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Reduction-Responsive Polymeric Micelles and Vesicles for Triggered Intracellular Drug Release

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Abstract

Significance: The therapeutic effects of current micellar and vesicular drug formulations are restricted by slow and inefficient drug release at the pathological site. The development of smart polymeric nanocarriers that release drugs upon arriving at the target site has received a tremendous amount of attention for cancer therapy. **Recent Advances:** Taking advantage of a high reducing potential in the tumor tissues and in particular inside the tumor cells, various reduction-sensitive polymeric micelles and vesicles have been designed and explored for triggered anticancer drug release. These reduction-responsive nanosystems have demonstrated several unique features, such as good stability under physiological conditions, fast response to intracellular reducing environment, triggering drug release right in the cytosol and cell nucleus, and significantly improved antitumor activity, compared to traditional reduction-insensitive counterparts. **Critical Issues:** Although reduction-sensitive micelles and polymersomes have accomplished rapid intracellular drug release and enhanced *in vitro* antitumor effect, their fate inside the cells including the mechanism, site, and rate of reduction reaction remains unclear. Moreover, the systemic fate and performance of reduction-sensitive polymeric drug formulations have to be investigated. **Future Directions:** Biophysical studies should be carried out to gain insight into the degradation and drug release behaviors of reduction-responsive nanocarriers inside the tumor cells. Furthermore, novel ligand-decorated reduction-sensitive nanoparticulate drug formulations should be designed and explored for targeted cancer therapy *in vivo*. *Antioxid. Redox Signal.* 21, 755–767.

Introduction

IN THE PAST DECADES, tumor-targeting nanodrug delivery systems, particularly biodegradable polymeric micelles with a typical core-shell structure and vesicles with a large aqueous interior, have been extensively explored for a potent delivery of anticancer drugs and proteins (16, 17, 19, 51, 54). The nanoscale polymeric micelles and vesicles self-assembled from amphiphilic copolymers bestow several unique advantages, such as prolonged circulation time, decreased adverse effects, improved drug availability, passive targeting to the tumor site *via* the enhanced permeability and retention effect, and better pharmacological profiles over the conventional clinical approaches. It should be noted, however, that the therapeutic effects of current micellar and vesicular drugs are usually restricted by slow and inefficient drug release at the pathological site (23, 42).

Recently, smart bioresponsive polymeric micelles and vesicles that are sufficiently stable under extracellular environments while are disrupted rapidly in response to an intracellular stimulus, such as pH, enzyme, and redox, have been developed to achieve fast and efficient drug release inside the tumor cells (10, 49, 52, 80). For instance, by virtue of the slightly acidic conditions in the endo/lysosomal compartments (pH 4.0–6.8), pH-sensitive micelles and polymersomes that are destabilized at mildly acidic pH have been extensively studied to achieve enhanced intracellular drug release (21, 22). In particular, the past several years have witnessed a booming development of reduction-sensitive micelles and polymersomes for active intracellular drug delivery (9, 50, 61), due to the presence of a high reducing potential in the cytosol and cell nucleus. Glutathione (GSH) tripeptide is the most abundant low-molecular-weight

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biological reducing agent and is kept reduced by NADPH and GSH reductase. Notably, GSH concentration in the cytosol and cell nucleus ranges from 2 to 10 mM, which is about 100–1000 times higher than that in body fluids and extracellular matrices (2–10 μ M) (2, 59, 83). It should further be noted that the endosomal compartment has also been reported to possess a high reducing potential, which is modulated by γ -interferon-inducible lysosomal thiol reductase (GILT) coexistence with reducing agents from GSH such as cysteine (Cys) (36). Moreover, tumor tissues are highly reductive with at least four times higher concentration of GSH compared to normal tissues (35). Therefore, reduction is a unique and ubiquitous signal that can be used as a fascinating trigger to accomplish rapid and efficient drug release in both cancer cells and tumor tissues.

This review provides an overview of recent progresses in reduction-sensitive micelles and polymersomes for active intracellular delivery of anticancer drugs as well as protein therapeutics. Depending on the location and number of cleavable disulfide or diselenide linkages and properties of constituting polymers, reduction-sensitive micelles and polymersomes may aggregate, disintegrate, dissolve, or swell in response to a reducing agent. Taking disulfide-containing reduction-sensitive micelles as an example, they can be classified accordingly into four different categories: (i) reduction-sensitive shell-sheddable micelles, (ii) reduction-sensitive disassemblable micelles with a reductively degradable core, (iii) reduction-sensitive reversibly shell-cross-linked (SCL) micelles, and (iv) reduction-sensitive reversibly core-cross-linked (CCL) micelles (Fig. 1). The versatile design and

FIG. 1. Illustration of reduction-sensitive micelles for active delivery of anticancer drugs. (A) Reduction-sensitive shell-sheddable micelles formed from amphiphilic block or graft copolymers containing intervening disulfide bonds would quickly shed off shells, disassemble, and form large aggregates under a reductive environment, resulting in fast and complete intracellular drug release. (B) Reduction-sensitive disassemblable micelles are prepared from amphiphilic copolymers with a reductively degradable hydrophobic block. The micelles would swell and eventually be disintegrated inside the tumor cells, giving rise to complete drug release. (C) Reduction-sensitive reversibly SCL micelles, although robust against dilution, are prone to fast de-cross-linking and dissociation at low concentrations (<CMC) in response to the intracellular reductive condition, elegantly resolving the extracellular stability and intracellular drug release dilemma. (D) Reduction-sensitive reversibly CCL micelles, although stable against dilution, are subject to de-cross-linking, micelle swelling, and dissociation at low concentrations (<CMC) under a reductive environment, which represents the other way to address the extracellular stability and intracellular drug release dilemma. CMC, critical micelle concentration; CCL, core-cross-linked; SCL, shell-cross-linked. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

construction of reduction-sensitive micelles and polymersomes render them particularly interesting for controlled delivery of various drugs.

