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Reversibly crosslinked nanocarriers for on-demand drug delivery in cancer treatment

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Abstract

Polymer micelles have proven to be one of the most versatile nanocarriers for anticancer drug delivery. However, the *in vitro* and *in vivo* stability of micelles remains a challenge due to the dynamic nature of these self-assembled systems, which leads to premature drug release and nonspecific biodistribution *in vivo*. Recently, reversibly crosslinked micelles have been developed to provide solutions to stabilize nanocarriers in blood circulation. Increased stability allows nanoparticles to accumulate at tumor sites efficiently via passive and/or active tumor targeting, while cleavage of the micelle crosslinkages, through internal or external stimuli, facilitates on-demand drug release. In this review, various crosslinking chemistries as well as the choices for reversible linkages in these nanocarriers will be introduced. Then, the development of reversibly crosslinked micelles for on-demand drug release in response to single or dual stimuli in the tumor microenvironment is discussed, for example, acidic pH, reducing microenvironment, enzymatic microenvironment, photoirradiation and the administration of competitive reagents postmicelle delivery.

Compared with normal organs, the unique leaky blood vessels in solid tumors [1] provide an opportunity to target nanotherapeutics to tumor sites based on the selective size effects of nanoparticles. In addition, the lack of the lymphatic vessels within a solid tumor enables the extended duration of nanoparticles within tumor sites, therefore increasing cancer killing of the payload drugs. This is called the enhanced permeability and retention (EPR) effects of nanoparticles for tumor targeting [1–2]. However, the hyperpermeability of blood vessels and the lack of lymphatic drainage lead to interstitial hypertension, which alters the convective transport of nanoparticles through solid tumors to size-dependent slow diffusion. In addition, the abnormal tumoral blood vessels [3] create hypoxia, low pH microenvironment, which induces the resistance of cancer cells to chemotherapy and radiation therapy. Hypoxia may also induce cellular changes resulting in cancer cells with more malignant potential. Therefore, the normalization of tumor blood vessels via antiangiogenic therapy to reduce interstitial pressure can sensitize endothelial cells and improve the intratumoral delivery of drug molecules in chemotherapy [4,5]. A recent simulation and experimental study revealed that the smaller nanomedicines with an approximate size of 12 nm are much more effective due to the superior tumor penetration in a murine mammary tumor model after antiangiogenic treatment [6]. Furthermore, the heterogeneity of solid tumors have been well recognized between different type of tumors as

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well as the stages of tumors and even the status of the same tumor during the treatment period. Therefore, the homogenous delivery of nanoparticles to a solid tumors is impaired by the heterogeneous physical barriers and chemical microenvironments within solid tumors. In addition, the physiological pore size of the different blood vessels in normal organs vary from a few nanometers to 100 nm, depending on the structure and function of the capillaries [7]. For example, the diameters of the fenestrae in the capillary walls of human hepatic sinusoidal capillaries are, on average, approximately 105 nm (ranging from 50 to 180 nm) [8]. This may lead to the physical entrapment and phago endocytosis of nanoparticles with large particle sizes in liver. Therefore, the size, surface chemistry and stability of nanocarriers need to be specifically designed and optimized for effective trans vascular and interstitial transport of chemodrug to tumor, other than to normal organs.

Compared with dendrimers, liposomes and other organic/inorganic nanoparticles, polymer micelles are much more versatile for delivery of a broad spectrum of therapeutics [9–12]. Numerous amphiphilic polymers with different chemistry and various architectures have been developed to form polymeric micelles. Therefore, the favorable properties of polymer micelles, such as stealth surface chemistry, high drug-loading capacity, multiple functionality and optimal particle sizes (10–100 nm), can be achieved through various chemistries. These optimized chemical and physical properties enable **tumor-targeted drug delivery** and minimize the side toxicity of payload drugs. However, the destabilization of micelles *in vitro* and *in vivo*, related to the inherent dynamic self-assembled systems, results in premature drug release, opsonization, reticuloendothelial system clearance and high uptake of the nanoparticles in the liver, spleen and lungs. These negative events reduce the efficiency of tumor-targeted drug delivery via the passive EPR effect and the active-targeting effects of a nanocarrier, thereby hindering *in vivo* **cancer treatment**. Strategies of reversible crosslinking have been employed to stabilize micellar nanocarriers in blood circulation as well as specifically releasing drugs in response to unique intra-cellular or extracellular stimuli within tumor microenvironments.

Tumors develop unique chemical microenvironments related to the uncontrolled, progressive multiplication of cancer cells. Increased glycolysis produces more lactic acid than normal cells [13–14], compounded with higher plasma membrane proton-pump activity of tumor cells, resulting in a slightly acidic pH (pH 6.5–7.2) in the extracellular space of solid tumors [15]. It is worthy to note that the pH in tumors is near neutral close to the blood vessels and within a distance of approximately 100 μm from the vessel wall. The pH drops to a value of approximately 6.6 at a distance of approximately 400 μm from the vessel wall [16]. Therefore, in order to access the acidic pH, the pH-sensitive nanoparticles need to be small in size for the deep penetration into acidic tumor tissue [17]. The overproduction of enzymes, such as matrix metalloproteinases, is also common in most tumors, which are important for angiogenesis, metastasis and other extracellular signaling events involved in tumor propagation [18]. The presence of hypoxia in human tumors, especially in solid tumors, is well known. Some regions in tumor tissues are poorly vascularized, resulting in low oxygen tension, pH and nutrient levels, as well as the overexpression of angiogenic factors and endogenous reductive enzymes [19]. Glutathione level is elevated in a number of different human cancer tissues [20] and many chemoresistant cancer biopsy tissues (tenfold higher than those before treatment) [21]. In addition, the pH in the intracellular compartments such as the later endosome and lysosome are significantly lower (pH 4.0–6.5) than extracellular environments. Furthermore, the intracellular concentration of reducing glutathione is known to be substantially higher than that in the cellular exterior ($\sim 10\text{ mM}$ vs $\sim 2\text{ }\mu\text{M}$). These extreme conditions provide opportunities for tumor-targeted drug delivery. In addition, some external stimuli such as magnetic field, photoirradiation and ultrasound can be precisely applied to some tumors to trigger the drug release locally within tumor sites.

