other groups [28–33]. Covalent cross-links between specific domains of the micelles are formed in order to improve the micelles' structural stability suitable to drug delivery rather than the weak non-covalent intermolecular hydrophobic interactions existing in the conventional polymeric micelles that facilitate polymer micelles assembly and integrity [27]. To be more effective, anticancer drugs should be released exclusively in tumor tissue or inside tumor cell. However, excessively stabilized micelles may prevent the drug from releasing to target sites, thus reducing the therapeutic efficacy [28,29]. Stimuli-responsive cross-linked micelles (SCMs) are introduced to improve the drug delivery [30–33]. SCMs exhibit unique stability in blood circulation and can better retain the drug contents. The utilization of environmentally

sensitive cross-linkers or assembling units makes SCMs responsive to single or multiple stimuli in the microenvironment of tumor site or inside the tumor cells [34,35] or the application of exogenous stimuli (Fig. 1). The cleavage of the intra-micellar cross-linkage or disassembly of the micelles responding to stimuli leads to exclusively drug release in the target site [36,37]. The special micelles are often called 'smart' or 'intelligent' micellar nanoparticles. This review briefly summarizes the recent advances in stimuli-responsive cross-linked micellar nanocarriers with the main focus on the design, characterization, cross-link strategy, protein interaction, stimuli-sensitive release mechanism and preclinical evaluation.

2. Main text

2.1. Design of stable SCMs with single or multiple responsive properties

The basic elements need to be considered in the design of SCMs include how and where to introduce cross-linkages to the micelles and how to endow the micelles with responsiveness to the microenvironments of the target sites or exogenous stimuli. The cross-linkage can be introduced at the hydrophilic shell [38,39], hydrophobic core [26,40,41] or core-shell interface [35,42] of the micelle via chemical cross-link, photo cross-link or polymerization after the micelle formation via self-assembly in aqueous solution. Recently, *in situ* cross-linking approach has been reported by several groups. In this case, one or two types of cross-linkable pendants are introduced to the assembly units so that the micelles can be cross-linked spontaneously during the micelle formation [43,44]. The reported stimuli for drug delivery include pH, temperature, enzymes, electrolytes, redox (reduction/oxidation), ultrasound, light and magnetic field [45–47]. Very recently, a few studies have been succeeding in systemically applying exogenous stimuli via FDA approved agents, such as N-Acetylcysteine (a reducing agent) and mannitol (a cis-diol) [43,44]. The responsiveness of SCMs refers to

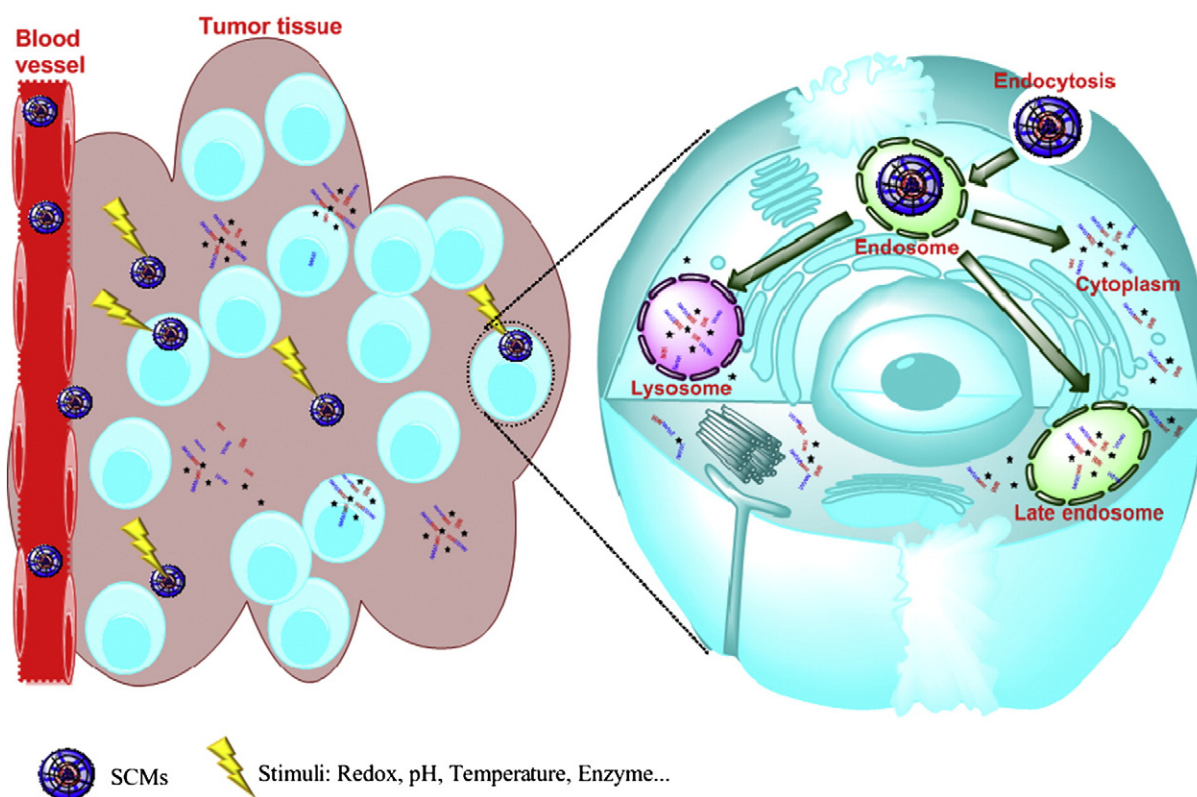


Fig. 1. Schematic illustration of stimuli-responsive cross-linked micelles (SCMs) for cancer therapy.

their ability to receive, transmit one or multiple types of above mentioned stimuli, and respond with desirable effects, including degradation, swelling/collapsing, dissolution/precipitation and eventually triggering the drug release from the nanocarriers. In order to make the cross-linked micelles responsive to tumor local environments, polymers with intrinsic responsive properties to pH, temperature, or redox conditions have been used to design SCMs. Furthermore, various cleavable cross-linkages (e.g. disulfide bonds [44], pH cleavable [48] or hydrolysable ester bonds [26]), have also been utilized to synthesize cross-linked micelles. An overview of the SCMs including the assembly units, preparation methods, cross-linking strategy, stimuli response and their applications are given in Table 1.

2.1.1. Hydrolysable SCMs

Several biodegradable polymers, such polylactate (PLA), polycaprolactone (PCL) have been exploited to design hydrolysable cross-linked micelles for anticancer drug delivery. Talelli *et al* reported such micelles via the self-assembly of poly(ethylene glycol)-*b*-poly[N-(2-hydroxypropyl) methacrylamide-lactate] (mPEG-*b*-p(HPMAm-Lacn)) diblock copolymers in aqueous solution when heated above the critical micelle temperature (CMT) [46,49]. 30–40% w/w of doxorubicin was loaded into the micelles though the conjugation of a DOX methacrylamide derivative (DOX-MA) to the micelles core followed by core cross-link via free radical polymerization. DOX-MA is hydrolysable under acidic conditions (mimicking the microenvironment within the tumor and in the intracellular organelles leading to micelle disintegration). An enhanced tumor accumulation and a prolonged circulation time were obtained after intravenous administration of these micelles because of their relatively small particle size (60–80 nm) and the intramolecular cross-linkages. In the drug release studies, it was observed

that the cross-linked micelles released the entire drug payload after 24 h incubation at pH 5 and 37 °C, while around 5% of doxorubicin was released during the same period of time when incubated at pH 7.4 and 37 °C. Furthermore, the cross-linked micelles with covalently bound DOX showed higher cytotoxicity against B16F10 and OVCAR-3 cells and better therapeutic efficacy in B16F10 melanoma carcinoma mice models than both free DOX and DOX-MA. Lee *et al* demonstrated a biocompatible and pH-hydrolyzable shell cross-linked polymer micelle for DOX delivery [50]. The micelles were self-assembled by triblock copolymer of poly(ethylene glycol)-poly(L-aspartic acid)-poly(L-phenylalanine) (PEG-PAsp-PPhe) forming core-shell-corona model with the PEG outer corona, the PAsp middle shell, and the PPhe inner core. Bifunctional ketal cross-linkers with the primary amines reacted with O-acylurea in Asp moieties forming cross-linkages at the shell of the micelles. Transmission electron microscopy (TEM) and dynamic light scattering (DLS) results implied that the PEG outer corona confines the shell cross-linking reaction within the PAsp middle shells, preventing the formation of intermicellar aggregates. The shell cross-links act as a diffusion barrier for low molecular weight compounds and may be effective in reducing the premature drug release at pH 7.4. It was found that the shell cross-linked micelles showed high physical stability even after the addition of strong detergent in fluorescence spectroscopy experiments. Meanwhile, the hydrolysis kinetics study demonstrated that the half-life of ketal cross-linked micelles at pH 5.0 was 74-fold faster than that at pH 7.4. As a result, the DOX release from the micelles was faster at pH 5.0 than that at pH 7.4.

2.1.2. Redox sensitive SCMs

Given the unique characteristics of redox state in the body, reducible linkages have been widely applied to smart micellar nanoparticles

Table 1

An overview of the reported stimuli responsive crosslinked micelle systems.