Reduction-Sensitive Shell-Sheddable Micelles and Polymersomes

Reduction-sensitive shell-sheddable micelles and polymersomes, which underwent shell-shedding, core/membrane aggregation, and rapid drug release in response to a reducing condition, are the most simple and yet effective platform for triggered drug release inside the tumor cells (Fig. 1A). In an attempt to enhance intracellular drug release of traditional biodegradable micelles, reduction-sensitive shell-sheddable micelles were designed and prepared from disulfide-linked poly(ethylene glycol)-*b*-poly(ϵ -caprolactone) (PEG-SS-PCL) or dextran-*b*-PCL (Dextran-SS-PCL) diblock copolymers (60, 62). Interestingly, these micelles formed large aggregates in a few hours in response to 10 mM dithiothreitol (DTT) due to shedding of hydrophilic PEG or dextran shells and accomplished much faster doxorubicin (DOX) release inside the mouse leukemic monocyte macrophage cells (RAW 264.7), inducing significantly enhanced antitumor activity compared to the corresponding reduction-insensitive controls. The subsequent studies showed that the intracellular drug release and therefore the antitumor activity of DOX-loaded PEG-*b*-PCL micelles can be precisely controlled by the extent of shell-shedding (74). Very recently, Zhong *et al.* reported that ligand-directed reduction-sensitive shell-sheddable micelles based on PEG-SS-PCL and galactose-PEG-PCL copolymers efficiently delivered and released DOX into the nuclei of target HepG2 cells, resulting in superior *in vitro* antitumor efficacy with a half maximal inhibitory concentration (IC₅₀) comparable to free DOX (97). Tang *et al.* prepared reduction-sensitive shell-sheddable micelles from disulfide-linked poly(ethyl ethylene phosphate)-*b*-PCL (PEEP-SS-PCL) diblock copolymer (67). These DOX-loaded micelles exhibited intracellular GSH-dependent DOX release behavior and demonstrated enhanced DOX retention as well as more rapid DOX release in DOX-resistant MCF-7 cells (Fig. 2A), thus effectively overcoming multi-drug resistance with an IC₅₀ decreased by 68% compared to

reduction-insensitive PEEP-*b*-PCL micelles following 72 h incubation (Fig. 2B) (77).

In the past several years, reduction-sensitive shell-shedding has been established as a general and effective approach to accomplish accelerated drug release inside the tumor cells. For example, Cui *et al.* reported that shell-sheddable PEG-SS-hexadecyl (PEG-SS-C₁₆) micelles with a size of 137 nm completely destructed into two populations at 14 and 196 nm following 20 min incubation with 10 mM DTT (13). Furthermore, DOX-loaded PEG-SS-C₁₆ micelles, although having similar cellular uptake efficiency with reduction-insensitive PEG-CC-C₁₆ micelles, displayed significantly higher cytotoxicity against HeLa cells. Zhao *et al.* conjugated polo-like kinase 1 (siPlk1) siRNA to α -tocopheryl polyethylene glycol succinate (TPGS) *via* a disulfide linkage (siPlk1-SS-TPGS) (96). The docetaxel (DTX)-loaded siPlk1-SS-TPGS/TPGS micelles exhibited enhanced cell inhibition compared to TPGS micelles, in which IC₅₀ in NIH3T3 cells after 48 h incubation was reduced by 99.5%. The reduction-sensitive shell-sheddable polypeptide micelles based on PEG-SS-poly(ϵ -benzyloxycarbonyl-L-lysine) (PEG-SS-PZLL) were reported to undergo rapid micellar rearrangement and fast DOX release in the presence of 10 mM GSH (18, 82). DOX-loaded micelles demonstrated higher cytotoxicity toward glutathione monoester (GSH-OEt)-pretreated HeLa and HepG2 cells. GSH-OEt is often used to artificially enhance the intracellular GSH level. Similarly, DOX-loaded PEG-SS-poly(leucine) (PEG-SS-PLeu) micelles exhibited accelerated DOX release in response to 10 mM DTT and enhanced antitumor efficacy in GSH-pretreated HepG2 cells (56). Thambi *et al.* discerned that shell-sheddable PEG-SS-poly(γ -benzyl L-glutamate) (PEG-SS-PBLG) micelles with high loading efficiency for camptothecin (CPT) and DOX revealed GSH-triggered drug release behavior and provoked higher cytotoxicity to SCC7 cancer cells than reduction-insensitive PEG-*b*-PBLG controls (69, 70). Reduction-sensitive shell-sheddable CPT-SS-PEG-SS-CPT prodrug micelles formed large aggregates with mean diameters over 1000 nm in response to 10 mM DTT and effectively inhibited cell growth under the tumor-relevant GSH concentration (39). We found that reduction and pH dual-sensitive micelles based on disulfide-linked PEG-*b*-poly(2,4,

FIG. 2. Intracellular drug release and antitumor activity of DOX-loaded PEEP-SS-PCL micelles in DOX-resistant MCF-7 cells. (A) CLSM images of MCF-7/ADR cells following 48 h incubation with free DOX or DOX-loaded micelles (DOX dosage: 5 μ g/ml). (B) IC₅₀ of free DOX and DOX-loaded micelles in MCF-7/ADR cells measured by MTT assay ($n=3$). DOX, doxorubicin; IC₅₀, half maximal inhibitory concentration. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

6-trimethoxybenzylidene-pentaerythritol carbonate) diblock copolymer exhibited the fastest DOX release at pH 5.0 with 10 mM GSH (94.2% DOX release in 10 h) (7). These dual-sensitive micelles efficiently transported DOX into the cell nuclei following 8 h incubation, provoking high antitumor activity with IC_{50} of 0.75 and 0.60 μ g DOX equiv./ml for HeLa and RAW 264.7 cells, respectively. Yuan *et al.* prepared reduction and thermo dual-sensitive supramolecular micelles from PEG-SS-poly(2-(*N,N*-dimethylamino)ethyl methacrylate) and α -cyclodextrin (PRX-SS-PDMA) (92). The conformation and DOX release behavior of these micelles could be fine tuned by temperature and DTT concentration.