Many non-crosslinked micelles (NCMs) with pH sensitivity or photosensitivity have been developed and reviewed extensively [22–24] for targeting tumor acidic microenvironment, or photoirradiation applied to tumor sites for efficient drug release. However, these NCMs may also encounter the same problem of premature drug release due to the dynamic nature of the self-assembled micelle system. This review focuses on the current development of reversibly crosslinked nanocarriers for tumor-targeted drug delivery. These nanocarriers are stable under physiological conditions and sensitive to the unique internal stimuli presented in the tumor microenvironment, as discussed above, or responsive to external stimuli applied to tumor sites, such as light, magnetic field and ultrasound, for **on-demand drug release**. First, the choice of labile chemistries for reversible micelle crosslinking will be introduced; second, the development of crosslinked nanocarriers for drug delivery will be summarized; then, the examples of **reversibly crosslinked micelles** for on-demand drug release will be discussed; And finally, we will introduce our published and on-going research in the development of various reversibly crosslinked telodendrimer micelles, which can respond to redox and acidic conditions, photoinitiation and *cis*-diol competition, respectively, for on-demand drug release for efficient cancer therapy will be presented.

Crosslinking chemistry & reversible linkages

Micelles are self-assembled systems and drug molecules are usually loaded in their core during micelle formation. Therefore, the crosslinking of the micelle should happen only after drug loading. Hetero- or homo-crosslinking groups should be preconjugated onto the polymer structure and crosslinking reactions are preferred to occur automatically after drug loading without any catalyst required or initiated only with clean reagents or physical stimuli, such as water molecules, oxygen in air, or mild thermo- or photo-initiation. However, in many studies, additional small molecular crosslinking reagents, catalysts or condensation reagents were added into the system to crosslink micelles; these may end up as chemical impurities for *in vitro* and *in vivo* application, especially for clinical applications. Extensive dialysis or size-exclusive chromatography is required to remove these excessive/remaining small-molecular reagents. In some cases, the hydrophobic impurities are trapped in the core of the crosslinked micelles and will prove very difficult to remove completely. At the same time, a certain amount of drug molecules will be lost during the purification and dialysis steps. Therefore, the clean, high-yield loaded and highly specific chemistries are desired to crosslink micelles for practical anticancer drug delivery.

The efficacy and simplicity of crosslinking chemistries will significantly impact the purity and stability of crosslinked nanoformulations, as well as the feasibility for future clinical development. Sensitivity and selectivity of stimuli-responsive **labile linkages** will determine the stability of the nanocarrier in systemic circulation as well as efficiency for on-demand drug release in tumors. In general, there are two ways to crosslink a micelle reversibly:

- The *in situ* crosslinking chemistry can be reversible in response to specific stimuli at the tumor site, such as the formation of a redox-sensitive disulfide bond [25–27], acid labile and *cis*-diol-sensitive boronate [28], photosensitive chemistries [24] and **enzyme-labile** phosphate linkages [29];
- Reversibly crosslinked micelles can be developed by incorporating labile linkages as spacers to introduce active crosslinking groups onto the polymer, which form permanent crosslinkages for efficient micelle crosslinking. The labile spacer linkages are commonly seen as redox-sensitive disulfide bonds [30], acid-labile orthoesters [31], ketals [32] or an enzymatic-labile peptide sequence [33].

The most commonly used chemistries in literature to form permanent crosslinkages in micelle crosslinking include amide bond formation [31,34], vinyl polymerization [35,36],

bioinspired silicification [37,38], thiol-ene [34] and [3+2] cyclization [39] click chemistry. Theoretically, any efficient chemistry can be used to crosslink micelles at the core, shell or intermediate layer. However, the reversible linkages need to be specific towards the unique tumor microenvironment, which have been widely used for prodrug constructions [40]. Therefore in **Table 1** [28,31,32,34,41–65], only the tumor-specific labile linkages, which have been applied or can be used to crosslink micelles, are summarized.

Crosslinked micelles for hydrophobic drug delivery

Nanoparticles from self-assembled block copolymers with core-shell structures and diameters of 10–100 nm show promise as vehicles for the targeted delivery of drugs, proteins, genes and imaging agents; this is a result of the EPR effect, where nanocarriers encapsulating drugs show long circulation time and large accumulation at tumor sites. A major problem for block copolymer micelles is their spontaneous dissociation at highly dilute concentrations, below their critical micelle concentration, which is typically encountered during intravenous administration. To overcome this issue, crosslinked micelles were explored to enhance self-assembled micelle stability. A range of strategies [66–68] have been reported to achieve crosslinked micelles, such as radiation-induced crosslinking [62,69–71], metalcatalyzed crosslinking [72], click crosslinking [73,74] and reaction with bifunctional crosslinking agents [75,76], which include carbodiimide coupling [77], activated esters [78,79] and disulfide-based crosslinkers [80,81]. More generally, crosslinked micelles can be categorized into three types based on variation of crosslinking position: shell crosslinking, core crosslinking and intermediate layer crosslinking.

