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Glutathione-responsive nano-vehicles as a promising platform for targeted intracellular drug and gene delivery

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ABSTRACT

The past couple of years have witnessed a tremendous progress in the development of glutathione-responsive nano-vehicles for targeted intracellular drug and gene delivery, as driven by the facts that (i) many therapeutics (e.g. anti-cancer drugs, photosensitizers, and anti-oxidants) and biotherapeutics (e.g. peptide and protein drugs, and siRNA) exert therapeutical effects only inside cells like the cytosol and cell nucleus, and (ii) several intracellular compartments such as cytosol, mitochondria, and cell nucleus contain a high concentration of glutathione (GSH) tripeptides (about 2–10 mM), which is 100 to 1000 times higher than that in the extracellular fluids and circulation (about 2–20 μM). Glutathione has been recognized as an ideal and ubiquitous internal stimulus for rapid destabilization of nano-carriers inside cells to accomplish efficient intracellular drug release. In this paper, we will review recent results on GSH-responsive nano-vehicles in particular micelles, nanoparticles, capsules, polymersomes, nanogels, dendritic and macromolecular drug conjugates, and nano-sized nucleic acid complexes for controlled delivery of anti-cancer drugs (e.g. doxorubicin and paclitaxel), photosensitizers, anti-oxidants, peptides, protein drugs, and nucleic acids (e.g. DNA, siRNA, and antisense oligodeoxynucleotide). The unique disulfide chemistry has enabled novel and versatile designs of multifunctional delivery systems addressing both intracellular and extracellular barriers. We are convinced that GSH-responsive nano-carrier systems have enormous potential in targeted cancer therapy.

1. Introduction

In the past two decades, tremendous efforts have been directed to the development of targeted drug delivery systems because they promise to resolve several key therapeutical issues associated with current clinical practice including low treatment efficacy and significant side effects [1,2]. However, despite that considerable progress has been made, few of the targeting systems have achieved optimal outcomes, which is very often due to a poor intracellular trafficking process and/or inefficient drug release inside targeted cells. It is realized that many therapeutics (e.g. anti-cancer drugs, photosensitizers, and anti-oxidants) and biotherapeutics (e.g. peptide and protein drugs, DNA and siRNA) have to be delivered and released into the cellular compartments such as the cytoplasm or cell nucleus, in order to exert therapeutic effects [3,4].

Potential targeted drug delivery systems, therefore, should be able to overcome not only extracellular barriers (long circulation time, preferential accumulation at diseased sites, selective binding to the targeted cells, etc.) but also equally important intracellular barriers (cellular internalization, endosomal escape, drug release, etc.). In the past years, design of novel bio-responsive nanocarriers that release drugs in response to an intracellular signal, in particular acidic pH and redox potential, has received great interest [5–7]. Nano-vehicles, which are pH sensitive, are usually designed to destabilize vehicles and release drugs in endosomal and/or lysosomal compartments, which have pH values typically as low as 5.5 and 4.5, respectively. In comparison, redox-responsive vehicles are mostly intended to disassemble and release drugs in the cytosol which contains 2 to 3 orders higher level of glutathione (GSH) tripeptide (approximately 2–10 mM) than the extracellular fluids (approximately 2–20 μM) (Scheme 1) [8]. GSH/glutathione disulfide (GSSG) is the major redox couple in animal cells that determines the anti-oxidative capacity of cells [9]. GSH/GSSG is kept reduced by NADPH and glutathione reductase. The intracellular level of GSH is also dependent on other redox couples such as $NADH/NAD^+$, $NADPH/NADP^+$ and $thioredoxin_{red}/thioredoxin_{ox}$ [9,10]. This significant difference in GSH level has rendered GSH-responsive nano-vehicles most appealing for targeted intracellular drug delivery. It should further be noted that endosomal compartment is also redox-active in which the redox potential is modulated by a specific reducing enzyme gamma-interferon-inducible lysosomal thiol reductase (GILT) in the co-presence of a reducing agent such as cysteine (but not GSH) [11].