Reduction-sensitive shell-sheddable micelles have also been constructed from amphiphilic star-block and graft copolymers. For instance, reduction-sensitive shell-sheddable micelles based on hexa-arm star-shaped 6s-PCL-SS-PEG copolymers, while stable under a normal condition, quickly aggregated and released DOX in response to 10 mM DTT (55). The DOX-loaded micelles exhibited GSH-dependent cytotoxicity toward MCF-7 cells. Liu *et al.* found that shell-sheddable micelles based on multiarm hyperbranched H40-*star*-PLA-SS-poly(2-ethoxy-2-oxo-1,3,2-dioxaphospholane) (H40-*star*-PLA-SS-PEP) copolymer formed large aggregates rapidly in the presence of 10 mM DTT and exhibited faster DOX release in GSH-OEt-pretreated HeLa cells, affording enhanced cell proliferation inhibition (45). Li *et al.* demonstrated that reduction-sensitive shell-sheddable micelles based on hyaluronic acid-*g*-SS-deoxycholic acid (HA-*g*-SS-DOCA) conjugates aggregated into irregular-shape and large-size fragments following 24 h incubation with 20 mM GSH (38). These paclitaxel (PTX)-loaded micelles entered into human breast adenocarcinoma cells (MDA-MB-231) *via* HA-receptor-mediated endocytosis and induced significant higher antitumor activity (IC_{50} = 25.6 ng/ml) following 72 h treatment than PTX-loaded HA-*g*-DOCA micelles (IC_{50} = 56.6 ng/ml) and Taxol (IC_{50} = 51.7 ng/ml). The *in vivo* studies in tumor-bearing mice revealed preferential accumulation of payloads in the tumor site at 24 h following injection. Similarly, reduction-sensitive shell-sheddable chondroitin sulfate-*g*-SS-cholesterol micelles displayed DTT-dependent quercetin release as a result of chondroitin shell shedding (89). DOX-loaded reduction-sensitive poly(α,β -(*N*-(2-hydroxyethyl)-L-aspartamide))-*g*-SS- C_{16} (PHEA-*g*-SS- C_{16}) micelles entered into HeLa cells *via* clathrin-mediated endocytosis and accomplished accelerated intracellular DOX release as well as enhanced antitumor efficacy compared to PHEA-*g*-CC- C_{16} control (14). Wu *et al.* reported that reduction-sensitive shell-sheddable CPT prodrug micelles based on poly(aspartic acid)-*g*-CPT/SS-PEG (PAsp-*g*-CPT/SS-PEG) were prone to aggregation and intense release of CPT as a result of DTT-induced shell detachment (85). DOX-loaded reduction-sensitive, shell-sheddable, magnetic micelles were prepared *via* Fe₃O₄ nanoparticle-induced self-assembly of PAsp-*g*-dopamine/SS-PEG copolymer (90). Thus formed micelles showed enhanced DOX fluorescence intensity in Bel-7402 cells and higher r_2 relaxation rate compared to reduction-insensitive PAsp-*g*-dopamine/PEG control and commercially available SPION. Chen *et al.* constructed robust reduction-sensitive shell-sheddable PCL-*g*-SS-PEG micelles with a low critical micelle concentration (CMC) in water (8). The DOX-loaded micelles induced

pronounced antitumor activity to HeLa cells with an IC_{50} close to that of free DOX.

The reduction-sensitive shell-shedding is also an intriguing pathway for polymersomes to achieve efficient and rapid intracellular protein release. For instance, Hubbell *et al.* discovered that reduction-sensitive shell-sheddable PEG-SS-poly(propylene sulfide) (PEG-SS-PPS) polymersomes displayed fast response to 0.7 mM Cys and yielded substantial cellular uptake as well as rapid calcein release inside mouse macrophage J774A-1 cells (3). Reduction-sensitive shell-sheddable chimeric polymersomes were recently developed based on PEG-SS-PCL and PEG-*b*-PCL-*b*-poly(2-(diethylamino)ethyl methacrylate) (PEG-*b*-PCL-*b*-PDEA) copolymers for facile loading and triggered intracellular release of proteins (75). Cell experiments demonstrated that the intracellular protein release and therefore cell apoptosis effect of cytochrome C (CC)-loaded chimeric polymersomes were closely related to the disulfide content. The reduction and pH dual-sensitive PEG-SS-PDEA polymersomes, although stable with minimal protein release under physiological conditions, rapidly dissolved or formed large aggregates in response to endosomal pH and/or intracellular-mimicking reductive environment, affording fast protein release (95). CC-loaded dual-sensitive polymersomes have demonstrated much faster release of CC inside the MCF-7 cells and significantly enhanced apoptosis (42.9% in 48 h with CC dosage of 80 μ g/ml) compared to free CC and CC-loaded reduction-insensitive PEG-*b*-PDEA control (18.3% and 29.1% apoptosis, respectively).