Core-crosslinked micelles

The core crosslinked method was originally reported by Liu and colleagues [62,63] in which a photosensitive cinnamoyl ethyl group was selectively incorporated into the core-forming block; upon UV irradiation, a crosslinked micelle core can be successfully achieved. Kataoka *et al.* [82] have shown micelle core crosslinking to be an effective way to avoid micellar disintegration upon dilution. They demonstrated that core crosslinked micelles of poly(ethylene glycol)-*b*-poly(*D,L*-lactide) possess high stability against dilution, temperature change and even dissolution of surfactants. Kissel's group [83] also developed novel core crosslinked micelles for drug delivery. Polymerization of crosslinkable double bonds in the hydrophobic domain enabled high polymeric micelle stability [84]. More recent stimulating work concerning core crosslinking of micelles was reported by Takahara's group [85], who synthesized linear diblock copolymers consisting of poly(methyl methacrylate) block and random copolymer block of methyl methacrylate and methacrylic esters with alkoxyamine moiety via atom transfer radical polymerization. The crosslinked nanogels were obtained by the radical crossover reaction of alkoxyamine moieties under heating conditions. A polymer micelle with a crosslinked ionic core was reported by Bronich *et al.* [86] The micelle core is composed of crosslinked polyions containing polymethacrylate anions and divalent metal cations, which is surrounded by the shell of hydrophilic polyethylene oxide chains. The reaction scheme in **Figure 1** summarizes some more examples of core crosslinked micelles [41,63,69,72,81,87–89].

Shell-crosslinked micelles

Since the founding work from Wooley group on shell crosslinked (SCL) micelles in 1996 [90], there has been increasing interest in this area [66]. SCL nanoparticles, by the self-assembly of block copolymers, possess an amphiphilic core-shell morphology consisting of a crosslinked shell and core domain. The crosslinking of the shell layer by chemical or physical interactions can reinforce the micellar architecture and result in high stability under environmental variations, such as ionic strength, solvent system and pH. Wooley's group

[91] described the preparation of a stable, SCL micelle, formed by condensation reactions between diamino crosslinkers and pendant carboxylic acid groups along the poly(acrylic acid) segments located in the periphery shell. Experimental results indicate that SCL micelles, in comparison to non-SCL micelles, continue to have stable nanostructures after ozonolysis-induced disintegration. In another case, Zhuo's group [92] reported preparation of SCL thermoresponsive hybrid micelles by crosslinking the hydrophilic shell with trimethoxysilyl residue via an acid-catalyzed sol-gel process. Drug-loading and *in vitro* drug-release data has shown that SCL micelles exhibit much greater encapsulation efficiency in addition to a slower release rate than noncrosslinked micelles. Some more examples of the typical reactions for core crosslinking strategies were summarized in **Figure 2** [73,90,93–96].

Intermediate-layer-crosslinked micelles

The major problem in the preparation of SCL micelles is that shell crosslinking may cause extensive intermicellar crosslinking, restricting commercial applications [97]. To solve this issue, new intermediate-layer-crosslinked micelles were developed by Liu and Armes [98,99], who reported triblock copolymers containing poly(2-(dimethylamino)ethylmethacrylate) of crosslinkable unit as a middle block. They demonstrated that ABC triblock copolymers offer great advantages over conventional diblock copolymers, because the former allows shell crosslinking with little or no intermicellar crosslinking. The thermally induced micellar self-assembly structure could selectively further crosslink the dimethyl adipimidate residues in the intermediate shell. Intermicellar crosslinking was negligible, since the coronal PEG chains ensured minimal interpenetration of the micelles due to steric stabilization. Wooley group [73] developed a new methodology for the preparation of intermediate layer click crosslinked nanoparticles. Herein, click reactions with the alkynyl groups to form covalent linkages throughout the block copolymer micelle corona were successfully incorporated into polymer systems for forming a crosslinked intermediate shell. **Figure 3** shows some samples [100] of intermediate layer crosslinked micelles and self-assembly illustration.

Reversibly crosslinked nanocarriers for hydrophilic therapeutic delivery

Nanogels

Nanogels are nanosized hydrated gel networks, which can potentially serve as delivery systems for water-soluble drug molecules such as proteins, siRNA, peptides and genes by incorporating them into the nanogel network [101]. Nanogels comprised of polyethyleneimine (PEI) are some of the most widely used cationic polymers in the area of drug delivery. Branched PEI can be easily modified with polymers or targeting ligands from the numerous primary and secondary amines. Vinogradov's group [102] reported crosslinked PEI nanogel via complexation with 5'-triphosphates of nucleoside analogues, an important group of anticancer chemotherapeutics. A drug-release mechanism triggered by the interaction of drug-loaded nanogels with the phospholipid bilayer is proposed. Kuckling *et al.* [103] reported a thermosensitive nanogel prepared by *N*-isopropyl-acrylamide and 2-dimethylmaleinimido ethylacrylamide, crosslinked under photoinitiated polymerization. Shi and Berkland [104] reported a polyvinylaminenanogel capsule, which is prepared by free radical polymerization in the presence of a novel acid-labile ketal-containing crosslinker. Jiang's group [105] also reported a novel chitosan nanogel crosslinked with ethylenediaminetetraacetic acid, which can reversibly switch surface charges based on pH stimuli. Change in external pH may have a significant influence on the release of drug molecules.

Polyionic complex micelles

Recent intracellular protein delivery is mostly based on the polycationic materials, which has been reviewed recently by Gu *et al.* [106]. Cationic amphiphile-based delivery reagent allows complex formation with proteins both via electrostatic and hydrophobic interactions [107]. Alternatively, a similar principle has also been applied in polyionic complex micelles using PEG–polycationic block copolymers to complex with polyanionic modified proteins [108–111]. In order to further stabilize the complex, disulfide bond chemistry has been applied to reversibly crosslinked nanoparticles [25,112,113], at the same time tethering protein molecules in the core of the micelle [112].