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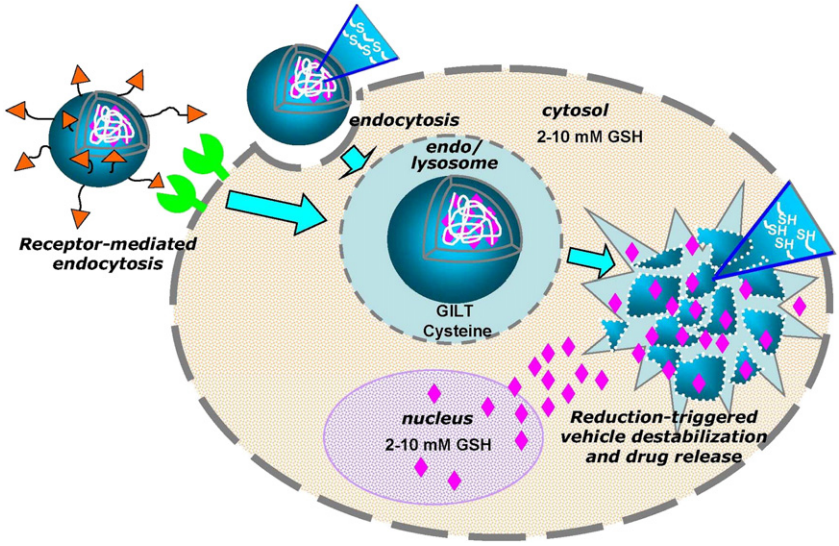


Scheme 1. Schematic illustration of the intracellular trafficking pathway of GSH-responsive nano-vehicles including steps of cellular internalization, endosomal escape, reduction-triggered vehicle degradation, and drug release. The redox potential of the cytosol is primarily determined by GSH/GSSG, while that of the endo/lysosome is modulated by a specific reducing enzyme GILT and co-factor cysteine. GSH-responsive nano-vehicles may also be partially degraded in the endo/lysosomal compartments.

Moreover, the redox-active lysosome contains also low-mass iron that is kept in a reduced state (Fe^{2+}) by the acidic interior and high concentrations of thiols such as cysteine within lysosome [12]. The reduction-sensitive polymers, bioconjugates and vehicles have attracted a lot of attention for diverse biomedical applications including controlled drug delivery, gene delivery and diagnostic imaging [13,14]. It has to be noted, however, that only in the last couple of years exploding progress has been made in the design of GSH-responsive nano-vehicles for triggered intracellular drug release.

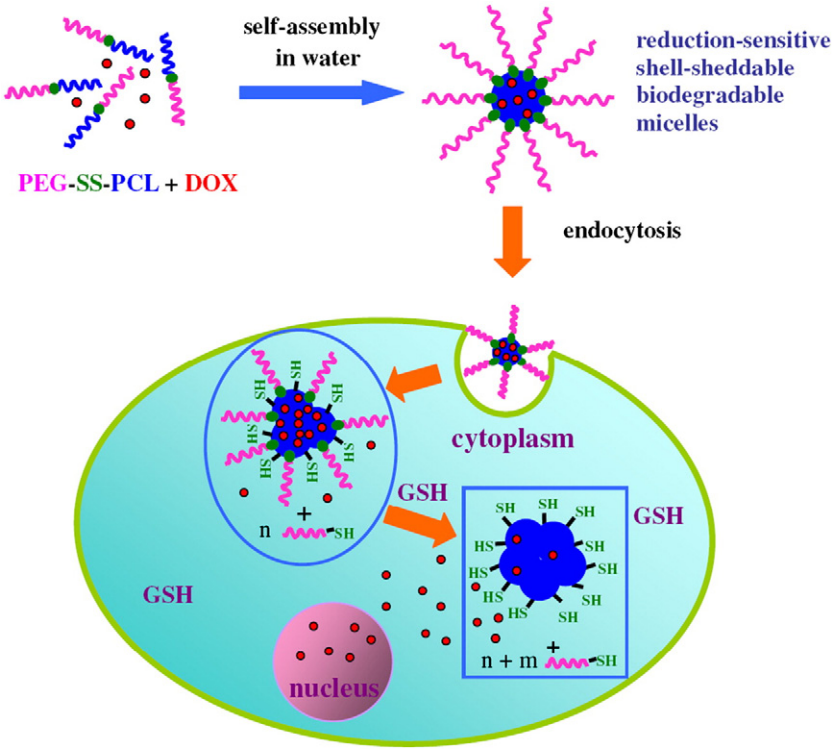
In this review, new developments in the field of GSH-responsive nano-vehicles such as micelles, nanoparticles, capsules, polymersomes, nanogels, dendrimers, and nano-sized nucleic acid complexes for controlled delivery of anti-cancer drugs (e.g. doxorubicin and paclitaxel), photosensitizers, anti-oxidants, peptide and protein drugs, or nucleic acids (e.g. DNA, siRNA, and antisense oligodeoxynucleotide) will be discussed. The unique disulfide chemistry has enabled novel and versatile design of multifunctional delivery systems to overcome both extracellular and intracellular barriers. It is anticipated that GSH-responsive nano-vehicles have enormous potential in targeted cancer therapy.