Reduction-Sensitive Disassemblable Micelles with a Reductively Degradable Core

Reduction-sensitive disassemblable micelles with a reductively degradable core are prepared from amphiphilic copolymers bearing one or multiple disulfide linkages embedded in the main chain or at the side chain of hydrophobic block (Fig. 1B). For example, reduction-sensitive core-degradable prodrug micelles based on PEG-*b*-PPS-SS-tioguanine conjugate released bioactive tioguanine in response to Cys and inhibited the growth of OVA-B16-F10 melanoma cells in a dose-dependent manner (71). The size of reduction-sensitive micelles based on disulfide-cored octa-armed SS-8s-PCL-*b*-PEG copolymer was reduced into half, and the DOX release rate was markedly increased upon the addition of 10 mM DTT (63). In a previous study, reduction and thermo dual-sensitive polypeptide micelles prepared from disulfide-cored tetra-armed polypeptide SS-4s-poly(diethylene glycol-L-glutamate) were reduced into half-sized micelles in response to 10 mM DTT (41). Reduction and pH dual-sensitive micelles were obtained from PEG-*b*-polyurethane-*b*-PEG triblock copolymer containing multiple disulfide bonds and tertiary amine groups in the polyurethane (PU) block (91). DOX-loaded dual-sensitive micelles exhibited GSH-dependent DOX release behavior and cell inhibition effect against HeLa and HepG2 cells. Notably, Ma *et al.* found that redox-sensitive micelles based on diselenide containing PEG-*b*-PUSeSe-*b*-PEG triblock copolymer revealed fast response to both reducing and oxidizing agents, wherein rhodamine B was rapidly released at 0.01 mg/ml GSH or 0.01% v/v H₂O₂ (47). Chen *et al.* prepared core-degradable micelles from reduction-sensitive poly(β -amino

ester)-*g*-PEG (SSPAE-*g*-PEG) copolymer (5). *In vitro* release studies indicated that thus formed micelles released about 80% of DOX within 6 h in the presence of 10 mM DTT, whereas only about 20% of DOX was released for reduction-insensitive control under otherwise the same conditions even after 24 h. DOX-loaded SSPAE-*g*-PEG micelles demonstrated significantly high antitumor efficacy against HepG2 cells with IC₅₀ two times lower than free DOX. The following study showed that reduction and pH dual-sensitive core-degradable SSPAE-*g*-PEG micelles accomplished accelerated DOX release at pH 6.5 or in the presence of 5 mM DTT at pH 7.4, and the fastest DOX release in response to both stimuli (pH 6.5 and 5 mM DTT) (4). These DOX-loaded dual-sensitive micelles could effectively release DOX into the nuclei of HepG2 cells, resulting in a low IC₅₀ close to that of free DOX.

Reductively degradable poly(amide amine) (SSPAA) polymers based on the Michael addition reaction of cystamine bisacrylamide and primary amines have been used for versatile construction of reduction-sensitive disassemblable micelles. Sun *et al.* reported that reduction-sensitive SSPAA-*g*-PEG micelles released DOX nearly quantitatively within 10 h in the presence of 1 mM DTT (65). These micelles effectively transported DOX into the nuclei of both HeLa and HepG2 cells, resulting in high antitumor efficacy. Furthermore, DOX-loaded SSPAA-*g*-PEG micelles exhibited significantly stronger accumulation at the tumor site, reduced distribution in normal organs, lower side effects, and higher antitumor efficacy compared to free DOX·HCl in nude mice bearing 4T1 breast carcinoma tumor xenograft (66). SSPAA-*g*-PEG/CPT prodrug micelles showed enhanced *in vitro* release of CPT at higher DTT concentration (20). Nam *et al.* found that reduction-sensitive SSPAA-*g*-arginine/SS-(PEG-PTX) prodrug micelles could efficiently condense plasmid DNA into polyplexes with sizes ranging from 125 to 210 nm (53). These polyplexes displayed improved transfection efficiency than 25 kDa bPEI, enhanced cellular uptake than SSPAA-*g*-arginine/DNA polyplexes, and had higher antitumor potency than free PTX. Reduction-sensitive polyion micelles obtained from SSPAA-*g*-PCL and PEG-COOH showed a DTT-dependent PTX release profile, in which about 30%, 75%, and 100% of PTX was released within 9 h in response to 0, 10, and 40 mM DTT, respectively (26). Reduction-sensitive micelles were also constructed from amphiphilic hyperbranched homopolyphosphates (HPHSEPs) with disulfide bonds in the backbone, which were prepared by self-condensing ring-opening polymerization of 2-((2-hydroxyethyl)-disulfanyl) ethoxy-2-oxo-1,3,2-dioxaphospholane (43). DOX-loaded HPHSEP micelles exhibited enhanced cell inhibition against GSH-OEt-pretreated HeLa cells (IC₅₀ = 0.08 µg/ml) and reduced inhibition toward buthionine sulfoximine-pretreated (an inhibitor for the intracellular synthesis of GSH) cells (IC₅₀ = 0.64 µg/ml) compared to the nontreated cells (IC₅₀ = 0.35 µg/ml). In a previous study, it was observed that amphiphilic hyperbranched multiarm HPHSEP-*star*-PEP copolyphosphate-based micelles decreased from 70–100 nm to 10 nm after 48 h incubation in 10 mM DTT (46). Moreover, DOX-loaded micelles were shown to rapidly release DOX into the nuclei of HeLa cells, resulting in enhanced antitumor efficacy. Multicore/shell-structured reduction-sensitive micelles constructed from hyper-

branched polydiselenide possessing alternative hydrophobic diselenide bonds and hydrophilic phosphate moieties in the backbone framework displayed fast response to reductive agents and achieved rapid DOX release into the nuclei of HeLa cells (44).