Assembly units	Crosslink strategy	Stimuli	Payload	Particle size (nm)	Ref.
Poly(amidoamine)-cholesterol conjugates	Disulfide	Reduction	Azobenzene, estradiol	100–300	[75]
PCL- <i>b</i> -PPESH- <i>b</i> -PEG	Disulfide	Reduction	Doxorubicin	50	[54]
PEG- <i>b</i> -PLys- <i>b</i> -PPha	Disulfide	Reduction	Methotrexate	25–50	[35]
PEI-(bromomethyl)phenylboronic acid	Disulfide	Reduction	pGL3 gene	200–300	[76]
PEG-oligocholeic acids (CA)	Disulfide	Reduction	Paclitaxel	28	[44]
PEG-oligocholeic acids (CA)	Disulfide	Reduction	Vincristine	16	[55]
PEI	Disulfide	Reduction	DNA		[56]
PEG-P(Lys)/PEG-P(Asp)	Disulfide	Reduction	siRNA	16	[57]
PEG-PLL	Disulfide	Reduction	pDNA	100	[77]
[PEG- <i>b</i> -(PLL-IM)]	Disulfide	Reduction	siRNA	60	[58]
PEG-L ₂ -PCL	Disulfide	Reduction	Doxorubicin	20–150	[78]
Dextran-lipoic acid derivatives	Disulfide	Reduction	Doxorubicin	20–35	[29]
mPEG-Cys-PCL	Disulfide	Reduction	Doxorubicin	191	[47]
Thiolated c(RGDfK)-poly(ethylene glycol)- <i>b</i> -poly(lysine) (PEG-PLys),	Disulfide	Reduction	pDNA	110	[79]
poly(L-cysteine)- <i>b</i> -poly(L-lactide)/PLC- <i>b</i> -PLLA	Disulfide	Reduction		50	[80]
PEG- <i>b</i> -P(DEA- <i>s</i> -TMSPMA)	Siloxane	pH		250	[64]
PEO- <i>b</i> -P(NIPAM-co-NAS)	Cystamine	Temperature and reduction		50–120	[41]
P(NIPAAm-co-MPMA)- <i>b</i> -P(DEA)	Silica-based cross-linking	Temperature and pH	Prednisone	70–220	[81]
PAGA ₁₈₀ - <i>b</i> -PNIPAAm ₃₅₀	Covalent	Temperature and pH		85–310	[67]
poly(hydroxyethyl acrylate)-poly(butyl acrylate) (PHEA)- <i>b</i> -PBA)	Divinyl acid labile cross-linker	pH	Doxorubicin	40–260	[40]
PEG-oligocholeic acids (CA)	Boronate	pH, cis-diol reduction	Paclitaxel	22–27	[43]
PEO- <i>b</i> -PMA/Ca ₂ ⁺	Disulfide		Doxorubicin	100–300	[59]
mPEO-PAPMA-PDPAEMA	Disulfide	pH, reduction		120–220	[63]
mPEG-Asp(MEA)-PAsp(DIP)	Disulfide	pH, reduction	Doxorubicin	45–550	[62]
mPEG- <i>b</i> -p(HEMA _m -Lac _n)	Hydrazone linker	Hydrolysable	Doxorubicin	68	[26,46,49]
PEG-PAsp-PPhe	Ketal-containing cross-linkers	Hydrolysable	Doxorubicin	50–55	[50]
P(AMPS- <i>b</i> -AAL))	Inter-polyelectrolyte	pH/Salt		27–70	[60]
PDMAEMA ₅₄ - <i>b</i> -P(MMA ₅₅ -co-MMA ₁₉)	Photoinduced dimerization of coumarin groups	Temperature and pH		30–50	[66]
mPEO-PAPMA-PDPAEMA	Imine	Temperature and pH	prednisolone 21-acetate	80–95	[61]

system. The concentration of a thiol-containing tripeptide called glutathione (GSH) in cytoplasm could be 100–1000 fold higher than that in the extracellular environments and blood pool [35,51,52]. Meanwhile, in some particular tumor cells, the concentration of GSH is about seven times higher than that in the normal cells [52,53]. The high concentration of GSH in cytoplasm could lead to the cleavage of disulfide bond to free thiols via the redox reaction, which is the mechanism of intracellular drug release for redox sensitive cross-linked micelles. So far, researchers have used the disulfide bond cross-linked smart micelles to deliver doxorubicin (DOX) [54], paclitaxel (PTX) [44], methotrexate (MTX) [35], vincristine (VCR) [55], DNA [56] and siRNA to the target site following the pioneer effort of Kataoka group. Lipoic acids have been utilized as cross-linkable groups to design reduction sensitive cross-linked nanoparticles [29]. For instance, Zhong et al reported a type of disulfide cross-linked nanoparticle assembled via dextran-lipoic acid conjugates followed by catalyzing the cross-linking reaction via 10 mol% dithiothreitol (DTT) relative to the lipoyl units in phosphate buffer (pH 7.4, 10 mM) [29]. It was found that monodispersed nanoparticles with average sizes ranged from 85.3 to 142.5 nm before cross-linking and shrunk by 20–35 nm after cross-linking. Notably, the cross-linked dextran nanoparticles (C-DNPs) exhibited superior drug loading efficiency in contrast with the non-cross-linked dextran nanoparticles. In the *in vitro* drug release studies, they found that the release of DOX from C-DNPs decreased to minimal about 10% even after extensive dilution, but over 90% of the DOX was released in 11 h after the addition of 10 mM DTT, which mimicks the intracellular reductive environment. Our group has recently developed a novel type of reduction-sensitive disulfide cross-linked micelles, which exhibit excellent stability, prolonged blood circulation time, and minimal drug premature release properties [44]. We introduced four cysteines to polylysine backbone of our previously reported linear dendritic polymer (called telodendrimer) PEG^{5k}-CA₈, resulting in a well-defined amphiphilic thiolated telodendrimer. The thiolated telodendrimers were then self-assembled to form micelles with particle size of around 20 nm followed by air oxidation of free thiols to intra-micellar disulfide cross-linkages (Fig. 2). Dynamic light scattering was applied to study the stability of the micelles via monitoring the change of micellar particle size. In the presence of sodium dodecyl sulfate (SDS), an ionic-detergent which is known to disintegrate polymeric micelles [35], the disulfide cross-linked micelles retained their particle size for days while the corresponding non-cross-linked micelles dissociated rapidly as evidenced by the immediate disappearance of the measured particle size signal (Fig. 3a). However, after the addition of SDS and GSH, the particle size of the cross-linked micelles was stable in the first 30 min but suddenly disintegrated within 10 s, indicating that the reduction of a critical number of disulfide bond leads to a rapid dissociation of the micelle. The level of cross-link and therefore *in vivo* stability of

such micelles can be fine-tuned conveniently by varying the ratio between thiolated and non-thiolated telodendrimers. These characteristics of the cross-linked micelles may endow the drug nanocarriers prolonged circulation time and triggered drug release at the tumor site and inside the cancer cells with high reductive potential. We found that the release of encapsulated paclitaxel from disulfide cross-linked micelles was slower than that from non-cross-linked micelles in PBS solution. The drug release rate of the non-cross-linked micelles remained unchanged after adding GSH (10 mM) at a specific release time (5 h), whereas that of the cross-linked micelles showed a dramatic increase after the addition of GSH to cleave the disulfide bond (Fig. 3b). In addition, N-acetylcysteine (NAC), an FDA approved reducing agent has been demonstrated to possess similar effect as GSH in breaking down the disulfide bond. So it has the potential to act as a triggering agent for the drug release from disulfide cross-linked micelles after these micelles have accumulated in the tumor site (Fig. 4). So far, several polymeric micelle systems with ionic core containing biodegradable cross-linkages such as disulfide bond have been designed, synthesized and applied for the delivery of gene [57,58] or chemotherapeutic agents [59]. A new class of polymeric micelle, called the polyion complex (PIC) micelle, has been developed by Kataoka et al. They have selectively added thiol groups to the lysine repeat units resulting in poly(ethylene glycol)-block-poly-(L-lysine) (PEG-P(Lys)) [57]. During the self-assembly of PEG-P(Lys) to a PIC micelle, the free thiols remained in the lysine core were oxidized to form disulfide bond. The disulfide bond can be reversibly cleaved by reducing agents such as GSH or DTT. Another kind of micelles with ionic core containing disulfide bond has been reported by Kim et al based on block ionomer complexes (BIC) of poly(ethylene oxide)-*b*-poly(methacrylic acid) (PEO-*b*-PMA) and divalent metal cations (Ca²⁺) as templates [59]. They used cystamine as a biodegradable cross-linker to form the disulfide bond in the ionic core. These micelles (with particle size of 100–220 nm in diameter) exhibited a superior doxorubicin loading (50% w/w) in the ionic core and significantly increased cytotoxicity against human A2780 ovarian carcinoma cell.

2.1.3. pH sensitive SCMs

In recent years, pH-sensitive micelles have emerged as an important class nanocarrier for drug delivery based on the subtle pH changes in the human body. For example, the pH in the stomach is pH 1–2 compared with that in intestine (pH 5–8). The pH of the extracellular in the tumor site is slightly acidic (pH 6.5–7.2) while the pH value in blood and normal tissue is about 7.4. The endosome has a pH ranges from 5.0 to 6.5 and the pH in lysosome is even lower varying from 4.5 to 5.0. The pH-sensitive SCMs can target tumor site or the organelles in the tumor cells and release loaded drug due to the pH difference existing in the body [60–63]. Chan and co-workers have reported a

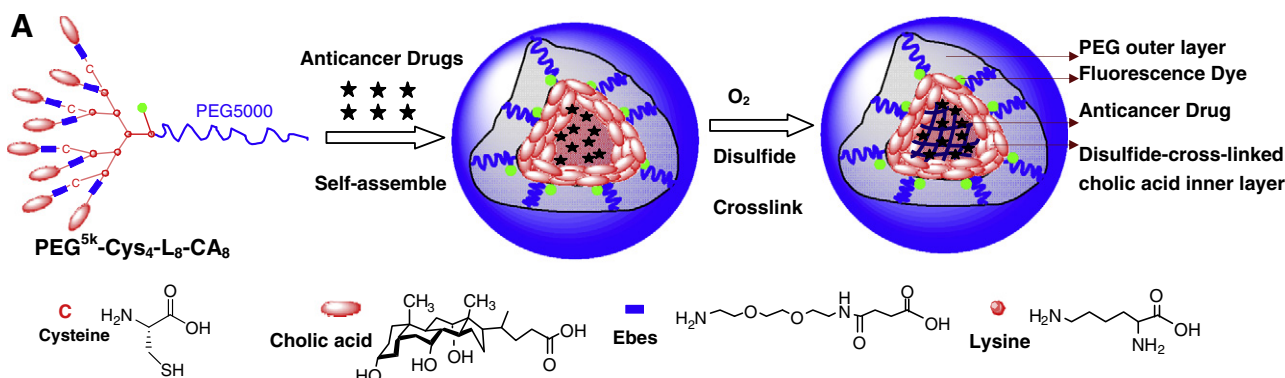


Fig. 2. Schematic representation of the disulfide cross-linked micelles formed by oxidation of thiolated telodendrimer PEG^{5k}-Cys₄-L₈-CA₈ after self-assembly. Reprinted from [44], Copyright (2011), with permission from Elsevier.