2. Glutathione-responsive micelles

In the past two decades, biodegradable micelles e.g. based on poly(ethylene glycol)–b-poly(ε-caprolactone) (PEG–PCL) block copolymers have received much attention for tumor-targeted anti-cancer drug delivery [15]. However, due to the gradual degradation of polyesters, sustained release of drugs over periods of days to weeks via a diffusion-controlled mechanism, which often results in reduced drug efficacy, is commonly observed. We recently reported that shell-sheddable micelles based on PEG-SS–PCL released DOX quantitatively within 12 h in a reductive environment analogous to that of the intracellular compartments such as cytosol and the cell nucleus (Scheme 2) [16]. In contrast, minimal drug release (<20%) was observed within 24 h for the reduction insensitive PEG–PCL micelles under the same conditions as well as for PEG-SS–PCL micelles under non-reductive conditions. PEG-SS–PCL micelles were shown to be sufficiently stable in water, but prone to fast aggregation in the presence of 10 mM dithiothreitol (DTT), most likely due to shedding of the PEG shells through reductive cleavage of the intermediate disulfide bonds. Interestingly, cell experiments using a mouse leukemic monocyte macrophage cell line (RAW 264.7) revealed that these shell-sheddable micelles released DOX much faster inside the cells and showed a higher antitumor efficacy as compared to the "traditional" reduction insensitive control. Very similar results were also shown for dextran-SS–PCL block copolymer micelles, for which cell viabilities of about 20 and 70% were observed for RAW 264.7 cells after 2 d treatment with DOX-loaded dextran-SS–PCL micelles and DOX-loaded dextran–PCL micelles, respectively [17]. Notably, Wang and coworkers reported that shell-detachable micelles based on disulfide-linked diblock copolymer of PCL and hydrophilic poly(ethyl ethylene phosphate) (PCL-SS–PEEP) displayed GSH-responsive release of DOX and led to enhanced growth inhibition of A549 tumor cells pretreated with glutathione monoester (GSH-OEt) [18]. GSH-OEt is often used to artificially enhance the intracellular GSH level. Thayumanavan and coworkers showed that GSH-sensitive micelles could be prepared from amphiphilic copolymers containing disulfide bonds in the hydrophobic segments, in which disassembly of micelles with concomitantly enhanced drug release took place in response to elevated GSH concentrations [19]. The drug release was relatively slow even in the presence of 70 mM GSH. The cytotoxicity of DOX-loaded micelles, however, demonstrated a clear correlation with the intracellular GSH level in MCF-7 cells.

Fan and coworkers prepared reductively degradable micelles from amphiphilic graft copolymers of disulfide-containing hydrophobic poly(amido amine) (SS-PAA) and PEG (SS-PAA-g–PEG) [20]. In vitro release studies showed that DOX was nearly quantitatively released in 10 h in response to 1 mM DTT due to reduction-sensitive degradation of the PAA main chain resulting in micelle disassembly, whereas only approximately 25% DOX was released in 24 h in the absence of DTT. The IC50 of the DOX-loaded SS-PAA-g–PEG micelles was determined to be 0.0647 μg/mL for HepG2 cells and 0.0494 mg/mL for HeLa cells, which are only slightly higher than the IC50 of free DOX. Huang and coworkers prepared reduction-degradable micelles by conjugating azide-functionalized camptothecin (CPT) and azide-terminated PEG to SS-PAA containing alkyne groups via click chemistry (SS-PAA-g–PEG/CPT) [21]. Over 85% copolymer was degraded into oligomers and small complexes in 7 d at 40 mM DTT. In vitro release studies showed enhanced release of CPT at higher DTT concentration.