Reduction-sensitive core-degradable micelles have also been designed from amphiphilic copolymers bearing multiple disulfide bonds at the side chain of hydrophobic regimen. For example, Ryu *et al.* prepared poly(acrylic acid)-*g*-(triethylene glycol monoethyl ether acrylate)/(undecyl dithiolethanol) graft copolymer containing multiple disulfide linkages between PAAc main chain and pendant hydrophobic undecyl dithiolethanol groups (57). The resulting micelles demonstrated GSH-dependent intracellular release of DOX as well as GSH-dependent cytotoxicity to MCF-7 cells. Reduction-sensitive PTX prodrug micelles based on PAAc-*g*-PEG/SS-PTX conjugate with PTX linking to PAAc backbone via a disulfide bond exhibited pronounced cytotoxicity to OS-RC-2 kidney tumor cells while low cytotoxicity to normal macrophage cells (6). Sun *et al.* obtained reduction-sensitive core-degradable micelles from poly(ethylene oxide)-*b*-poly(*N*-methacryloyl-*N'*-(*t*-butoxycarbonyl) cystamine) (PEO-*b*-PMABC) diblock copolymers that possess multiple disulfide bonds at the side chain of PMABC block (64). The resulting micelles, although stable under normal physiological conditions, were rapidly degraded into free polymers in the presence of DTT, triggering fast DOX release and provoking higher cytotoxicity toward T-24 cells than the reduction-insensitive controls. Jiang *et al.* found that reduction and thermo dual-sensitive core-degradable micelles based on PEG-*b*-poly(PEG methyl methacrylate-*co*-MABC) PEG-*b*-P(PEGMMA-*co*-MABC) diblock copolymer achieved higher cell inhibition in HepG2 cells than free PTX (29). Subsequently, reduction, pH, and thermo triple-sensitive core-degradable micelles were constructed from PEG-*b*-P(PEGMMA-*co*-MABC-*co*-(vinyl imidazole)) diblock copolymer (27). These micelles revealed faster PTX release at pH 5.0 or in the presence of 10 mM DTT at pH 7.4. Khorsand *et al.* reported that DOX-loaded reduction-sensitive micelles based on PEO-*b*-polymethacrylate containing pendant disulfide groups (PEO-*b*-PHMssEt) were quickly destabilized in response to 10 mM GSH and induced similar cytotoxicity to free DOX toward 10 mM GSH-OEt-pretreated HeLa cells (Fig. 3) (31). Wang *et al.* constructed reduction-sensitive disassemblable micelles from poly(6-*O*-methacryloyl-*D*-galactopyranose-*co*-DMA)-*b*-poly(pyridyl disulfide ethyl methylacrylate) (P(MAGP-*co*-DMA)-*b*-PPDSEMA) diblock copolymer (78). The micelles size decreased from 136 to 12 nm and about 75% of DOX was released in 48 h in the presence of 10 mM GSH.

Reduction-Sensitive Reversibly SCL Micelles and Polymersomes

In addition to slow intracellular drug release, low *in vivo* stability that leads to premature drug release and diminishing drug accumulation in the tumor sites is another critical issue for current micellar and vesicular drug formulations. In the past several years, reduction-sensitive SCL micelles and polymersomes as well as CCL micelles have been explored simultaneously to address the extracellular stability and intracellular drug release problems (Fig. 1C, D).

FIG. 3. PEO-*b*-PHMssEt micelles exhibiting enhanced DOX release in response to intracellular GSH in cancer cells. GSH, glutathione. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

Reduction-sensitive SCL micelles and polymersomes are usually prepared by cross-linking of amine, carboxylic acid, or thiol functional groups in their shells *via* the carbodiimide chemistry or oxidative cross-linking. Koo *et al.* developed reduction-sensitive SCL polypeptide micelles *via* self-assembly of PEG-*b*-poly(lysine)-*b*-poly(L-phenylalanine) (PEG-*b*-PLys-*b*-PPhe) triblock copolymer in water followed by cross-linking with 3,3'-dithiobis(sulfosuccinimidylpropionate) *via* carbodiimide chemistry (33). The resulting SCL micelles, although demonstrating improved stability in serum and sodium dodecyl sulfate solution as well as significantly inhibited drug release of methotrexate (MTX) and DTX, exhibited increased drug release upon increasing GSH concentrations. The *in vivo* studies in MDA-MB-231 tumor-bearing mice showed enhanced tumor accumulation and more effective tumor growth inhibition of DTX-loaded SCL micelles compared to the noncross-linked (NCL) controls (34). PEG-SS-PLys-*b*-PLEu SCL micelles cross-linked by 3,3'-dithiodipropionic acid showed much faster CPT release in the presence of 10 mM DTT compared to irreversible SCL micelles under otherwise the same conditions (72). Moreover, CPT-loaded reversible SCL micelles revealed two times stronger CPT fluorescence and higher cytotoxicity in GSH-OEt-pretreated HeLa cells. Reduction-sensitive SCL micelles based on Y-shaped PEG-*b*-PLG-*b*-(PLA)₂ block copolymers with cystamine as a cross-linker have demonstrated enhanced DOX loading efficiency and improved *in vitro* stability than NCL counterparts (93). DOX-loaded disulfide-cross-linked micelles showed similar cytotoxicity against HeLa cells as well as longer blood circulation life *in vivo* compared to free DOX and NCL micelles. Samarajeewa *et al.* reported that reduction-sensitive SCL micelles self-assembled from poly(oligo(ethylene glycol) methyl ether acrylate-*co*-*N*-acryloyloxysuccinimide)-*b*-PDLLA (P(OEGMA-*co*-NAS)-*b*-PDLLA) diblock copolymer following cross-linking with cystamine exhibited accelerated PTX release in the presence of GSH and pro-

teinase K (58). Notably, these PTX-loaded SCL micelles showed remarkable cell inhibition effect, with about threefold lower IC₅₀ value than free PTX in KB cells following 2 h incubation and ~11-fold lower IC₅₀ than Taxol in OVCAR-3 cells after 72 h incubation. Tao *et al.* obtained SCL Pluronic micelles from thiolated Pluronic F127 and P123 using gold nanoparticles as a cross-linker (Au-PF-PTX) for PTX delivery (68). Au-PF-PTX SCL micelles demonstrated enhanced antitumor efficacy against GSH-OEt pretreated U87 cells than nontreated cells as well as preferable accumulation in spleen and liver following 24 h i.v. injection. Reduction and pH dual-sensitive SCL micelles were developed *via* self-assembly of aldehyde-containing P(OEGMA-*co*-p-(methacryloxyethoxy)benzaldehyde)-*b*-PCL diblock copolymer in NH₄OAc buffer followed by cross-linking using difunctional dithiolbis (propanoic dihydrazide) (25). These dual-sensitive SCL micelles, although greatly retarded CPT release at pH 7.4, exhibited the fastest CPT release in response to 10 mM DTT at pH 5.0, which was comparable to that of NCL control. Furthermore, drug-loaded dual-sensitive SCL micelles could efficiently transport CPT or DOX into cell nuclei, resulting in effective cell apoptosis.