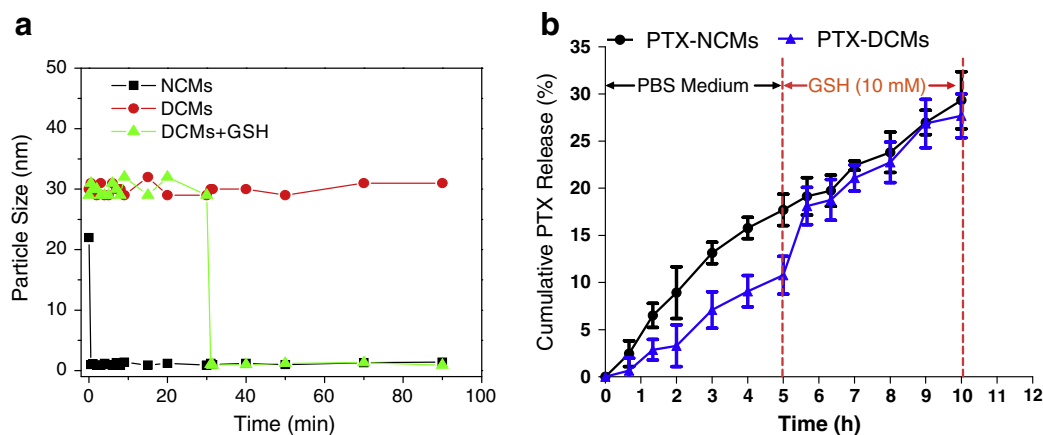


Fig. 3. (a) The stability in particle size of NCMs and DCMs in the presence of 2.5 mg/mL SDS measured by DLS. (b) GSH-responsive PTX release profiles of PTX-DCMs by adding GSH (10 mM) at a specific release time (5 h) comparing with PTX-NCMs. Reprinted from [44], Copyright (2011), with permission from Elsevier.

type of acid-labile core cross-linked micelles for pH-triggered drug release composed of poly(hydroxyethyl acrylate)-block-poly(*n*-butyl acrylate) (PHEA-*b*-PBA) [40]. The core of the micelles was cross-linked via the reversible addition-fragmentation chain transfer (RAFT) polymerization of a divinyl functionalized degradable monomer from the

living polymer chains which can be triggered to disintegrate by a pH-dependent manner. There was no big change in the particle size and morphology of the cross-linked micelles compared with the original non-cross-linked micelles determined by TEM and DLS. During the cross-linking process, high drug loading capacity (up to 60 wt.% of the copolymer weight) was achieved because of the π - π interactions between doxorubicin and the cross-linker's phenyl ring. Both cleavage and drug release rates at acidic pH were significantly higher than those at neutral pH. The pH-sensitive micelles capable of releasing drugs at slightly acidic pHs such as those found in extracellular fluids or intracellular endosomes of tumors are promising for anti-tumor chemotherapy. Du and colleagues reported a type of shape-persistent polymeric vesicle with pH-tunable membrane permeability. The vesicles were formed by the self-assembly of a pH-responsive, hydrolytically self-cross-linkable copolymer, poly(ethylene glycol)-*b*-poly((2-(diethylamino) ethyl methacrylate)-*s*-(3-(trimethoxysilyl) propyl methacrylate)) (PEG-*b*-P(DEA-*s*-TMSPMA)) [64]. Siloxane was utilized to cross-link the polymeric vesicle based on the further reaction of $-\text{Si}(\text{OD})_3$ groups. The mean diameter (D_h) of the vesicles is 630 ± 250 nm at neutral pH while the D_h increased monotonically under pH value below 7. Furthermore, the walls of the vesicles swell along with the protonation of the DEA residues.

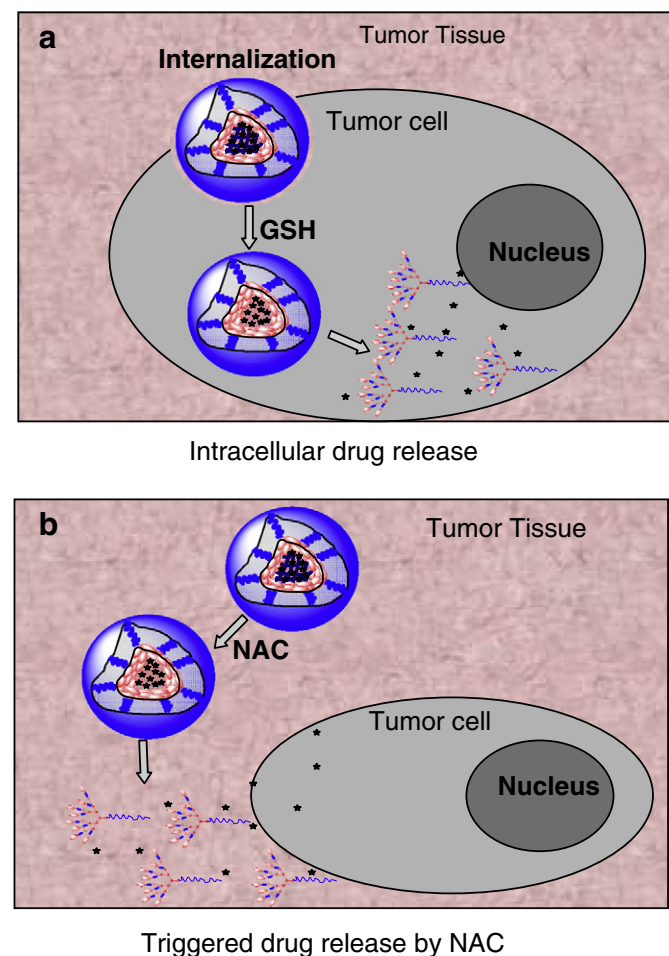


Fig. 4. Schematic illustration of the hypothesized mechanisms for reducing agents (a: GSH, b: NAC) mediated drug release once the PTX-DCMs accumulated at tumor sites. Reprinted from [44], Copyright (2011), with permission from Elsevier.

2.1.4. Multi-responsive SCMs

Recently, novel SCMs with multi responsiveness properties have been designed to deliver drugs to the complicate *in vivo* microenvironments. The SCMs' ability to respond to multiple stimuli, for instance, pH/reduction, temperature/reduction, temperature/pH and pH/cis-diol are introduced in the following section.

2.1.4.1. pH/reduction sensitive SCMs. A novel class of SCMs that are responsive to acidic and reductive stimuli was synthesized by Chen and co-workers [65]. The micelles with particle size of 60–100 nm were self-assembled via the polymers of PEG and 2,2'-dithio-diethanol diacrylate, 4,4'-trimethylene dipiperidine. The disulfide bonds and tertiary amines on the backbone of poly(β -amino ester) make SCMs have the characteristic of pH and reduction dual-sensitivity. The drug release profile of the DOX-loaded SCMs was monitored by UV-vis spectrometry at 480 nm at different time. About 30% of the entrapped DOX was released within 48 h in PBS solution at pH 7.4, while at pH 6.5, a 50% release in the first 6 h and up to 75% release in 48 h was observed. These results indicated that the tertiary amines in the core prevented the loaded DOX from diffusing to the outside of the smart micelles at physiological pH which maintained the core-shell structure of the micelles. The difference response to pH ranging from 7.4 to 6.5

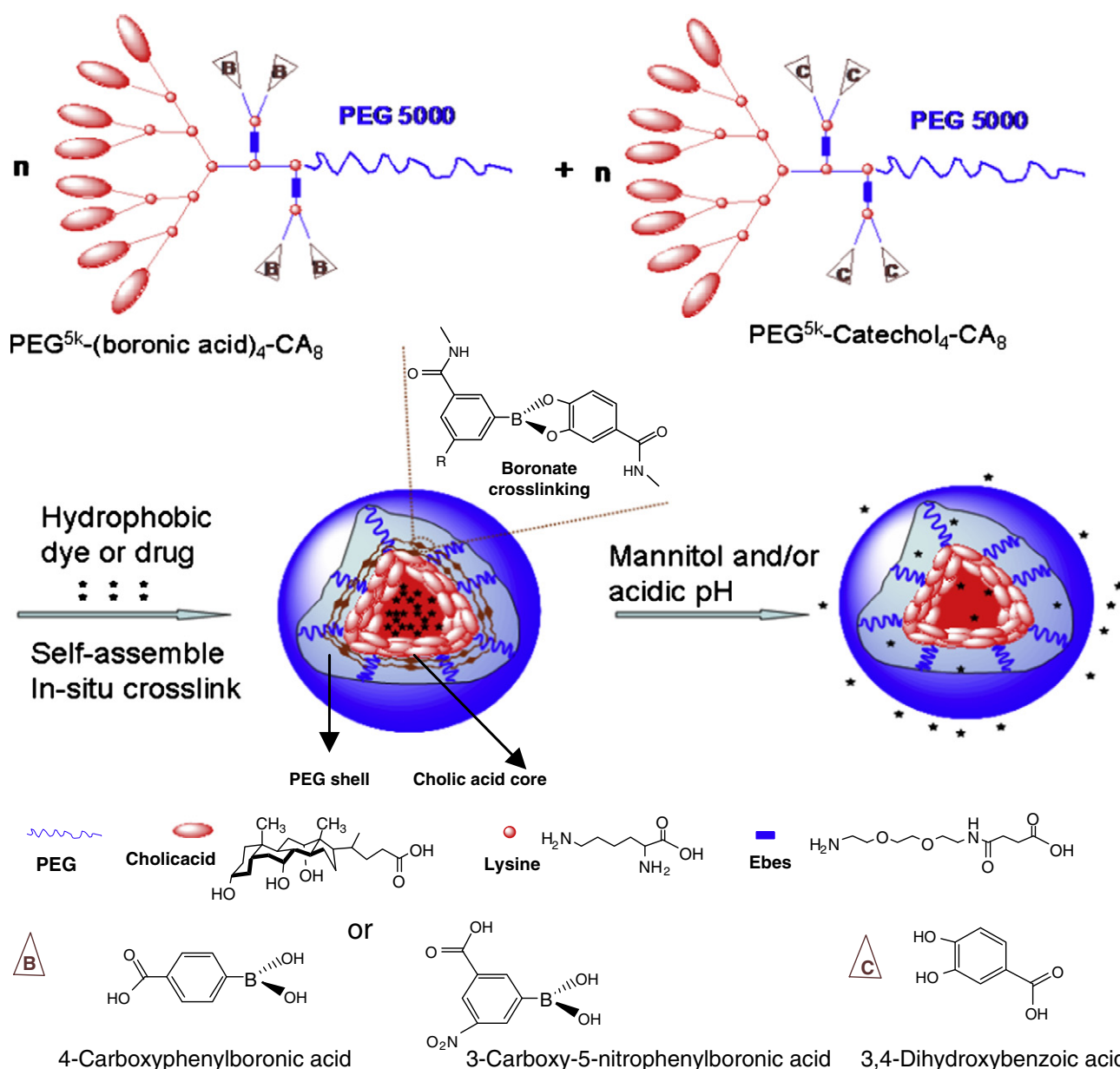


Fig. 5. Schematic representation of the telodendrimer pair [PEG^{5k}-(boronic acid/catechol)₄-CA₈] and the resulting boronate crosslinked micelles (BCMs) in response to mannitol and/or acidic pH [43]. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission from [43].