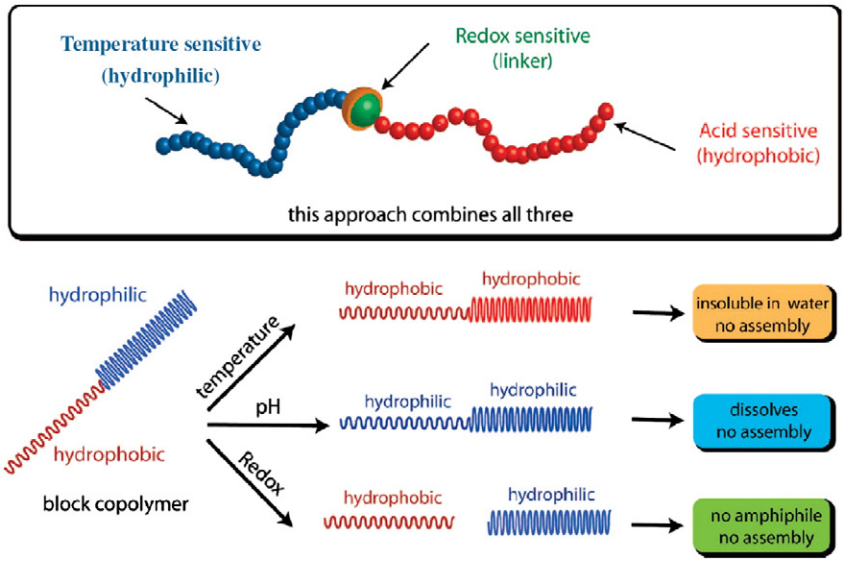
Thayumanavan and coworkers reported triple-stimuli sensitive micelles of PNIPAM-SS-P(THP-HEMA) that respond to changes in temperature, pH and redox potential (THP-HEMA: tetrahydropyran



Scheme 2. Schematic illustration of reduction-sensitive shell-sheddable biodegradable micelles based on PEG-SS–PCL block copolymer for efficient intracellular release of DOX triggered by GSH [16].

(THP)-protected 2-hydroxyethyl methacrylate) (Scheme 3) [22]. Micelles were disassembled under the following conditions: (i) above the LCST, the PNIPAM block becomes hydrophobic, rendering the copolymer insoluble in water and hence leading to loss of assembly; (ii) by lowering the pH, the P(THP-HEMA) block becomes hydrophilic, resulting in dissolution of the assembly; and (iii) in a reducing environment, the block copolymer is cleaved into individual homopolymers and hence the assembly is disrupted. This multi-stimuli behavior might provide a unique possibility to fine-tune the release kinetics of the encapsulated hydrophobic guest molecules. The authors have shown that while the pH and redox stimulus by itself exerted slow or incomplete release of nile red over a long period of time, combination of both stimuli resulted in significantly accelerated and more complete release of nile red. Zhang and coworkers developed novel redox-sensitive diselenide-containing block copolymer micelles that were rapidly disassembled in response to a low concentration of reducing agent (GSH, 0.01 mg/mL) as well as oxidant (H_2O_2, 0.01% v/v) [23].

As for other supramolecular structures, one practical issue of micelles is their spontaneous dissociation at concentrations below

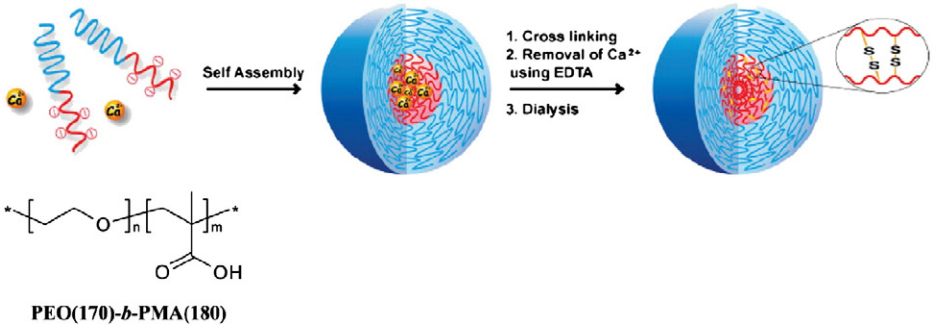


Scheme 3. Schematic representation of amphiphilic block copolymer which can respond to three stimuli: pH, temperature and redox [22].