Single-protein nanogel

In addition, single-protein nanogels with polymer coating shells of approximately 2–5 nm have been developed to deliver protein therapeutics with significant enhancement in stability. Protein molecules were first chemically modified with vinyl monomers, and then *in situ* interfacial polymerization was initiated to form a nanogel to encapsulate a single protein. Various protein therapeutics have been encapsulated using this method: α -chymotrypsin [114], trypsin [114], horseradish peroxidase [115], bovine carbonic anhydrase [116], lipase [117,118], and ribonuclease A. Yan *et al.* [115] demonstrated that horseradish peroxidase nanogels exhibit similar bio-catalytic behavior and significantly improved stability at high temperature to a free enzyme. However, these nanogels were covalently coated to the protein surface, preventing the release of native protein molecules, therein limiting functions of the enzyme involving the protein–protein interactions. Instead, an alternative approach has been developed to encapsulate the unmodified protein in a reversibly crosslinked polymer nanogel capsule [30,59,119–121]. The adapted approach is the first to absorb numbers of positively charged monomers physically via electrostatic adsorption onto the proteins, then polymerize the monomers in the presence of divinyl crosslinking molecules *in situ*. A reductive-labile disulfide bond [30] or enzyme–labile peptide spacer [59] was introduced into the crosslinker, allowing the intracellular release of native protein efficiently in response to a specific-tumor microenvironment.

The above positively charged delivery system induces the cellular uptake of the encapsulated protein via endocytosis, followed by dissociation of the complex to disrupt the endolysosome to release the protein into the cytosol. However, nonspecific cytotoxicity remains a severe concern for positively charged delivery systems.

On-demand drug release

The concept of a ‘magic bullet’ in drug delivery was raised approximately 100 years ago by Paul Ehrlich in hopes of having an ideal carrier to drop therapeutics only at disease sites, where a drug is on-demand. The advance of nanotechnology, especially the development of a stimuli-responsive crosslinked nano carrier makes this dream closer to becoming a reality in cancer treatment. These nanocarriers need to be stable as well as innocuous under physiological conditions, also responsive to chemical (pH, reducing glutathione and tumor-specific enzymes) or physical (photoirradiation, magnetic field and ultrasound) stimuli in the tumor microenvironment. Timko *et al.* recently summarized the remotely triggerable drug-delivery systems, including nanoparticles, microparticles, membranes and devices that can respond to visible light, near-IR (NIR) light, ultrasound or magnetic fields to efficiently release drugs [122]. Magnetic field and ultrasound have been used to heat or increase the mobility of the nanocarriers via action on the co-loaded inorganic nanoparticles, therefore increasing drug-release rate [123–126]. However, they are rarely applied to trigger the reversibly crosslinked nanocarrier systems for on-demand drug release in cancer treatment. This is due to their relatively low energy, which is not strong enough to break chemical

crosslinkages. UV lights have the proper energy level to tailor certain chemical bonds, such as cleavage and formation of cyclobutane structure, which can also be achieved via a two-photon mechanism of NIR irradiation [127].

In addition, some US FDA-approved drug molecules can be administrated systemically after nanoparticles have concentrated at tumor sites, via EPR or active-targeting effects, to decrosslink micelles, especially for the portion of nanoparticles trapped in the tumor extracellular space. This action will release the drug locally, allowing deep penetration of chemodrugs for efficient cancer treatment. These chemicals include reducing *N*-acetylcystein [48,49] and *cis*-diol containing mannitol [28] for disulfide and boronate-crosslinked micelles, respectively. These studies will be discussed in detail later. Now, focus will be on the reversibly crosslinked micelles in response to single or multiple stimuli, for example, photoirradiation, acidic pH, reducing reagents, tumor-specific enzymes and competing chemicals administrated systemically, for on-demand drug release in cancer treatment.

pH-sensitive micelles

Many non-crosslinked block copolymer **pH-sensitive micelles** have been developed to target the acidic tumor microenvironment, which have been discussed in recent reviews [22,23]. In common, these block copolymers contain segments that are present as hydrophobic blocks in physiological conditions, such as polyhistidine [128–131] and polysulfonamide [132]. These segments will be converted into hydrophilic chains upon protonation at the tumor site. Therefore micelles are destabilized and drug molecules release efficiently at tumor sites. However, these NCMs cannot avoid premature drug release due to the dynamic nature of assembled micelles.

pH-sensitive reversibly crosslinked micelles

As shown in **Table 1**, various acid-labile crosslinkages can be utilized to crosslink micelles reversibly for tumor-acidic extracellular microenvironments and even acidic intracellular compartments. Kim's group [133] reported that acid-labile imine linkers were incorporated into polymer systems composed of polyethylenimine and glutaraldehyde. The experimental studies showed that the acid-labile crosslinked PEI was much less toxic compared with non-crosslinked PEI. It can be rapidly degraded into nontoxic, low-molecular-weight PEI in the acidic endosome. Recently, Knorr *et al.* reported that two types of acid-degradable gene carriers polymerized by oligoethylenimine with a pH-sensitive ketal crosslinker 2,2-*bis*(*N*-maleimidoethyloxy) propane or the 4-methoxybenzaldehyde *bis*acrylate acetal crosslinker 1,1-*bis*-(2-acryloyloxy ethoxy)-[4-methoxy-phenyl]methane) [134]. The experimental results showed that polyplexes with acid-labile linkages have improved toxicity, *in vitro* and *in vivo*, compared with those made with acid-stable polycations.

Reductive-sensitive-crosslinked micelles

Disulfide chemistry exists in protein structures, which can be tailored naturally by reducing glutathione presented in the cytosol of tumor cells [135]. Therefore, disulfide bonds are often incorporated into the structure of a micelle for reversible crosslinking. Cleavage of the disulfide to corresponding thiols in reducing conditions causes micelle dissociation. In addition, it has been demonstrated that the administration of high dosage of biocompatible *N*-acetylcystine (FDA-approved drug) can efficiently break disulfide bonds *in vitro* and *in vivo* to trigger drug release efficiently [48,49]. Since Trubetskoy *et al.* first reported reductive pDNA polyplex particles based on crosslinkers with a disulfide bond prepared through template polycondensation [136], McKenzie's group has further developed a new class of reductively activated peptide gene delivery polyplexes [137]. Using disulfide bonds as crosslinkers, these polyplexes exhibit significantly enhanced transfection efficacies

compared with noncrosslinked polyplexes. Recently, Liu and co-workers developed a new reductive-responsive-crosslinked polyethylenimine through click reaction of azide-terminated PEI and a disulfide-containing dialkyne crosslinker [138]. In this work, they demonstrated that reducible-cationic polymers with a disulfide-based linker were less cytotoxic and more effective in gene transfection compared with nondegradable PEI.