In contrast to carbodiimide chemistry that requires the use of a cross-linker, lipoyl and thiol-functionalized micelles are prone to auto-cross-linking. Xu *et al.* found that SCL micelles based on PEG-*b*-PCL diblock copolymer with two lipoyl groups at its junction (PEG-L₂-PCL) showed markedly enhanced stability against dilution, whereas released DOX rapidly in response to 10 mM DTT with about 75% release in 9 h under otherwise the same conditions (87). Wang *et al.* prepared reduction-sensitive SCL micelles from pendant thiol-containing PEG-*b*-poly(thioethyl ethylene phosphate)-*b*-PCL (PEG-*b*-PEEP_{SH}-*b*-PCL) triblock copolymer followed by oxidative cross-linking in the H₂O₂ aqueous solution (76). Thus formed SCL micelles revealed enhanced stability against

FIG. 4. SCL PEG-*b*-Cys₄-*b*-PDLLA micelles for enhanced tumor-targeting delivery of DOX. (A) Fluorescent image of tissue distribution of Cy5.5-labeled SA and DS micelles at 1 day after injection. (B) Relative tumor volume (the ratio of tumor volume to initial size before treatment) for M109 tumor as a function of time after intravenously injected DOX formulations (2 mg/kg) at day 0 and 4 ($n = 6-8$). * $p < 0.1$; ** $p < 0.005$. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

the DMF addition and accelerated intracellular DOX release in GSH-OEt-pretreated A549 cells. In a similar way, reduction-sensitive SCL micelles demonstrating enhanced stability under severe conditions accelerated intracellular drug release, and higher antitumor activity were also obtained from thiol-containing PEG-*b*-poly(Cys)-*b*-PPhe (PEG-*b*-PCys-*b*-PPhe) triblock copolymer (73). Recently, Lee *et al.* reported that SCL-biodegradable PEG-*b*-Cys₄-*b*-PDLLA (DS) micelles stably retained DOX during circulation with half-life of about 11.6 h and delivered 7-fold higher DOX to the tumor while 1.9-fold lower in the heart compared to NCL PEG-*b*-PDLLA (SA) micelles (37). Cy5.5-labeled DS micelles demonstrated twofold higher fluorescence intensity in the tumor tissue than that of SA micelles at 1 day postinjection (Fig. 4A). Notably, these DOX-loaded SCL (DOX/DS) micelles almost completely inhibited M109 tumor growth in mice within 14 days after initial treatment with 2 mg DOX/kg at day 0 and 4 (Fig. 4B). Reduction and pH dual-sensitive SCL micelles based on PEG-*b*-poly(Asp-*g*-2-mercaptoethylamine)-*b*-poly(Asp-*g*-2-(diisopropylamino)ethylamine) triblock copolymer, while stable with low drug leakage during storage and blood circulation, displayed about 95% DOX release in 5 h under an acidic and reductive environment (15). Furthermore, *in vivo* studies *via* measuring tumor size, body weight, and survival rate revealed that DOX-loaded dual-sensitive SCL micelles had significantly better therapeutic effects than free DOX and DOX-loaded PEG-*b*-PCL micelles.

Recently, we prepared reduction and thermo dual-sensitive SCL polymersomes based on water-soluble PEG-*b*-PAAc-*b*-poly(*N*-isopropylacrylamide) triblock copolymer with cystamine as a cross-linker for facile encapsulation of proteins under mild conditions and rapid release of proteins in cancer cells (11, 86). The resulting SCL polymersomes, while showing remarkable stability against dilution, organic solvent, high salt conditions, and change of temperature in water, were otherwise completely dissociated in 0.5 h upon incubation with 10 mM DTT at pH 7.4 and 37°C. These reversible SCL polymersomes with high protein loading efficiency showed much faster CC release into cytosol of MCF-7

cells, inducing enhanced cell apoptosis compared to free CC and the irreversibly cross-linked controls.