corresponding to the change from extracellular pH to early endosomal pH suggested those SCMs are a suitable intracellular drug delivery system. As described before, the reducing agents can trigger cleavage of the disulfide bonds and lead to a burst release of loaded-drug. DTT, a reducing agent, was used in the reduction-sensitive study of the smart micelles. The DOX released at a low rate similar to that in PBS at pH 7.4 in 0.1 mM DTT solution compared to more than 40% DOX release in 1 mM DTT. When the concentration of DTT reached 5 mM, about 60% DOX was released within first the 6 h and up to 80% DOX release was measured with 48 h. Shuai et al also demonstrated a highly packed interlayer-cross-linked micelle (HP-ICM) with pH and reduction dual sensitivity prepared from a triblock copolymer of monomethoxy polyethylene glycol (mPEG), 2-mercaptoethylamine (MEA)-grafted poly(l-aspartic acid) (PAsp(MEA)), and 2-(diisopropylamino)ethylamine (DIP)-grafted poly(l-aspartic acid) (PAsp(DIP)) [62]. Poly(BLA) aminolysis with MEA and DIP were utilized to introduce, disulfide bonds and imine bonds responding to acidic pH (pH 5.5) and a reducing

agent tris(2-carboxyethyl)phosphine (TCEP), respectively. The HP-ICM was highly stable and drug release rate was extremely low at pH 7.4. However, in the presence of reducing agent and an acidic pH (~5), the HP-ICM was dissociated and the loaded drug was released significantly faster.

2.1.4.2. Temperature/pH sensitive SCMs. A novel type of temperature and pH sensitive cross-linked micelles was developed by Babin and co-workers using an amphiphilic diblock copolymer of PDMAEMA₅₄-b-P(MMA₅₅-co-MMA₁₉) [66]. The shell cross-linked reverse micelles (SRCM) were obtained via photo-cross-linking upon UV irradiation at $\lambda > 310$ nm. The halide groups of SRCM initiated the grafting of styrene and dimethylaminoethyl methacrylate (DMAEMA) on the surface of the nanoparticles via atom transfer radical polymerization (ATRP). The resulting nanoparticles were sensitive to the changes in pH and temperature as well as UV irradiation. Zhang et al have reported the synthesis of dual pH- and temperature-sensitive SCMs via the self-assembly of

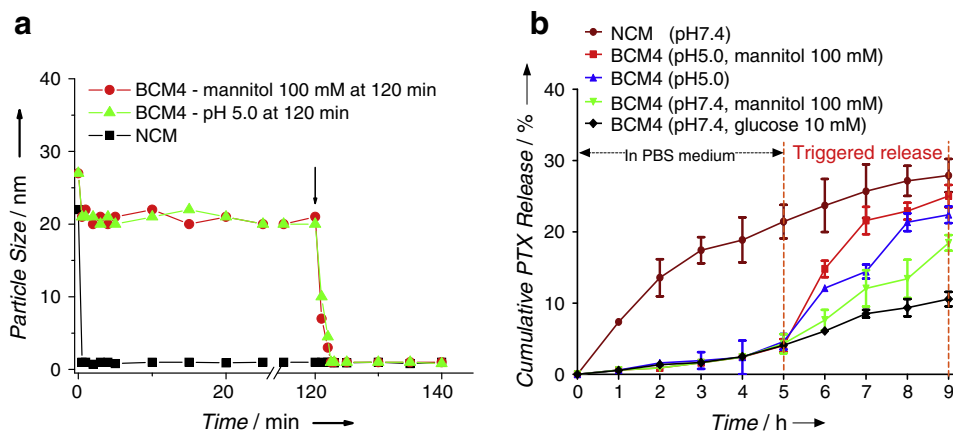


Fig. 6. (a) Continuous dynamic light scattering measurements of NCMs in SDS and BCM4 in SDS for 120 min, at which time mannitol was added or pH of the solution was adjusted to 5.0 (see arrow). (b) pH- and diol-responsive PTX release profiles of BCM4 by treating with diols (mannitol and glucose) and/or pH 5.0 at 5 h compared with that of NCMs [43]. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission from [43].

thermoresponsive block copolymer (PAGA₁₈₀-b-PNIPAAm₃₅₀) [67]. 3,9-divinyl-2,4,8,10-tetraoxaspiro[5.5]-undecane was used as an acid-labile cross-linking agent to generate the dual sensitive cross-linked nanoparticles. The resulting micelles were very stable at pH ranging from 6 and 8.2 but could be degraded at acidic conditions.

2.1.4.3. Temperature/reductive sensitive SCMs. A core cross-linked micelle system possessing sensitivity to temperature and reduction was reported by Jiang et al. These micelles were synthesized in a one-pot manner via RAFT copolymerization of *N*-isopropylacrylamide (NIPAM) and bis(2-methacryloyloxyethyl) disulfide (DSDMA) difunctional monomers using PAEMA as the macromolecular RAFT agent [41]. The obtained micelles were biocompatible and bioactive, since the amino groups in outer coronas of the micelles were further conjugated with carbohydrate and biotin as the targeting moieties. The temperature and reduction sensitivity of the micelles were demonstrated via the rupture of disulfide bonds in the presence of DTT and the shrinking of micelle cores when heating above the phase transition temperature of PNIPAM. These results indicated that the temperature and reductive sensitive SCMs have the potential to be used for targeted and controlled drug delivery.

2.1.4.4. pH/cis-diol sensitive SCMs. Boronate esters formed by boronic acids and diols possess dual sensitivity to pH value and competing diols [68–71]. Catechols are considered as an excellent reactant in the forming process of boronate esters. We have developed a novel type pH/cis-diol sensitive SCMs based on this principle for anticancer drug delivery [43]. A series of boronate cross-linked micelles with particle size of 20–30 nm were synthesized using equal molar ratios of the boronic acid- and catechol-containing telodendrimers (Fig. 5). Among them, BCM4 formed by the telodendrimer pair of PEG^{5k}-NBA₄-CA₈ and PEG^{5k}-Catechol₄-CA₈ showed superior stability with no significant particle size changes in the presence of either 50% (v/v) human plasma in 24 h or 2.5 mg/mL SDS solution. However, a rapid decrease of particle size occurred in SDS when the external pH value decreased to 5.0 or mannitol (100 mM, a safe FDA approved drug for diuresis) was added into the solution, indicating that the cleavage of critical percentage of boronate (Fig. 6a). PTX was used as model drug to compare the release kinetics of the reversible boronate cross-linked micelles and the non-cross-linked micelles (NCM). As the boronate formed by boronic acids and catechols could be disassembled in acidic environment or in the presence of mannitol, it was suggested that the drug release from these micelles could be triggered inside endosomes of tumor cells with an acidic pH or at the tumor sites with exogenously administered mannitol. Within the first 5 h, the PTX release of BCM4 was much

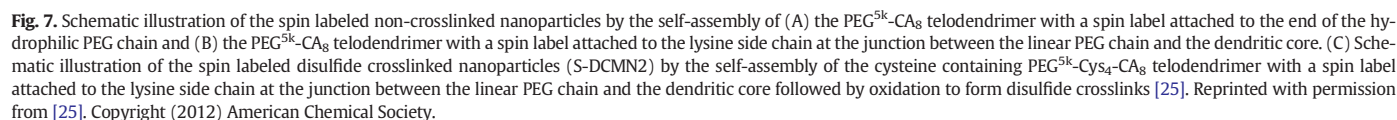
slower than that of NCM as shown in Fig. 6b. However, the BCM4 showed a burst PTX release upon the addition of 100 mM mannitol or the pH of the medium was adjusted to 5.0 at the 5 h time point. Furthermore, the combination of acidic pH and 100 mM mannitol could greatly accelerate the PTX release of the BCM4 micelles. This novel dual pH/cis-diol responsive nanocarriers to exhibited great promise for drug delivery accompanying with minimal premature drug release at physiological glucose level (2–10 mM) and physiological pH values (pH 7.4) in blood circulation but can be activated to release drug on demand at the acidic tumor microenvironment or in the acidic cellular compartments upon uptake into target tumor cells.

The SCM-based nanocarriers take advantage of the unique endogenous and exogenous stimuli and incorporate them into micelle-based drug delivery systems to precisely regulate the drug release. Although promising, addition of stimuli-responsive cross-linkers to the nanocarrier will add complexity and cost to their preparation. Therefore, robust processes that facilitate scale-up synthesis and manufacturing will undoubtedly accelerate the translation of SCMs into the clinics.

2.2. Characterizations of the interaction between SCMs and blood proteins

Blood proteins or lipoprotein particles may interact strongly with micellar nanoparticles, resulting in the dissociation of the nanoparticles and premature drug release. To get a better understanding of the interaction between SCMs and blood proteins, site-specific labeling with spin probes and fluorophores combined with electron paramagnetic resonance (EPR) spectroscopy and fluorescence resonance energy transfer (FRET) measurements were applied to monitor the assembly structure and dynamics within nanoparticles. EPR is a sensitive spectroscopic technique for studying materials with unpaired electrons. FRET, often call “molecular ruler”, is a distance dependent physical process by which an excited donor chromophore may transfer energy to an acceptor chromophore through nonradiative dipole–dipole coupling. Both EPR and FRET provide unique capabilities to probe the order within nanoparticles in a highly sensitive manner [25].