Lam *et al.* have developed a disulfide crosslinked micelle (DCM) system by introducing cysteines to the backbone of the dendritic oligo-cholic acid in an amphiphilic telodendrimer [48,49]. The disulfide crosslinkages were formed by the oxidization of thiol groups via air (oxygen) bubbling after loaded with superior amount paclitaxel (PTX) (up to 35.5% [w/w, drug/micelle]). Crosslinking of the micelles within the core reduced their apparent critical micelle concentration and greatly enhanced their stability in nonreductive physiological conditions as well as severe micelle-disrupting conditions. The release of PTX from the DCMs was significantly slower than that from NCMs, but can be gradually facilitated by increasing the concentration of the reducing agent (glutathione) to an intracellular reductive level. The DCMs demonstrated a longer *in vivo* blood circulation time, less hemolytic activities and superior toxicity profiles in nude mice when compared with NCMs. DCMs were found to be able to preferentially accumulate at the tumor site in nude mice bearing SKOV-3 ovarian cancer xeno-graft. It was also demonstrated that the DCM formulation of PTX (PTX-DCMs) was more efficacious than both free drug and the noncrosslinked formulation of PTX at equivalent doses of PTX in the ovarian cancer xenograft mouse model. The anti-tumor effect of PTX-DCMs can be further enhanced by triggering the release of PTX on-demand by the administration of the FDA-approved reducing agent, *N*-acetylcysteine, at a very high dose after PTX-DCMs have reached the tumor site to efficiently decrosslink micelles and release drugs efficiently within tumor extracellular space. The strategy of *N*-acetylcysteine post administration can be applied to other DCM systems to facilitate drug release after nanotherapeutics target to tumor sites.

Photosensitive crosslinked micelles

Some of the **photosensitive micelle** systems have been recently reviewed in detail for example, Zhao amongst others [24,139–141]. In common, these block copolymers contain segments that represent as hydrophobic blocks at physiological condition, such as hydrophobic esters of pyrene [142], *O*-nitrobenzyl [143] and coumarin [144]. These segments will be converted into hydrophilic chains after photo-initiated hydrolysis induced by the application of a specific wavelength of light applied at a tumor. Therefore, micelles are destabilized and drug molecules release efficiently at tumor sites. Kohane *et al.* reported a novel spiropyran-containing nanoparticulate for drug delivery that undergoes reversible volume change from 150 to 40 nm upon photo-triggering isomerization of spiropyran with UV light [145]. The volume change of these monodispersed nanoparticles enables repetitive dosing from a single administration and enhances tissue penetration. However, these NCMs are suspected to be less stable in blood circulation and premature drug release may occur due to the dynamic nature of the self-assembled system.

Some photosensitive chemicals are capable of undergoing a reversible formation and cleavage of a chemical bond upon exposure to lights at different wavelengths. These photosensitive molecules such as anthracene [146,147], thymine [148,149] and coumarin [60,70,150] have been widely employed as photo-crosslinkers to reversibly crosslink polymeric micelles and hydrogels. Zhao's group [65,151,152] first reported the use of coumarin moieties incorporated in the diblock copolymer to crosslink assembled micelles by photo-dimerization under irradiation at UV wavelength >300 nm [153]. The photo-cleavage of coumarin dimer can be easily achieved upon exposure to a UV light with short wavelength <280 nm, therefore, decrosslinking the micelle efficiently. Raghupathi *et al.*

[154] further used coumarine moieties as a photo-crosslinker to develop a class of nanoparticles based on an amphiphilic dendrimer. They pointed out that the extent of guest-molecule release can be precisely controlled by manipulating the extent of crosslinking in the nanoassemblies.

Dual-responsive-crosslinked micelles for on-demand drug release

Thermosensitive dual-responsive-polymeric nanoparticles—Thermosensitive polymeric nanoparticles or devices have been extensively reviewed elsewhere [155,156]. Poly(*N*-isopropylacrylamide) abruptly changes from hydrophilic to hydrophobic at a lower critical solution temperature (LCST) of 32°C, which will serve as the micelle's hydrophobic core, formed by poly(*N*-isopropylacrylamide) containing block copolymer for hydrophobic molecule loading *in vitro*. The micelle will be destabilized at a temperature lower than LCST, causing drug release *in vitro*. Thermoresponsive polymeric systems have been successfully applied in hydrogel formation for controlled release of hydrophilic drugs or proteins [155,157]. However, only a few LCST-containing micelles have shown success in accelerating drug release under hyperthermic conditions (41–42°C)[158,159]. In literature, many thermosensitive crosslinked micelle systems have been incorporated with another stimulus-responsive property, such as pH or reducing sensitivity. However, these dual-responsive crosslinked micelles may only respond to a single stimulus other than temperature [160–165], since no studies have reported the feasibility to apply hypothermia (<LCST) at tumor sites to trigger efficient on-demand drug release.

Redox- and photo-sensitive micelles—Zhao's group has reported a new amphiphilic ABA-type triblock copolymer with hydrophobic B block containing two types of stimuli-sensitive functionalities (disulfide and *o*-nitrobenzyle methyl ester) via a two-step click chemistry approach [166]. The NCMs formed via such block copolymers respond to reducing agents and light, respectively and can be disintegrated either rapidly via photo-cleavage of *o*-nitrobenzyl methyl esters or slowly through cleavage of disulfide groups by a reducing agent in the micellar solution. Therefore, UV light will induce the burst release of hydrophobic drug loaded inside micelles, where reducing agents can release drug molecules slowly.