Reduction-Sensitive Reversibly CCL Micelles

Reduction-sensitive reversibly CCL micelles are usually prepared from amphiphilic copolymers that possessed pendant or end-capped functional groups, such as hydrazide, carboxylic acid, alkynyl, dithiopyridine, lipoyl, and thiol in the hydrophobic block. The de-cross-linking of the micellar core under reductive environments results in swelling or dissociation of micelles, facilitating intracellular drug release (Fig. 1D). Wei *et al.* constructed reduction and pH dual-sensitive DOX-conjugated CCL micelles from PEG-*b*-poly(methacrylic acid-*g*-hydrazone-DOX) diblock copolymer containing pendant hydrazide groups (PEG-*b*-P(MA-*g*-Hyd-DOX)) using dithiodiethanoic acid as a cross-linker (79). The resulting CCL micelles exhibited the fastest DOX release in response to both stimuli (pH 4.0 and 15 mM DTT). Reduction-sensitive CCL polyion PEO-*b*-PMA micelles cross-linked with cystamine revealed accelerated DOX release in the presence of 10 mM GSH, for which the release rate (24.6 $\mu\text{g/h}$) was about 12.6- and 30.8-fold faster than that of irreversibly cross-linked counterparts under otherwise the same conditions and reversible CCL micelles in the absence of GSH, respectively (32). These DOX-loaded CCL micelles demonstrated much higher cytotoxicity with IC₅₀ at least six times lower than irreversibly cross-linked controls in human A2780 ovarian cancer cells. Cheng *et al.* designed reduction-sensitive CCL micelles *via* click reaction of PEG-*b*-poly(γ -propargyl-L-glutamate) diblock copolymer based micelles with *bis*(2-azidoethyl)disulfide cross-linker (12). Thus formed CCL micelles swelled gradually in response to GSH likely due to the reduction-triggered de-cross-linking and promoted intracellular DOX release inside GSH-pretreated HeLa cells, resulting in enhanced cell inhibition efficacy. Reduction and pH dual-sensitive DOX-conjugated CCL micelles with an average size of 60 nm were prepared from poly(*N*-(2-hydroxypropyl)methacrylamide)-*b*-PPDSEMA (PHPMA-*b*-PPDSEMA) block copolymers by

simultaneous DOX conjugation to the micellar core *via* acid cleavable hydrazone bonds and core-cross-linking *via* disulfide bonds under catalysis of *tris*(2-carboxyethyl)phosphine (28). Heffernan *et al.* assembled reduction-sensitive CCL polyeion micelles *via* electrostatic self-assembly of 2-pyridyldisulfide (PDS)-functionalized negatively charged proteins with PEG-*b*-PLys-PDS block copolymer followed by cross-linking with 3,6-dioxa-1,8-octanedithiol through disulfide exchange reaction (24). These CCL micelles exhibited high stability in serum and were explored for controlled delivery of vaccines, including ovalbumin and immunostimulatory CpG-DNA.

Wei *et al.* reported that reduction-sensitive CCL micelles based on lipoic acid (LA)-functionalized PEG-*b*-PHPMA (PEG-*b*-PHPMA-LA) conjugates, although highly stable under extensive dilution and concentrated salt condition, swelled to over 800 nm in response to 10 mM DTT likely due to the increased hydrophilicity of micellar core upon de-cross-linking (81). These DOX-loaded CCL micelles induced pronounced antitumor effect following 48 h incubation in HeLa and HepG2 cells with IC_{50} values of 6.7 and 12.8 μ g DOX/ml, respectively. With a similar strategy, reduction-sensitive CCL micelles demonstrating accelerated intracellular drug release and enhanced cell inhibition were also obtained from starch-g-PEG/LA copolymers (94). Wu *et al.* developed reduction and pH dual-sensitive CCL micelles from LA- and *cis*-1,2-cyclohexanedicarboxylic acid (CCA)-decorated PEG-*b*-PLys (PEG-*b*-PLys-CCA/LA) copolymer (Fig. 5) (84). The resulting robust CCL micelles quickly dissociated into unimers under a reductive environment and released DOX rapidly in response to 10 mM GSH at pH 7.4 and 5.0 with 86.0% and 96.7% of release in 24 h, respectively. Cell experiments revealed that DOX-loaded CCL PEG-*b*-PLys-CCA/LA micelles efficiently transported DOX into the cell nuclei leading to pronounced cytotoxicity effects to HeLa and HepG2 cells.

Abdullah Al *et al.* found that PTX-loaded CCL micelles based on thiolated Pluronic F127 bearing pendant thiol groups in the PPO block displayed a comparable cytotoxic effect to free PTX, with a cell viability of 39% in A549 cells following 24 h incubation at a PTX dosage of 1.5 μ g/ml (1). Yan *et al.* obtained reduction-sensitive CCL micelles from thiol-containing PEG-*b*-poly(LA-co-(2-mercaptoethyl-5-methyl-2-oxo-1,3-dioxane-5-carboxylate)) copolymer through self-assembly in aqueous solution and oxidation of thiols (88). These DOX-loaded CCL micelles exhibited GSH-dependent drug release and cell proliferation inhibition, wherein cell viability of GSH-pretreated MCF-7 cells decreased from more than 60% to less than 30% upon increasing GSH concentration from 5 to 20 mM. Li *et al.* described that reduction-sensitive CCL micelles based on thiolated telodendrimers composed of a linear PEG and a Cys-containing dendritic cluster of cholic acids (CAs), PEG-*b*-Cys₄-Lys₈-CA₈, had superior PTX-loading capacity, enhanced micellar stability, prolonged *in vivo* circulation time, and preferential accumulation at the tumor site in nude mice bearing SKOV-3 ovarian cancer xenograft (40). The release of PTX from micelles, while inhibited by core-cross-linking, could be gradually facilitated under a reductive environment. This CCL micellar PTX exhibited more effective tumor growth inhibition and longer survival time at a dose of 10 mg PTX/kg than the NCL counterparts and Taxol. In a previous study, similar *in vitro* release profile and cell cytotoxicity were observed for vincristine (VCR)-loaded CCL PEG-*b*-Cys₄-Lys₈-CA₈ micelles (30). The *in vivo* studies using nude mice bearing non-Hodgkin's lymphoma xenograft showed better tolerance, improved efficacy, and absence of acute neurotoxicity for VCR-loaded CCL micelles at a dose of 2.5 mg VCR/kg. Reduction-sensitive CCL prodrug micelles were readily prepared based on poly(methacryloyloxyethylphosphorylcholine)-*b*-poly(2-hydroxyethylmethacrylate-dihydrolipoic acid-CPT) block copolymers (48), resulting in

FIG. 5. Reduction and pH dual-sensitive CCL PEG-*b*-PLys-CCA/LA micelles for active loading and triggered intracellular release of DOX. (i) Self-assembly of PEG-*b*-PLys-CCA/LA copolymer and active loading of DOX. (ii) Core-cross-linking of micelles under catalytic amount of DTT to yield reduction and pH dual-sensitive cross-linked micelles. (iii) Endosomal pH-triggered cleavage of amide bond of CCA and partial drug release. (iv) GSH-triggered de-crosslinking, micelle dissociation, and complete drug release. DTT, dithiothreitol; LA, lipoic acid. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

3–9 μM against MCF-7 and COLO205 cells after 72 h incubation.