their critical micelle concentration (CMC) [24]. It has been reported that micelles rapidly dissociate upon intravenous administration, most probably due to large volume dilution as well as interactions with cells and biomolecules present in the blood. This in turn leads to premature drug release and inferior tumor-targetability. In the past decade, it has been shown that crosslinking of micelles effectively overcomes the instability problem [25]. It should be noted, nevertheless, that overly stable micelles are not ideal either because the release of drugs is prohibited after the micelles arrive at the target sites, resulting in low drug efficacy. The use of intracellularly reversible disulfide crosslinks is an attractive strategy to elegantly solve the stability problem of micelles. Bronich and coworkers prepared poly(ethylene oxide)–b-poly(methacrylic acid) (PEO–b-PMA) micelles using divalent metal cations (Ca^{2+}) as templates followed by crosslinking the ionic cores with cystamine (Scheme 4) [26]. Interestingly, these micelles showed a high level of DOX loading (50% w/w). In vitro release studies demonstrated significant acceleration of DOX release from cystamine-crosslinked micelles in the presence of GSH or cysteine in the release media, wherein about 75% of DOX was released in 1 h in response to 10 mM GSH. An MTT assay revealed that DOX-loaded cystamine-crosslinked micelles were much more cytotoxic for human A2780 ovarian carcinoma cells, with IC50 value at least six times lower, as compared to the stably crosslinked control. Stenzel and coworkers obtained stable nucleosides-containing block copolymer micelles by sequential reversible addition-fragmentation chain transfer (RAFT) copolymerization of polyethylene glycol methyl ether methacrylate, 5′-O-methacryloyluridine, and bis(2-methacryloyloxyethyl)disulfide (DSDMA, bioreducible crosslinker) [27]. The resulting core-crosslinked (CCL) micelles readily hydrolyzed into free block copolymers in the presence of 0.65 mM DTT in less than 1 h. As expected, CCL micelles showed a rather slow release of riboflavin (about 30% release in 7 h). The addition of 0.65 mM DTT, however, induced fast drug release, with a release profile similar to that of the un-crosslinked control (about 60–70% release in 7 h). Liu and coworkers employing RAFT polymerization prepared two types of degradable thermoresponsive CCL micelles [28,29]. In one approach, double hydrophilic block copolymer, poly (ethylene oxide)–b-poly(N-isopropylacrylamide-co-N-acryloxysuccinimide), existing as unimers in water at room temperature, formed micelles upon increasing the temperature to above its LCST, which after crosslinking with cystamine yielded stable CCL micelles [28]. The disulfide crosslinks could be cleaved in a strong reducing environment. Moreover, these micelles showed tunable swelling/deswelling behavior in response to change of temperature. In the other approach, CCL micelles were obtained in a one-pot manner via RAFT copolymerization of N-isopropylacrylamide (NIPAM) and DSDMA employing poly(N-(2-aminoethyl)methacrylamide) as a macro-RAFT agent [29]. These micelles could be disintegrated into unimers upon addition of 15.4 mM DTT. The authors have shown that coronas of CCL micelles could be further functionalized with biocompatible and/or bioactive molecules such as biotin and galactose.