Enzymatic-photosensitive reversibly crosslinked micelles—Photo-crosslinkable facially amphiphilic dendrimers have been synthesized to form micelles in aqueous condition [167]. Photosensitive coumarin was introduced into the dendrimer via an ester bond. Therefore, the crosslinked micelles can respond to both UV irradiation and esterase (porcine liver esterase) concentration. The release of the chemically conjugated coumarin and the physical-loaded fluorescent probe molecules can be tuned by photo-chemical crosslinking degree, which can limit the accessibility of the enzyme to the crosslinked core for hydrolysis.

pH- & redox-sensitive reversibly crosslinked micelles—pH and reduction dual-sensitive micelles are of great interest for anticancer drug delivery owing to the fact that tumor tissues have both a low pH and high reduction environment. Several studies have incorporated pH-sensitive linkages for drug conjugation in a polymeric micelle and then crosslink micelles with a disulfide-containing crosslinker [168–171]. Therefore, drug release from the resulting crosslinked micelles was controlled by both pH and reducing conditions. Wang *et al.* utilized a hydrazine functionalized PEG-*b*-PMAA block copolymer to attach adriamycin (ADR) through the formation of hydrazone, then the as-prepared ADR-conjugated block copolymer micelles were crosslinked by dithiodiethanoic acid. ADR was found to release most efficiently under both low pH and reductive conditions [169]. Zhong *et al.* have developed a main chain degradable polymer with disulfide bonds in each repeat

unit of poly(β -amino ester) via Michael addition polymerization [170]. The NCMs will be dissociated upon disulfide bond cleavage under reducing conditions and protonation of the polymer chains at slightly acidic pH 6.5. doxorubicin released faster from micelles in a weakly acidic environment than at pH 7.4 or in the presence of a higher concentration (5 mM) of reducing agent dithiothreitol. The *in vitro* studies showed that the micelles can be quickly internalized, effectively deliver the drugs into nuclei and inhibit cell growth. However, the *in vivo* stability and efficacy of this non-crosslinked system was not evaluated.

Cis-diol & acidic pH dual-responsive boronatecrosslinked micelles

Based on our previously developed telodendrimer systems (**Figure 4**) for efficient *in vivo* anticancer drug delivery [28,172–176], Luo, Lam and co-workers have developed two classes of smart crosslinked micelle systems: first was DCM as discussed previously [48,49], second was dual-responsive-reversible boronatecrosslinked micelles (BCM) [28,177], for targeted drug delivery (**Figure 5**). Telodendrimer is a linear-dendritic block copolymer containing linear PEG and dendritic polylysine with the peripheral amines capped with facial amphiphilic cholic acid. Telodendrimer is synthesized via step-wise peptide chemistry, which features telo dendrimers with well-defined structures and tunable functionality, as well as physical properties. Numerous orthogonal functional groups can be introduced into telodendrimer at different sites, and therefore displayed at different positions of the micelles, such as the surface, intermediate section or in the core of the micelle for ligand decoration, probe or drug molecule conjugation, or micelle crosslinking, respectively, as shown in **Figure 4**. Telodendrimer micelles have been loaded with a number of chemodrugs, for example, PTX [28,175,176], doxorubicin [173] and vincristine [49], and have shown superior anticancer efficacy and decreased side toxicity *in vivo* compared with the parent drugs.

In the BCM system, numerous of boronic acids and catechol have been introduced at the adjacent sites of different telodendrimers, respectively, in the separated preparations. These two kinds of telodendrimers were mixed homogenously together during the drug-loading process to form drug-loaded hybrid telodendrimer micelles. The crosslinking reaction between boronic acid and catechol occurred simultaneously after micelle formation. The advantages of this boronate crosslinking include: clean, automatic crosslinking without any catalyst required; orthogonal and specific chemistry and, pH and *cis*-diol dual-responsive drug release. By tuning the pK_a and numbers of boronic acids and catechols in the telodendrimers, the stability of the resulting BCMs as well as their stimuli-response to *cis*-diols and acidic pH values were optimized. The release of PTX from BCMs was significantly slower than that from non-crosslinked micelles, but can be accelerated by the acidic condition (pH 5) and/or mannitol (100 mM). The FRET assay demonstrated enhanced *in vivo* stability of the crosslinked micelles. This novel nanocarrier shows great promise for drug delivery 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 drugs on demand in the acidic tumor microenvironment or in the acidic cellular compartments upon uptake to target tumor cells. In addition, a novel approach can be potentially applied *in vivo* to trigger drug release from the nanoparticles trapped in the extracellular tumor space via additional intravenous administration of mannitol as an on-demand triggering agent after nanotherapeutics have concentrated at the tumor site. Mannitol has three pairs of *cis*-diol in its structure, which attributes to the significantly higher affinity to boronic acids than that of glucose at the same concentration in physiological conditions. Mannitol is a safe FDA-approved drug for diuresis. A high blood level of mannitol (>50 mM) can be achieved clinically based on the recommended dose, which can break down boronate crosslink-ages efficiently, accelerating drug release from BCMs in tumor sites.

Due to the engineerable nature of telodendrimer, other reversible crosslinking chemistries can be introduced into telodendrimer easily to develop clean and efficient reversibly crosslinked micelles, such as photosensitive building blocks. In addition, telodendrimer micelles can be crosslinked with a permanent linkage through highly specific and efficient green chemistries, which are introduced into telodendrimer via various labile-spacer molecules, such as disulfide linkages, peptide linkage and acidic-labile linkages. Therefore, the micelles can be crosslinked automatically and efficiently as well as respond to different stimuli in solid tumors.