We combined site-specific nitroxide spin labeling and fluorescence labeling techniques with EPR spectroscopy and FRET technique, respectively, to probe the multiple dynamic processes occurring within nanoparticles during interaction with blood proteins. These techniques enable us to quantitatively analyze the dynamic changes in assembly structure, local stability and cargo diffusion of a class of novel telodendrimer-based micellar nanoparticles in human plasma and individual plasma components [25]. The site-specific spin label reporter system was developed via attaching 2,2,6,6-tetramethylpiperidinyloxy (TEMPO) to two specific sites on the PEG^{5k}-CA₈ telodendrimers (Fig. 7)



assembly order indicating a signal of increased intensity after incubation in human plasma. However, the EPR signal of S-NCMN1 and S-DCMN2 increased slightly over time. LDL, HDL, VLDL, chylomicron and human serum albumin were chosen as the blood components to investigate their interactions with the micelles. The results indicated that lipoprotein particles e.g. LDL, VLDL and chylomicron could interact with non-cross-linked micelles strongly and eventually broke down the micelles (Fig. 8). We also revealed that we could minimize the interactions of

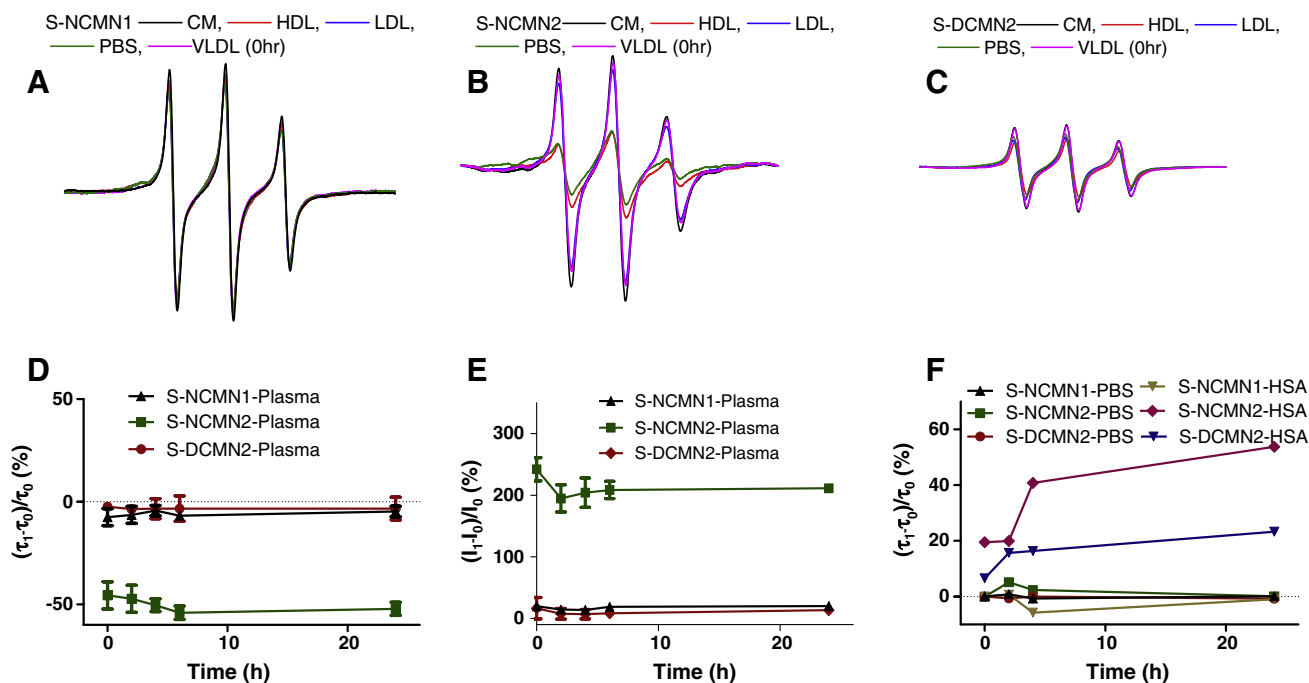


Fig. 8. Representative time-resolved EPR spectra S-NCMN1 (A), S-NCMN2 (B) and S-DCMN2 (C) in the presence of CM (1.0 mg/mL), HDL (2.0 mg/mL), LDL (2.0 mg/mL), PBS and VLDL (1.0 mg/mL). The percentage changes in rotational correlation time $((\tau_1 - \tau_0) / \tau_0)$ of the three types of spin labeled nanoparticles in human plasma at different time points (D), where τ_0 is the rotational correlation time of the sample in PBS while τ_1 is the rotational correlation time of the corresponding peak in a different media under identical instruments conditions, values reported are the mean diameter \pm SD for duplicate samples; The percentage of EPR intensity changes $((I_1 - I_0) / I_0)$ of the three types of spin labeled nanoparticles in human plasma at different time points (E), where I_0 is the intensity of the highest peak in the EPR spectrum of the sample in PBS while I_1 is the intensity of the corresponding peak in a different media under identical instruments conditions, values reported are the mean diameter \pm SD for duplicate samples; The $((\tau_1 - \tau_0) / \tau_0)$ value of the three types of spin labeled nanoparticles in PBS and HSA at different time points (F) [25]. Reprinted with permission from [25]. Copyright (2012) American Chemical Society.

micellar nanoparticles with blood proteins though introducing disulfide cross-linkages into the nanoparticles and activate these interactions by using reducing agents to cleave the disulfide bonds subsequently.

We constructed three position-specific FRET-based fluorescence reporter systems to monitor the dynamic changes in local proximity within nanoparticles upon interaction with blood proteins (Fig. 9). The first FRET system was developed by a green carbocyanine dye DiO (donor) physically encapsulated in the core of the disulfide cross-linked smart micelles without spin labeled and a red-orange dye rhodamine B (acceptor) covalently conjugated to the telodendrimer units. The second FRET system consisted of DiO (donor) and a red-orange carbocyanine dye DiI (acceptor) both physically encapsulated in the core of the SCMs. The third system comprised of a green dye FITC (donor) and rhodamine B (acceptor) both covalently conjugated to the telodendrimer units of the nanoparticles. A dramatic decrease in FRET signal for the non-cross-linked micelles occurred within 30 min in the presence of human plasma indicated that human plasma can disrupt the non-cross-linked nanoparticles. It was also observed that all the four major groups of lipoprotein particles (chylomicron, HDL, LDL and VLDL) cause a rapid decrease of FRET efficiency of FRET-NCMN1 while serum albumin (HSA) and immunoglobulin gamma (IgG, the most abundant antibody in blood) have minimal effect on the FRET efficiency. The FRET ratio of the disulfide cross-linked micelles decreased much slower than that of non-cross-linked micelles using the same FRET pair in the presence of HDL, LDL and VLDL indicating the disulfide cross-linked micelles have better stability and less premature drug release. However, the FRET signal of the disulfide cross-linked micelles dropped dramatically in the presence of human plasma and reducing agents (glutathione (GSH) and N-acetylcysteine (NAC)), indicating their reduction sensitivity. Furthermore, the disulfide cross-linked micelles were demonstrated to have significantly increased stability in animal models in comparison with their parent non-cross-linked micelles utilizing FRET technique [25].

The FRET technique has also been utilized to characterize the above mentioned reversible boronate cross-linked micelles [43]. We have used DiO and rhodamine B as a FRET pair to investigate the stability of NCM and BCM4. The efficient energy transfer from DiO to rhodamine B upon excitation of DiO at 480 nm indicated that the proximity between DiO and rhodamine B was within the FRET range. In the *in vivo* FRET studies, the FRET ratio of NCM decreased rapidly to 46% within 1 min post-injection and dropped to 21% 24 min after injection into nude mice via tail vein. However, the FRET ratio of BCM4 decreased much slower than that of NCM at the same micelle concentration, indicating the boronate cross-linking greatly enhanced the *in vivo* stability of the micelles, therefore, will decrease premature payload release.

As demonstrated above, SCMs are able to minimize the interaction of micellar nanoparticles with blood proteins (e.g. lipoproteins), improve the stability of micelles in the blood, prevent premature drug release, and enhance the delivery of drug to the tumor sites.

2.3. Pharmacokinetics and biodistribution of SCMs

Recently, stimuli-responsive cross-linked micelles have shown several attractive and distinct advantages compared to the non-cross-linked micelles. Ideally, the cross-linking could minimize the interactions between micelles and blood components such as plasma proteins and lipoproteins and prevent the micelles from premature dissociation upon extreme dilution in blood stream. Therefore, SCMs may have prolonged blood circulation time and eventually deliver more drugs into tumor tissue or tumor cells. In order to optimize the regimen for clinical applications, it is very important to monitor the pharmacokinetics (PK) and biodistribution of SCMs *in vivo*. Several approaches such as radio-labeling or fluorescent-labeling of polymer carriers and/or drug payloads are the most efficient way so far to determine the *in vivo* fate of polymeric micelles.