Bulmus and coworkers developed DOX-conjugated CCL micelles based on N-(2-hydroxypropyl)methacrylamide (HPMA) and 2-(2-pyridyldisulfide)ethyl methacrylate (PDSM) block copolymers, wherein DOX conjugation to the micellar core via acid cleavable hydrazone bonds and core-crosslinking via reducible disulfide bonds took place simultaneously [30]. These micelles were disintegrated into unimers upon treatment with tri(2-carboxyethyl)phosphine hydrochloride(TCEP). In vitro release studies showed that 72% and 21% of DOX were released in 23.5 h from CCL micelles at pH 5.0 and pH 7.4, respectively. Murthy and Heffernan reported the preparation of disulfide-crosslinked polyion micelles by electrostatic self-assembly of PEG–poly(L-lysine) (PEG–PLL) block copolymer with negatively charged proteins, both of which contained dithiopyridine functions, followed by disulfide crosslinking [31]. In this way, proteins were chemically tethered to the micellar core via a disulfide bond, resulting in a high degree of protein retention under SDS-PAGE. Vaccine delivery systems were prepared with ovalbumin and immunostimulatory CpG-DNA, which were designed to release the vaccine intracellularly through reduction of disulfide crosslinks. These micelles were also investigated as a long-circulating enzyme carrier that maintains the enzymatic activity of the anti-oxidant enzyme catalase within the micelle core.

Lee and coworkers designed shell crosslinked (SCL) micelles through self-assembly of PEG–b-poly(L-lysine)–b-poly(L-phenylalanine) triblock copolymers followed by crosslinking of the poly (L-lysine) block with 3,3′-dithiobis(sulfosuccinimidylpropionate) (DTSSP) [32]. These SCL micelles demonstrated enhanced stability against sodium dodecyl sulfate (SDS) and the release of methotrexate (MTX) was greatly retarded as compared to the non-crosslinked counterparts. The rate of drug release from CCL micelles increased with increasing GSH concentrations in the media. The toxicity of MTX-loaded CCL micelles in A549 cells revealed a clear correlation with the intracellular GSH level. Wang and coworkers developed reversible SCL micelles based on PEG–b-PPE_{SH}–b-PCL triblock copolymer (PPE_{SH}: thiol-functionalized polyphosphoester) [33]. These SCL micelles exhibited enhanced stability against dilution and addition of N,N′-dimethylformamide (DMF). Drug release was retarded by the cross-linking and accelerated in a reductive environment (20 mM DTT). The toxicity of DOX-loaded SCL micelles to A549 cells increased with increasing intracellular GSH level as shown by the MTT assay. We recently reported reduction-responsive reversibly crosslinked biodegradable micelles based on PEG–PCL diblock copolymer containing two lipoyl functional groups at their interface (PEG–L_2–PCL) [34]. The micelles were readily crosslinked by adding 7.6 mol% DTT relative to the lipoyl groups. Notably, micelles after crosslinking demonstrated a markedly enhanced stability against dilution, physiological salt concentration, as well as organic solvent. In the presence of 10 mM DTT, however, micelles were subject to rapid de-crosslinking. In vitro release studies showed minimal release of DOX