Future perspective

Understanding the heterogeneous and chaotic nature of solid tumor microenvironment and the physical and chemical barriers in delivering chemodrugs to a solid tumor is crucial for a chemist in designing an efficient nanocarrier for improved cancer treatment. Some properties of solid tumors are listed in box 1. From a material point of view, to prepare a biocompatible well-defined, size-tunable, reversible crosslinked stable nanocarrier with high capacity remains challenging. The *in vitro* and *in vivo* stability of nanoparticles will directly determine the deposition, efficacy and toxicity of the drug molecules. Developing reversibly crosslinked micelles is the primary strategy being utilized to minimize premature drug release and to achieve on-demand drug release in tumor sites for efficient cancer treatment. Crosslinking efficiency, decrosslinking sensitivity and specificity to single or dual stimuli will determine *in vivo* stability, biodistribution and efficacy of these nanotherapeutics in cancer treatment. Continuous efforts in engineering self-assembled polymers are needed to define the response of resultant reversibly crosslinked nanocarriers to the stimuli in tumor microenvironments. box 2 lists several stimuli approaches for on-demand drug release. As a typical example, the photo-labile crosslinking of micelles represent the most promising strategy for on-demand drug delivery, featured as a clean and remotely controlled tailor of chemical bonds. UV light has the highest power and can break a chemical bond more easily, but can also damage surrounding tissue and skin. Instead, visible light and NIR light offer less harmful alternatives to UV, and deeper tissue penetration. Two photon mechanism has been proven to effectively cleave a chemical bond for drug release [127] by absorbing two NIR photons at once. Furthermore, the upconverting nanoparticles can be encapsulated into photosensitive crosslinked micelles [178], which absorb NIR light and convert it to higher-energy photons in the UV and visible regions, therefore cleaving crosslinkages efficiently to trigger burst drug release in tumor. Although the NIR light-responsive nanoparticles are relatively new, it seems to be one of the most promising systems to control drug delivery.

Ultimately, for potential clinical use of a stimuli-triggered on-demand drug-delivery system in cancer patient treatment, one needs to develop a biocompatible micellar nanocarrier with low cytotoxicity, high drug-loading capacity, good stability and long blood circulation time. Then nanocarriers can be selectively accumulated in tumors and disrupted by stimuli at tumor sites in a controlled or even quantitative manner. In addition, given the marginally improved anticancer efficacy of Abraxane® and Doxil® compared with their parent formulations due to their relative large particle sizes (130 and 150 nm, respectively), limiting their penetration into tumor tissue from peripheral extravascular space, the relative small and tunable particle sizes of nanocarriers are preferred for effective transvascular and interstitial transport. The creative design of the polymer and the reversible crosslinking chemistry is needed to develop a clean and efficient reversibly crosslinked nanocarrier with a well-defined structure, capable of scale up with good reproducibility.

The traditional approach to develop a micellar nanocarrier for drug delivery is to prepare a new block copolymer via the random or relatively controlled polymerization of a new synthetic monomer. The optimization of the polymer is usually limited to vary the molecular

weight of polymer chain. Some polymers, with functional groups in the monomeric units, can be further conjugated with different pendant groups for property modification of micelles. However, due to the distinct limitations of these conventional polymers, control of the functionalization and structural engineering of polymer chains is fairly poor. Therefore, it is difficult to optimize the drug-loading capacity and stability in conventional polymer micelles systemically, via structure engineering. As discussed previously [172–176], we have developed a telodendrimer nanocarrier platform was developed, which has an engineerable structure prepared via stepwise condensation. Therefore, different building blocks, functional groups and probe molecules can be easily introduced into telodendrimer at specific sites with desired numbers to optimize the nanocarrier efficiently for the delivery of different drugs. Various spacer molecules and crosslinking groups can be introduced into telodendrimer to develop stimuli responsive reversibly crosslinked micelles. As reported in previous studies, disulfide bond crosslinkage [48,49] and boronate crosslinkage [28,177] have been incorporated in the telodendrimer micelle system to develop two highly efficient reversibly crosslinked micelles for tumor-targeted drug delivery and on-demand drug release. Other different crosslinking strategies, such as photo-crosslinking and the high-spring-loaded green chemistry with labile spacers, are actively investigated to develop patient-friendly, highly-efficient, well-defined, reversibly crosslinked nanocarriers to improve the efficacy of chemotherapy in cancer treatment.

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Key Terms

Tumor-targeted drug delivery: Drug molecules are delivered specifically to tumor site via the conjugation with a tumor-homing molecule or via encapsulation in a nanocarrier to target tumor via enhanced permeability and retention effects.

Cancer treatment:

Representing cancer therapy designed to potentially cure cancer including surgery, radiation, chemical agents, or biological therapies.

On-demand drug release:

Allowing a controlled on–off switchable release of the drug in target sites under internal or external stimuli.

Reversibly crosslinked micelles: Micelles are crosslinked via a labile chemical bond or physical interactions, while has the ability of being cleaved by stimuli, such as pH change, reduction and so on.

Labile linkages: Chemical bonds that could be cleaved under certain chemical or physical conditions.

Enzyme-labile: Property of a chemical bond that can be cleaved via the catalysis of a specific enzyme.

Key Terms

pH-sensitive micelles:

Chemical structure and/or physical properties of a micelle could respond to the changes in the pH of the surrounding medium causing the swelling, shrinking, cleaving or dissociation of nanoparticle structures to change the drug release profile.

Photosensitive micelles:

Micelles that can undergo chemical modification or physical transformation in response to irradiation at a specific wavelength. As a result, the stability and the drug release profile are modified accordingly.

Box 1. Tumor microenvironment

- Leaky blood vessels, acidic pH and hypoxia condition within solid tumor tissue provide the opportunity for the targeted delivery of nanotherapeutics to solid tumors.
- On the contrary, the heterogeneous microenvironments and high interstitial fluid pressure pose barriers for the transvascular and interstitial transport of nanoparticles.

Box 2. Available stimuli in solid tumors for on-demand drug release

- Endogenous stimuli within solid tumors for cleavage of reversible-crosslinked nanocarriers include acidic pH, hypoxia extracellular and the reducing intracellular microenvironment.
- External stimuli applied to tumors for cleavage of reversible crosslinked nanocarriers include photo-irradiation, ultrasound, magnetic field and hyperthermia, as well as the post-administrated reducing reagents (e.g., *N*-acetylcystein) or competing reagents (e.g., mannitol), and so on, for decrosslinking of nanocarriers.