Conclusions and Perspectives

The past several years have witnessed a dramatic advancement of diverse reduction-sensitive polymeric micelles and vesicles for triggered intracellular release of potent anticancer drugs and protein biotherapeutics. These smart drug delivery systems have demonstrated high stability under physiological conditions, fast response to intracellular reducing environment, triggering drug release right in the cytosol and cell nucleus, and pronounced antitumor effect. The findings that intracellular drug release from biodegradable micelles and polymersomes can be much accelerated by simply incorporating a single disulfide bond into amphiphilic biodegradable block copolymers are remarkable. The minimal chemical alternation renders these reduction-sensitive shell-sheddable biodegradable micelles and polymersomes particularly favorable for further advancement to clinical studies. The fast response observed for shell-sheddable micelles and polymersomes is due to excellent access of reducing agents to the interfacial disulfide bonds. In comparison, disassemblable micelles show somewhat lower response rate in that multiple disulfide bonds are embedded in the hydrophobic micellar core. The reduction-sensitivity might be enhanced by incorporating pH-sensitivity that leads to swelling of micelles before trafficking to the cytosol. It should be noted, however, that both shell-sheddable and disassemblable micelles and polymersomes, like most self-assembled nanosystems, might encounter *in vivo* stability problem. In this sense, reduction-sensitive cross-linked micelles and polymersomes are more ideal as they exhibit excellent stability in circulation and diminishing premature drug release while are prone to fast de-cross-linking and dissociation to unload drugs in response to intracellular level of GSH. Undoubtedly, disulfide-cross-linked micelles and polymersomes are one of the very few fascinating vehicles that are able to overcome the extracellular stability *versus* intracellular drug release dilemma for cancer therapy. Notably, several *in vivo* studies corroborate that disulfide-cross-linked micellar and vesicular drugs have achieved prolonged circulation time, enhanced accumulation at the tumor tissue, lower side effects, and higher tumor inhibition.

It should be noted, however, that the intracellular and systemic fate of reduction-sensitive micelles and polymersomes, including the mechanism, site, and rate of reduction reaction, remains unclear. To progress the clinical applications, biophysical studies on reduction-responsive micelles and polymersomes should be performed to achieve insight into the degradation and drug release behaviors inside the tumor cells. Moreover, multifunctional reduction-sensitive biocompatible and biodegradable micelles and polymersomes, which possess high drug loading capacity as well as site-specific targeting ligands, should be explored for targeted cancer therapy *in vivo*. We are convinced that reduction-sensitive degradable micelles and polymersomes will play a particular role in targeted cancer chemotherapy.

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Abbreviations Used

C_{16} = hexadecyl
 CA = cholic acid
 CC = cytochrome C
 CCA = *cis*-1,2-cyclohexanedicarboxylic acid
 CCL = core-cross-linked
 CMC = critical micelle concentration
 CPT = camptothecin
 Cys = cysteine
 DOCA = deoxycholic acid
 DOX = doxorubicin
 DS = PEG-*b*-Cys₄-*b*-PDLLA
 DTT = dithiothreitol
 DTX = docetaxel
 GILT = γ -interferon-inducible lysosomal thiol reductase
 GSH = glutathione
 GSH-OEt = glutathione monoester
 HA = hyaluronic acid
 HPHSEP = hyperbranched homopolyphosphates
 PEG = poly(ethylene glycol)
 PEGMMA = PEG methyl methacrylate
 PEO = poly(ethylene oxide)
 PEP = poly(2-ethoxy-2-oxo-1,3,2-dioxaphospholane)
 PHEA = poly(α,β -(*N*-(2-hydroxyethyl)-L-aspartamide))
 PHPMA = poly(*N*-(2-hydroxypropyl)methacrylamide)
 PLeu = poly(leucine)
 PLys = poly(lysine)
 PPDSEMA = poly(pyridyl disulfide ethyl methacrylate)

PPhe = poly(L-phenylalanine)
 PU = polyurethane
 IC₅₀ = half maximal inhibitory concentration
 L₂ = lipoyl
 LA = lipoic acid
 MA = methacrylic acid
 MABC = *N*-methacryloyl-*N'*-(*t*-butyloxycarbonyl)cystamine
 MTX = methotrexate
 NCL = noncross-linked
 OEGMA = oligo(ethylene glycol) methyl ether acrylate
 PAAc = poly(acrylic acid)
 PAsp = poly(aspartic acid)
 PBLG = poly(γ -benzyl L-glutamate)
 PCL = poly(ϵ -caprolactone)
 PDEA = poly(2-(diethyl amino)ethyl methacrylate)
 PDMA = poly(2-(*N,N*-dimethylamino) ethyl methacrylate)
 PDS = 2-pyridyldisulfide
 PEEP = poly(ethyl ethylene phosphate)
 PTX = paclitaxel
 PZLL = poly(ϵ -benzyloxycarbonyl-L-lysine)
 SA = PEG-*b*-PDLLA
 SCL = shell-cross-linked
 siPlk1 = polo-like kinase 1
 SS = disulfide bond/linkage
 SSPAA = reductively degradable poly(amide amine)
 SSPAE = reduction-sensitive poly(β -amino ester)
 TPGS = α -tocopheryl polyethylene glycol succinate
 VCR = vincristine