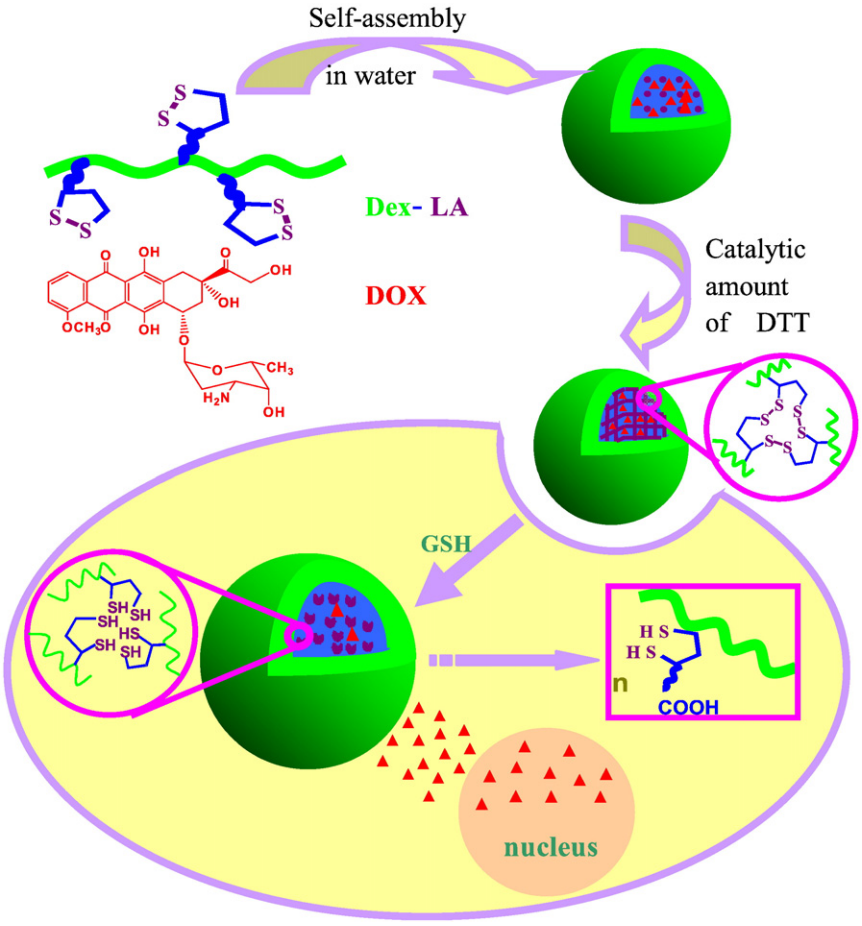


Scheme 4. Synthesis of cystamine-crosslinked PEO–b-PMA micelles [26].

from crosslinked micelles even at a particularly low micelle concentration (C<CMC of uncrosslinked micelles, analogous to intravenous injection). In the presence of 10 mM DTT mimicking an intracellular reductive environment, sustained release of DOX from crosslinked micelles was achieved, in which approximately 75% of the DOX was released in 9 h. Ahn and coworkers prepared reversibly crosslinked biodegradable micelles using PEG–PCL block copolymer linked by a peptide comprising three cysteine residues (PEG–Cys3–PCL) [35]. The disulfide-stabilized micelles remained stable at high dilution. The in vitro release profile showed a sustained release of DOX below the CAC at 37 °C (<20% release in 24 h), while addition of 1 mM DTT triggered a burst release of DOX. Zhao and coworkers reported novel reversible SCL micelles based on an alkynylated surfactant crosslinked via click reaction with a diazide containing cleavable disulfide, geminal diol or acetal bond [36]. Hydrophobic guests such as pyrene could be readily loaded into the SCL micelles and the micelles remained robust even after significant dilution to a concentration below the CMC of the surfactant. The entrapped pyrene was, however, completely released from disulfide-crosslinked micelles in ca. 1 min upon addition of just 1 equiv. or 20 μM DTT. Notably, acid-triggered pyrene release from acetal-crosslinked micelles was found to be much slower. McCormick and coworkers reported the fabrication of SCL micelles from pH-responsive triblock copolymer, PEO–b-poly(N-(3-aminopropyl) methacrylamide)-β-poly(2-(diisopropylamino)ethyl methacrylate) (mPEO–PAPMA–PDPAEMA), which was soluble in water at low pH (<5.0) but self-assembled into micelles above pH 6.0 [37]. The micelles were readily crosslinked with dimethyl 3,3′-dithiobispropionimidate (DTBP). The treatment of SCL micelles with 9.4 mM DTT for 1 h at room temperature resulted in rapid de-crosslinking.

3. Glutathione-responsive nanoparticles

Biodegradable nanoparticles have been extensively investigated for controlled drug delivery in vitro and in vivo [2,38]. To obtain nanoparticles with high extra-cellular stability and fast intracellular drug release, we recently developed reversibly stabilized multifunctional dextran nanoparticles based on dextran–lipoic acid derivatives (Dex–LAs) (Scheme 5) [39]. Dextran is a natural analog of PEG while lipoic acid is produced naturally in the human body and commonly used as an antioxidant drug for treating diseases such as diabetes and HIV. The nanoparticles after crosslinking with a catalytic amount of DTT were robust against dilution and a high salt concentration. The release of DOX was minimal (ca. 10%) even under extensive dilution, while over 90% of the DOX was released in 11 h in response to 10 mM DTT. Confocal laser scanning microscopy (CLSM) studies using HeLa and RAW 264.7 cells revealed a rapid and efficient delivery of DOX into the cell nucleus. MTT assays showed that DOX-loaded crosslinked nanoparticles had a similar drug efficacy as the non-cross-linked counterparts. It is anticipated that these smart nanoparticles will have tremendous potential for tumor-targeted chemotherapy. Jiang and coworkers were able to prepare reduction-sensitive nanoparticles by introducing disulfide bridges into the side chains of a thermosensitive polymer, p(PEG-MEMA-co-Boc-Cyst-MMAm) and simply heating the aqueous solution to above its LCST (LCST varied from 20 to 57 °C depending on copolymer compositions) [40]. These nanoparticles remained stable in the presence of 2 μM DTT for 24 h at 37 °C, but rapidly collapsed in response to 3 mM DTT, likely