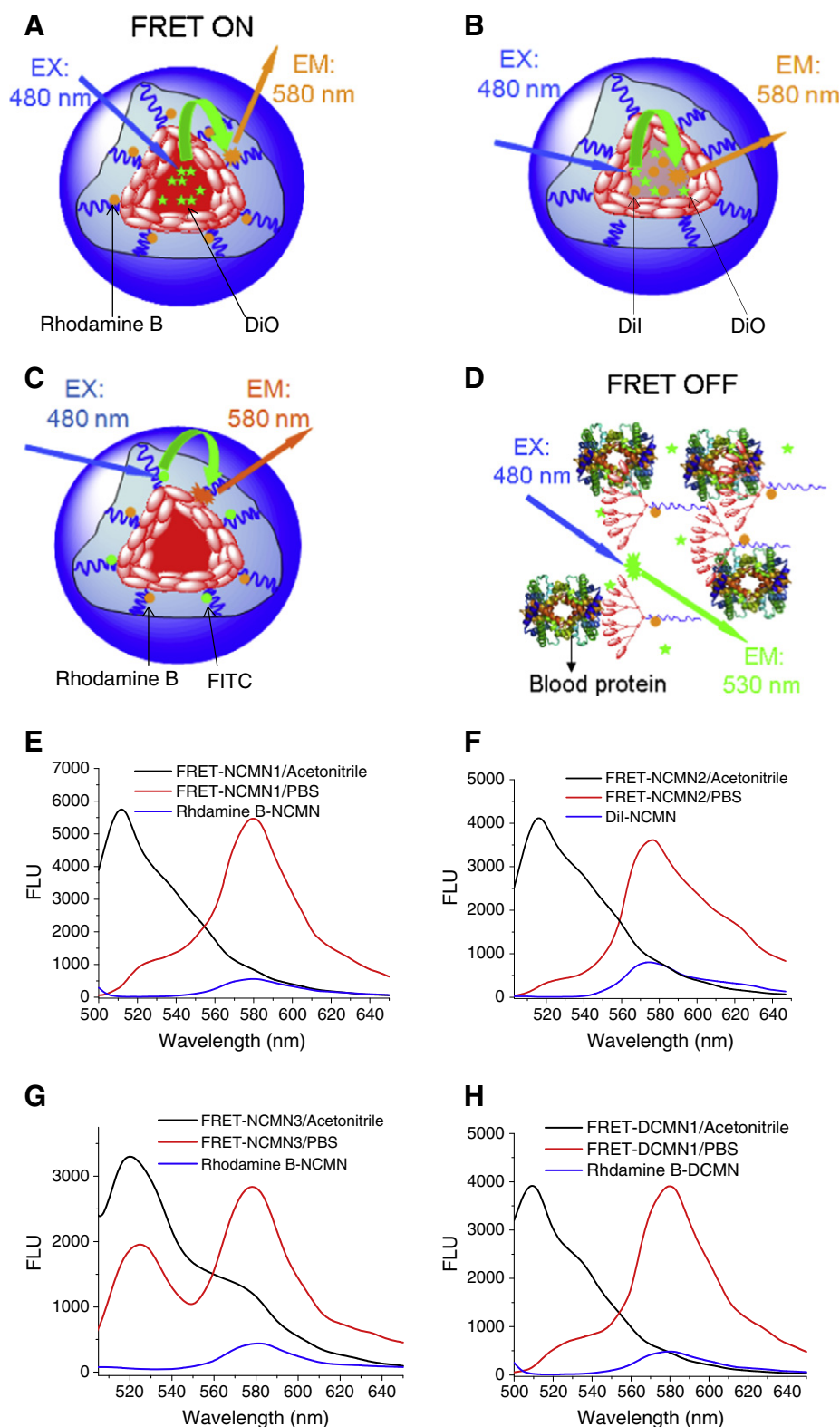


Fig. 9. Schematic illustration of non-crosslinked FRET-based nanoparticles FRET-NCMN1 (DiO and rhodamine B pair) (A), FRET-NCMN2 (DiO and DiI pair) (B), FRET-NCMN3 (FITC and rhodamine B pair) in PBS (C) and FRET-NCMN1 (DiO and rhodamine B pair) in human plasma (D); representative fluorescence spectra of FRET-NCMN1 (DiO loading: 2.5%, rhodamine B conjugated PEG^{5k}-CA₈: 5.0 mg) in PBS (red line) and in acetonitrile (black line), NCMN with rhodamine B alone (rhodamine B conjugated PEG^{5k}-CA₈: 5.0 mg) (blue line) with 480 nm excitation (E); representative fluorescence spectra of FRET-NCMN2 (DiO loading: 2.5%, DiI loading: 2.5%) in PBS (red line) and in acetonitrile (black line), NCMN with DiI alone (DiI loading: 2.5%) (blue line) with 480 nm excitation (F); representative fluorescence spectra of FRET-NCMN3 (FITC conjugated PEG^{5k}-CA₈: 5.0 mg, rhodamine B conjugated PEG^{5k}-CA₈: 5.0 mg) in PBS (red line) and in acetonitrile (black line), NCMN with rhodamine B alone (rhodamine B conjugated PEG^{5k}-CA₈: 5.0 mg) with 480 nm excitation (G); representative fluorescence spectra of FRET-DCMN1 (DiO loading: 2.5%, rhodamine B conjugated PEG^{5k}-Cys₄-CA₈: 5.0 mg) in PBS (red line) and in acetonitrile (black line), DCMN with rhodamine B alone (rhodamine B conjugated PEG^{5k}-Cys₄-CA₈: 5.0 mg) (blue line) with 480 nm excitation (H) [25]. Reprinted with permission from [25]. Copyright (2012) American Chemical Society.

A type of pH-responsive core cross-linked micelles also termed “polyion core micelles” synthesized by PEO-b-PMA block copolymers exhibit unique pharmacokinetics and biodistribution [72,73]. The core of the micelles comprises a network of the cross-linked polyanions formed by poly(methacrylic acid) with Ca^{2+} , which is surrounded by the hydrophilic PEO shell. To examine accumulation of CDDP-loaded micelles in cancer cells, the total Pt(II) content was measured in human ovarian carcinoma A2780 cells following their exposure to 50 μM free CDDP or CDDP-loaded micelles (with 20% degree of cross-linking) by ICP-MS. The net accumulation of Pt(II) was substantially higher in cells treated with CDDP-loaded and cross-linked micelles compared to cells treated with the free drug. These data demonstrated that the pH-responsive cross-linked micelles have an increased chemical stability, unique responsive drug release and accumulation in tumor site.

Another type of core-cross-linked (CCL) micelles based on mPEG5000 and N-(2-hydroxyethyl)methacrylamide-oligolactates (mPEG-b-p(HEMAm-Lacn)) was labeled with ^3H to investigate its circulation kinetics and biodistribution in ^{14}C (head and neck squamous-cell carcinoma line)-tumor bearing mice [26]. The non-cross-linked (NCL) micelles were rapidly eliminated from the circulation with only 6% of the injected dose (ID) present after 4 h whereas CCL micelles exhibited prolonged circulation times and more than 50% ID still present in the systemic circulation after 6 h. Hence, the area under curve (0–24 h) of CCL was substantially larger than that of NCL micelles (990 versus 136% ID \times h/mL blood). Although NCMs exhibited some initial tumor uptake at 4 h post-injection (2.5% ID), only 1.1% ID/g was recovered in the tumors after 24 h. In contrast, CCL micelles showed almost 6 times higher uptake in the tumors with more than 2-fold lower accumulation in the liver than NCMs at 24 h post-injection. More importantly, CCL micelles still remained a considerable tumor accumulation after 48 h (5.8% ID/g) by when the blood levels of ^3H -labeled CCL micelles were negligible. Therefore, the CCL micelles indicated prolonged circulation, better extravasation and tumor retention compared with the NCL micelles.

The *in vivo* blood elimination of the reversible boronate cross-linked micelles (BCMs) labeled with rhodamine was also monitored in our previous study [43]. After intravenous injection, the rhodamine B signal of non-cross-linked micelles decreased into background level with 10 h post injection, whereas the signal of BCMs was 6 times higher at 10 h and lasted for more than 24 h. These data demonstrated BCMs exhibited slower elimination during the blood circulation than the corresponding non-cross-linked micelles, which may significantly improve the pharmacokinetic profile of conventional water-insoluble anticancer drugs.

We have also investigated the blood elimination profiles and biodistribution of disulfide cross-linked micelles (DCMs) in comparison

with the corresponding non-cross-linked micelles (NCMs) [44]. Two separate types of near infra-red fluorescent (NIRF) dye-labeled NCMs and DCMs were developed to track the *in vivo* fate of the carriers and drug. BODIPY 650/665, an organic near infrared dye, was conjugated to nanocarriers and DiD, a hydrophobic near infra-red dye, was physically encapsulated into the core of the micelles as a drug surrogate. The signal of BODIPY 650/665 and DiD in NCMs was found to decrease faster than that of DCMs by monitoring the fluorescence intensity of BODIPY or DiD in the blood samples collected from tumor free nude mice at different time points post intravenous injection. BODIPY signal of DCMs in blood was up to 8 times higher than that of NCMs within 8 h post-injection and sustained up to 24 h. A similar trend of circulation kinetics was observed for the DiD-loaded NCMs and DCMs (Fig. 10a). The results indicated that DCMs have had longer blood circulation time than NCMs. Furthermore, in the *in vivo* NIRF optical imaging studies in SKOV-3 ovarian cancer xenograft mouse model, DiD-loaded DCMs were found to preferentially accumulate in the tumor site. A significant difference of fluorescence signal between tumor and background occurred at 4 h after administration and sustained up to 72 h. *Ex vivo* imaging at 72 h post-injection further confirmed the preferential uptake of DCMs in tumor compared to normal organs (Fig. 10b).

To summarize, the few studies published so far on pharmacokinetics and biodistribution demonstrate that stimuli-responsive cross-linking of micelles is able to significantly improve their stability in the blood, prolong their blood circulation, and enhance their tumor accumulation. After reaching the tumor site, SCMs are able to structurally respond to tumor micro-environments, such as low pH, and reductive condition, resulting in fast release of drug payload locally in the tumor tissue or inside the cancer cells.

2.4. Preclinical efficacy of SCMs

The physico-chemical property, pharmacokinetics and biodistribution of SCMs have been introduced in the previous section. Ultimately, the SCMs should be able to efficiently kill cancer cells *in vitro* and significantly inhibit the tumor growth *in vivo*. In the present section, we will discuss the *in vitro* and *in vivo* anti-cancer therapeutic efficacy of drug-loaded SCMs.

2.4.1. *In vitro* anti-tumor activity

As described in the previous section, a novel class of reversible stimuli-responsive disulfide cross-linked micelle was exploited by our laboratory based on thiolated telodendrimers [44]. The disulfide cross-linked micelles (DCMs) had a higher PTX-loading efficiency and slower drug release profile than the non-cross-linked counterpart,

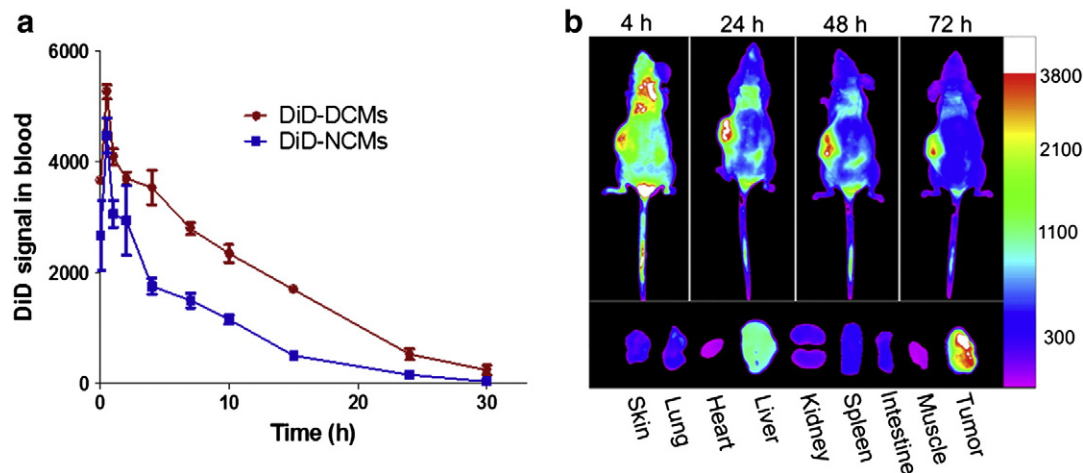


Fig. 10. *In vivo* pharmacokinetics (a) and biodistribution (b) of DiD-loaded DCMs and NCMs in nude mice bearing SKOV-3 ovarian cancer xenograft [44]. Reprinted from [44], Copyright (2011), with permission from Elsevier.