Executive summary

Background

■ Specific tumor microenvironment provides opportunities for nanoparticle-based tumor-targeted anticancer drug delivery by taking advantage of enhanced permeability and retention effects. However, it requires the nanoparticles to have good enough *in vivo* stability and biocompatibility, in order to continuously accumulate in tumor sites.

Crosslinking chemistry & reversible linkages

■ The reversible crosslinking of micelles is able to improve the stability of micelles in blood circulation and to selectively release drug molecules within tumor sites in response to certain stimuli, such as acidic pH, redox condition, photo-irradiation or certain competitive chemicals.

Crosslinked micelles for hydrophobic drug delivery

■ Micelles, formed with amphiphilic polymers, were designed to encapsulate poor water-soluble drugs in their hydrophobic cores. Then the micellar nanoparticles can be crosslinked at different sites, such as in the shell, core or the intermediate layer of micelles. Numerous chemistries can be applied to crosslink micelles reversibly, for on-demand drug delivery.

Reversibly crosslinked nanocarriers for hydrophilic therapeutic delivery

■ Nanocarriers including nanogels, polyionic complex micelles and single protein nanogel were used as delivery systems for water-soluble drug molecules such as proteins, siRNA, peptides and genes. These nanocarriers can be crosslinked via various labile linkages in response acidic pH, enzymes or reducing reagents and so on, to release hydrophilic payloads efficiently.

On-demand drug release

■ Various stimuli-responsive crosslinked nanocarriers were summarized, including pH-, reductive-, photosensitive-crosslinked micelles and several dual-responsive-crosslinked micelles.

Future perspective

■ Biocompatible, well-defined, size-tunable, reversibly crosslinked nanocarriers need to be developed as a new generation of drug-delivery systems. ‘Smart’, ‘feasibility’ and ‘green’ features of the crosslinking chemistry should be considered in the design of nanocarriers for *in vivo* anticancer drug delivery for efficient cancer treatment.

Figure 1. Chemistries used in form of core crosslinked micelles

(A) Micelles with crosslinked ionic cores; (B) core-crosslinked micelles containing near-IR fluorescent dyes; (C) polyphosphoester-core-crosslinked nanogel; (D) acid-labile-core-crosslinked micelles; (E) UV-induced core-crosslinked micelles; (F) cinnamoyl-based core-crosslinked micelles; (G) core-crosslinked micelles via click chemistry; and (H) micelles with a disulfidecrosslinked core.

(A) Data from [87]; (B) Data from [88]; (C) Data from [72]; (D) Data from [41]; (E) Data from [69]; (F) Data from [63]; (G) Data from [89]; (H) Data from [81].

Figure 2. Reaction strategies of shell-crosslinked micelles

(A) Shell-crosslinked micelles via acid-catalyzed sol-gel process; (B) shell-crosslinked micelles using diamine as linker; (C) shell click-crosslinked nanoparticles; (D) reversible-shell-crosslinked micelles; (E) crosslinking through the styrenyl side chains; (F) shell crosslinking via RAFT method; and (G) reduction-sensitive shell-crosslinked micelles. (A) Data from [93]; (B) Data from [94]; (C) Data from [73]; (D&G) Data from [95]; (E) Data from [90]; (F) Data from [96].

Figure 3. Intermediate layer crosslinked micelles

(A) Micelles formed by pH-responsive triblock copolymer; **(B)** crosslinked micelles via one-pot synthesis method; and **(C)** reversible-boronate-crosslinked micelles.

(A) Data from [97]; **(B)** Data from [98]; **(C)** Data from [28].

Figure 4. Structure and functionalization of telodendrimer
DCM: Dichloromethane; TFA: Trifluoroacetic acid.

Figure 5. Boronic acid and catechol-containing telodendrimer and the crosslinking and decrosslinking of reversible-boronate-crosslinked micelles for paclitaxel delivery.

The labile chemistries for reversible crosslinking of polymer micelles.

Structure	Stimuli	Applications	Ref.
Acid-labile linkages			
Ketal	pH 5.0	<i>In vitro</i> intracellular doxorubicin delivery	[32]
Acetal	pH 5.0	<i>In vitro</i> doxorubicin encapsulation and release	[41–42]
Orthoester	pH 5.4–6.4	pH-triggered release of paclitaxel	[31]
Imine	pH<6.0	Thermosensitive crosslinked micelle for pH-triggered <i>in vitro</i> drug release	[43]
Acylhydrazone	pH 5.3	For covalent conjugation of doxorubicin in polymer scaffold	[44]
<i>cis</i> -aconityl	pH 5.0	Covalent drug conjugation for acid-sensitive drug release	[45–47]
Boronate	pH 5.0	Efficiently crosslink telodendrimer micelles for pH and <i>cis</i> -diol dual-responsive drug release	[28]
Redox-labile linkages			
Disulfide —S—S—	Glutathione	Crosslinking micelles for drug, protein and gene delivery and release payload in response to reducing intracellular glutathione	[48–55]
Enzyme-labile linkages			
Peptide Ac-PLQLX-; GPLGIAGQX	MMP-2 and MM-9	Efficiently crosslinking hydrogel for tumor-specific, enzyme-responsive drug release	[56–58]
Peptide -DEVD-	Caspase-3	Crosslinking single-protein nanocapsules, which release protein in response to intracellular caspase-3	[59]
Photo-sensitive crosslinkages			

Structure	Stimuli	Applications	Ref.
Coumarin	Dimerization (> 300 nm); de-dimerization (<280 nm)	Efficiently crosslinking micelles or nanogels via photo-initiation and release payload upon short wavelength UV light	[60]
Cinnamic acid	Dimerization (260–290 nm)	Crosslinking 'onion-like' micelle in the middle layer, however the de-dimerization was not studied	[61–65]