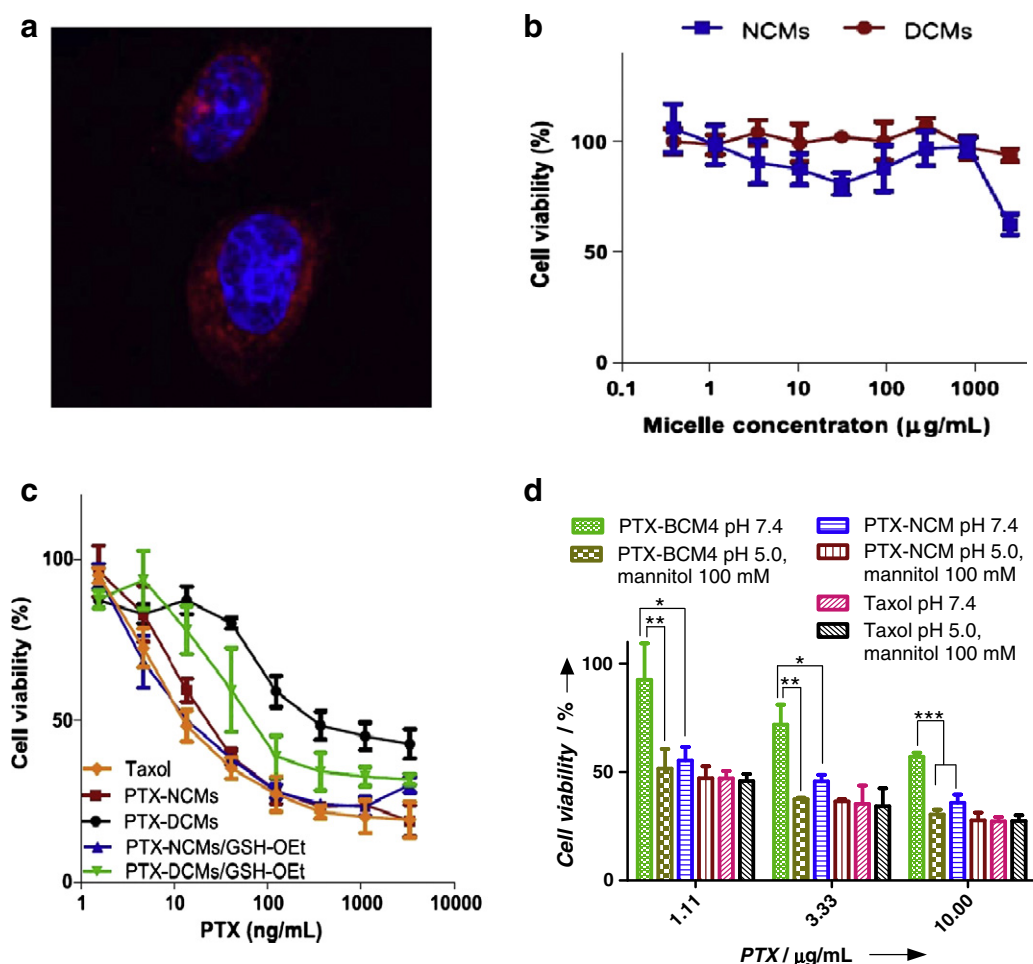


Fig. 11. (a) Cellular uptake of DiD-labeled DCMs in SKOV-3 ovarian cancer cells after 3 h incubation time, observed by confocal microscopy. The cell viability of SKOV-3 cells treated with (b) different concentrations of blank NCMs and DCMs, (c) Taxol®, PTX-NCMs and PTX-DCMs with and without pre-treatment of 20 mM GSH-OEt, and (d) Taxol®, PTX-NCMs and PTX-BCM4 with or without treatment with 100 mM mannitol at pH5.0 [43,44]. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission from [43]. Reprinted from [44], Copyright (2011), with permission from Elsevier.

however, the drug release rate from PTX-DCMs could be gradually facilitated by GSH at the intracellular concentration. The intracellular uptake of DiD fluorescent labeled DCMs in SKOV-3 ovarian cancer cells was observed under the confocal microscopy. As shown in the Fig. 11a, the DiD fluorescence intensity increased gradually during the first 3 h of incubation, and the internalized DCMs were found to localize mainly in the cytoplasmic region. The cell viability of blank and PTX-loaded DCMs were measure by MTT assay. Blank NCMs and DCMs exhibited no significant cytotoxicity against SKOV-3 ovarian cancer cells at the concentration up to 1.0 mg/mL. When loaded with PTX, PTX-NCMs had comparable *in vitro* cytotoxicity against SKOV-3 ovarian cells with Taxol®, whereas PTX-DCMs showed lower anti-cancer activity than both PTX-NCMs and Taxol®, which was likely due to the slower drug release profile from PTX-DCMs (Fig. 11b). However, PTX-DCMs exhibited enhanced cytotoxicity when glutathione monoethyl ester (GSH-OEt) was introduced to enhance intracellular GSH concentration which facilitated intracellular drug release due to the cleavage of intra-micellar disulfide bridges of DCMs (Fig. 11c).

The anti-tumor activity of the PTX-loaded reversibly boronate cross-linked micelles (PTX-BCM4) were also evaluated in comparison with PTX-loaded non-cross-linked micelles and free Taxol® [43]. They were incubated with SKOV-3 ovarian cancer cell for 1 h followed by PBS washing and then 23 h further incubation. PTX-NCMs and Taxol® showed similar anti-cancer activity, whereas PTX-BCM4 exhibited considerably lower cytotoxicity at equal PTX dose levels (Fig. 11d). Under

the tissue culture medium condition of pH 7.4, 5.5 mM glucose and 10% fetal calf serum (containing lipoproteins), BCM4 remained stable and level of drug release was limited. However, under acidic environment and in the presence of mannitol, drug released from BCM4 accelerated and therefore cytotoxicity was found to match that of PTX-NCMs and Taxol® (Fig. 11d). This result is consistent with previous discussion that acidic pH and mannitol can lead to the disintegration of the cross-linking boronates of BCM4 facilitating the release of drug from the nanoparticles.

2.4.2. *In vivo* therapeutic efficacy

SCMs have been widely studied because of their prolonged circulation time, good stability, high drug-loading capacity and on-demand drug release under certain stimulating environment. SCMs are expected to serve as a promising nanoparticle drug delivery system. Surprisingly, there are only a few reports in the literatures that have demonstrated the *in vivo* anti-tumor efficacy of SCMs.

For example, the *in vivo* anti-tumor efficacy of pH and redox dual-sensitive interfacially cross-linked micelles based on (mPEG-PASP (MEA)-PASP(DIP)) triblock copolymers prepared by Shuai et al. was evaluated in nude mice bearing the Bel-7402 tumor. The cross-linked micelle formulation of DOX was demonstrated to be more efficacious than free DOX [62]. The cross-linked mPEG-b-p(HPMAm-Lacn) micelles with covalently entrapped doxorubicin developed by Talelli et al. also

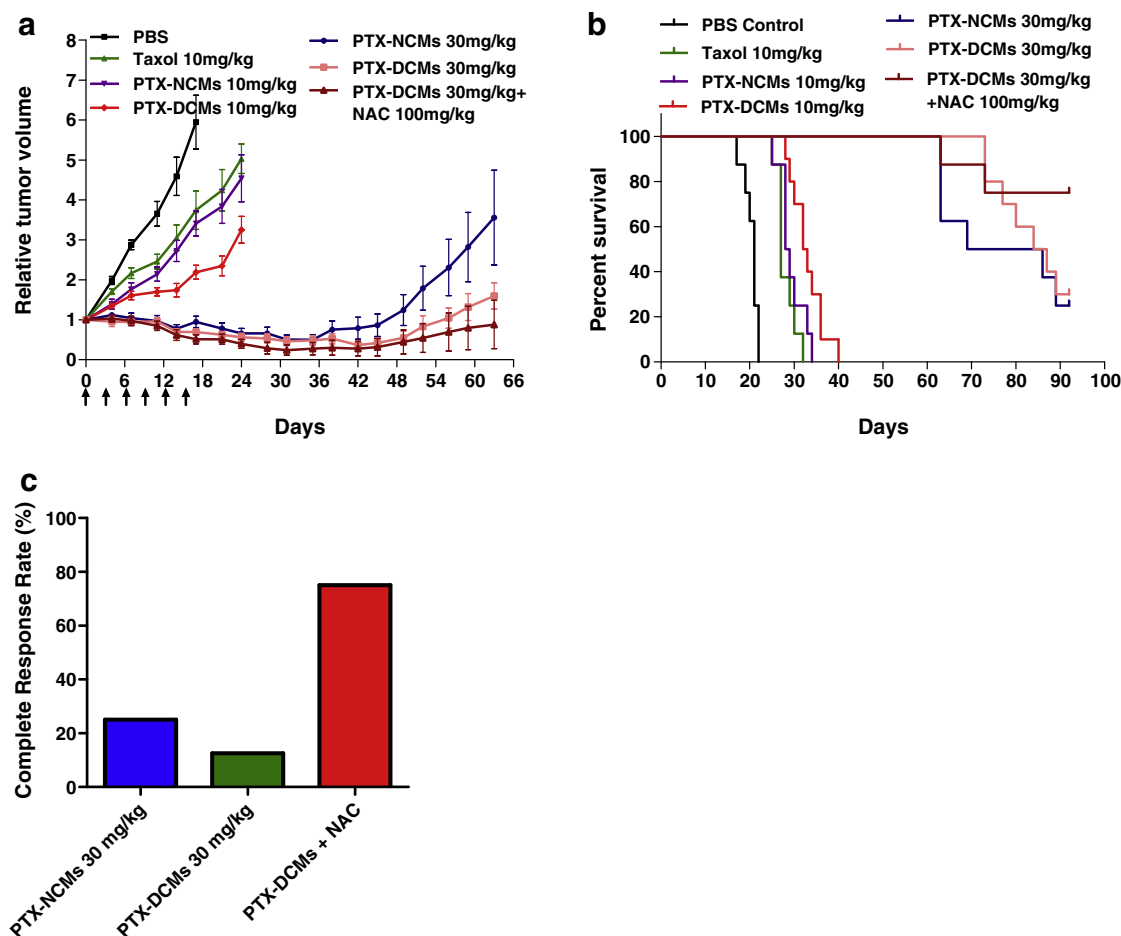


Fig. 12. (a) *In vivo* anti-tumor efficacy after intravenous treatment of different PTX formulations in the subcutaneous mouse model of SKOV-3 ovarian cancer. Tumor bearing mice were administered i.v. with PBS (control) and different PTX formulations on days 0, 3, 6, 9, 12, 15 when tumor volume reached about 100 ~ 200 mm³ (n = 8). (b) Survival of mice in different treatment groups. (c) Complete tumor response rate of mice treated with 30 mg/kg PTX-NCMs and PTX-DCMs with or without the trigger release by NAC [44]. Reprinted from [44], Copyright (2011), with permission from Elsevier.

showed better anti-tumor activity than free DOX in mice bearing B16F10 melanoma carcinoma [46].

The anti-cancer efficacy and toxicity profiles of our previously developed reversible disulfide cross-linked micelles loaded with PTX and VCR were evaluated in ovarian cancer and B-cell lymphoma mouse models, respectively [44,55]. Subcutaneous SKOV-3 tumor xenograft bearing mice was chosen in the therapeutic efficacy study of disulfide cross-linked micelles (DCM) in comparison with the non-cross-linked micelles (NCM) and paclitaxel (Taxol®). Compared to the control group, all the three treatment groups (clinical formulation of Taxol®, PTX-DCMs and PTX-NCMs) at 10 mg PTX/kg showed significant tumor growth inhibition ($p < 0.05$) (Fig. 12a) and prolonged survival time, (Fig. 12b). However, the tumor growth rate of mice in the PTX-DCMs groups was much lower than that in the PTX-NCMs group at both 10 mg PTX/kg and 30 mg PTX/kg dose. This can easily be explained by the lower premature drug release in the PTX-DCMs group. We have also tested the combination of PTX-DCMs with NAC (a reducing agent approved by FDA as a mucolytic agent and for the management of acetaminophen (Tylenol) overdose) given 24 h after each given dose of PTX-DCMs. NAC was given on-demand to facilitate drug release at the tumor site. Importantly, this treatment arm is the most efficacious to inhibit tumor growth (Fig. 12c). No palpable tumors were detected in 6 of the 8 mice by the end of the therapeutic studies. The result provides the preclinical “proof-of-concept” that exogenous agent such as NAC can be used for on-demand drug release of SCMs in cancer therapy.

In our another study, stimuli-responsive disulfide cross-linked micelles were applied for the targeted delivery of vincristine (VCR) to B-

cell lymphoma *in vivo* [55]. VCR encapsulated disulfide cross-linked micelles with a size of about 16 nm were formed by PEG^{5k}-Cys₄-L₈-CA₈ telodendrimers. Non-Hodgkin lymphoma xenograft bearing mice were treated with PBS, conventional VCR (1 mg/kg), DCM-VCR (1 mg/kg) with or without 100 mg/kg NAC given 24 h after the nanotherapeutic dose, or high dose DCM-VCR (2.5 mg/kg) groups. Significant inhibition in tumor growth was observed for all VCR treatment groups compared with the PBS controlled group ($p < 0.05$). The mice treated with 1 mg/kg DCM-VCR showed a significantly less weight lost than those treated with free VCR (1 mg/kg). Furthermore, 1 mg/kg DCM-VCR followed by 100 mg/kg NAC 24 h later showed superior anti-tumor effect to the free VCR group. High dose (2.5 mg/kg) of DCM-VCR exhibited even better tumor growth inhibition, and more importantly, it was well tolerated, with an equivalent amount of weight lost as the 1 mg/kg conventional VCR group.

Thus far, the number of papers reporting *in vivo* therapeutic studies of SCMs is rather limited; however, the results are convincingly unanimous in showing that SCMs are able to deliver anti-cancer drugs to tumor tissue more efficiently, and enhance anti-cancer therapeutic efficacy in preclinical animal models, when compared to non-cross-linked micelles.

2.5. Conclusion and future outlook

The research on SCMs addresses a critical issue in cancer research to reduce drug adverse toxicities and increase drug efficacy. State-of-the-art design of SCMs with enhanced stability and stimuli sensitivity

represents the frontier development of nanomedicine. It is a convenient and efficacious approach to prevent pre-mature drug release during circulation and deliver high concentration of drug to tumor sites on-demand. It shows great promise in formulating many existing and new drugs to enhance their therapeutic efficacies and lower their side-effects against cancers and other diseases.

Although the main focus of the nanodelivery field has been in cancer imaging and therapy, one ought not ignore its applications in the treatment of many benign diseases. With the appropriate targeting ligands, one may be able to deliver nanotherapeutics to target tissues of interest, such as liver, lung, adrenal glands, bone, stroke sites, ischemic myocardium, and brain endothelial cells. Recent work in our laboratory demonstrated that dexamethasone-loaded in nanomicelles is more efficacious than free dexamethasone in the treatment of experimentally induced asthma in murine model [82]. Ligands that can facilitate the crossing of the blood–brain-barrier may allow one to deliver SCMs to the brain tissues efficiently, bypassing the need to give intra-theal drugs. SCMs encapsulated with potent antibiotics and decorated with specific ligands against cell surface proteins of microbes and parasites may revolutionize the treatment of many globally important infectious diseases. Ligands that recognize host cells such as monocytes and macrophages may allow delivery of potent antibiotics for the treatment and eradication of mycobacteria from the patients. In order to realize the promise of using small RNAs for the treatment of many diseases, one needs to develop an efficient delivery system. We believe nanocarrier equipped with appropriate targeting ligands and endosomal releasing molecules is the likely solution.

To translate SCMs into efficacious therapies in the clinic will require more than just stable formulation with minimal drug release during the circulation [74]. The other following important factors will also need to be considered: 1) the nanocarrier itself needs to be biodegradable, biocompatible, and nontoxic; 2) scale-up production of the nanoparticle drugs needs to be economical, reproducible and robust; 3) formulation procedures by the pharmacist at the clinic needs to be simple and reproducible; 4) the shelf-life of the nanoformulation needs to be measured in months; 5) the particle size distribution needs to be narrow; 5) the uptake by the reticuloendothelial system in the liver, spleen, lung and bone marrow needs to be minimal; 6) To be fully effective, the nanoparticle drugs needs to be delivered intracellularly, which can be achieved by decorating nanocarriers with cancer cell targeting ligands that facilitate receptor-mediated endocytosis. Future effort should be focused on the development of nanocarriers that fulfill all the above mentioned criteria.

Taken all together, a bright future may be foreseen for nanoparticle drug delivery system, especially for stimuli-responsive cross-linked and cancer specific targeting nanocarriers. Many drugs that had failed previously because of formulation issues or toxicity issues may possibly be resurrected by incorporating and adopting the stimuli-responsive cross-linked nanocarriers technology for efficient delivery.

List of all abbreviations

EPR	Electron paramagnetic resonance
FRET	Fluorescence resonance energy transfer
MDR	Multidrug resistance
CMC	Critical micelle concentration
HDL	High density lipoprotein
LDL	Low density lipoprotein
VLDL	Very low density lipoprotein
DCMs	Disulide cross-linked micelles
NCMs	Non-cross-linked micelles
BCMs	Boronate cross-linked micelles
EPR	Enhanced permeability and retention
PTX	Paclitaxel
DOX	Doxorubicin

VCR	Vincristine
MTX	Methotrexate
GSH	Glutathione
RAFT	Reversible addition-fragmentation chain transfer
PEG- <i>b</i> -PHPMA-LA	Poly(ethylene glycol)- <i>b</i> -poly(<i>N</i> -2-hydroxypropyl methacrylamide)-lipoic acid
DTT	Dithiothreitol
LAM	Lipoic acids
SDS	Sodium dodecyl sulfate
BIC	Block ionomer complexes
PEO- <i>b</i> -PMA	Poly(ethylene oxide)- <i>b</i> -poly(methacrylic acid)
HEC- <i>g</i> -PAA	Cellulose- <i>g</i> -poly(acrylic acid)
PHEAb-PBA	Poly(hydroxyethyl acrylate)- <i>block</i> -poly(<i>n</i> -butyl acrylate)
PEG- <i>b</i> -P(DEA- <i>s</i> -TMSPMA	Poly(ethylene glycol)- <i>b</i> -poly((2-(diethylamino) ethyl methacrylate)- <i>s</i> -(3-(trimethoxysilyl) propyl methacrylate))
PLA	Poly lactate
PCL	Polycaprolactone
mPEG- <i>b</i> -p(HPMA _m -Lac _n)	Poly(ethylene glycol)- <i>b</i> -poly[N-(2-hydroxypropyl) methacrylamide-lactate]
DOX-MA	DOX methacrylamide derivative
PEG-PAsp-PPhe	Poly(ethylene glycol)-poly(L-aspartic acid)-poly(L-phenylalanine)
DLS	Dynamic light scattering
KSV	Stern-Volmer quenching constant
PEG- <i>b</i> -PLys- <i>b</i> -PPha	Poly(ethylene glycol)- <i>b</i> -poly(L-lysine)- <i>b</i> -poly(L-phenylalanine)
DTSSP	3,3'-dithiobis(sulfosuccinimidylpropionate)
TCEP	Tris(2-carboxyethyl)phosphine
mPEO-PAPMA-PDPAEMA	Methoxy poly(ethylene oxide)- <i>b</i> -poly(<i>N</i> -(3-aminopropyl) methacrylamide)-poly(2-(diisopropylamino)ethyl methacrylate)
DTBP	Dimethyl 3,3-dithiobispropionimidate
PAEMA	Poly(2-aminoethylmethacrylamide)
NIPAM	<i>N</i> -isopropylacrylamide
DSDMA	Bis(2-methacryloyloxyethyl) disulfide
ATRP	Atom transfer radical polymerization
SCRM	Shell cross-linked reverse micelles
DMAEMA	Dimethylaminoethyl methacrylate

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