Scheme 5. Schematic illustration of the preparation and intracellular fate of reversibly stabilized, multifunctional dextran–lipoic acid nanoparticles [39].

due to enhanced water solubility after cleavage of disulfide bonds. Bulmus and coworkers developed disulfide cross-linked PEG-streptavidin hybrid particles from biotin-PEG–b-PPDSM block copolymers [41]. The micellar core functionalization (e.g. with a maleimide derivative of a green fluorophore) and cross-linking could take place concomitantly, to afford fluorescent CCL micelles with a diameter of ca. 54 nm and 75 mol % biotin functionality exposed on the micelle corona. The micelles could be readily decorated with streptavidin, yielding polymer–protein hybrid particles with tunable dimensions of 350 nm–2 μm. Hennink and coworkers prepared disulfide-crosslinked positively charged nanoparticles from partially thiolated trimethylated chitosan (TMC) and thiolated hyaluronic acid [42]. The crosslinked nanoparticles were stable in 0.8 M NaCl. In contrast, particles made from non-thiolated polymers dissociated under otherwise the same conditions.

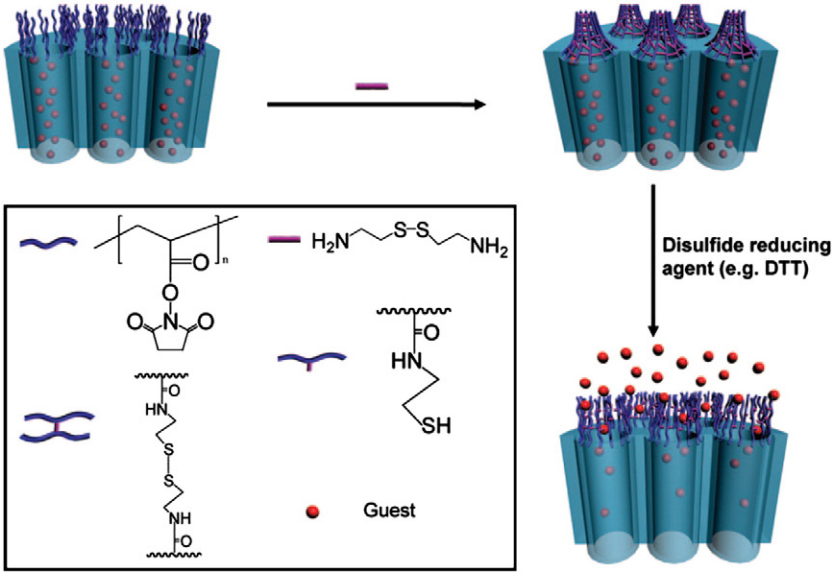
Feng and coworkers reported redox-responsive nanogated mesoporous silica nanoparticles (MSN) by grafting poly(N-acryloxysuccinimide) (PNAS) to the pore entrance of MSN particles followed by crosslinking with cystamine (Scheme 6) [43]. The polymer coating around MSN was uniform and 2 nm thick. The release studies demonstrated that loaded rhodamine B was rapidly released in response to 21.6 mM DTT, in contrast with a slow release in 0.216 mM DTT media. The release rate of rhodamine B was dependent on the DTT concentration. In comparison, irreversibly cross-linked ensembles (with 1,6-hexadiamine) showed no induced release with the addition of DTT. In a following study, the authors designed multi-responsive nanogated MSN by immobilizing β-CD to PNAS coated MSN via the disulfide bonds followed by crosslinking with diazo-linker [44]. The release studies showed that without external stimuli, no release of entrapped calcein from nanogated MSN was observed, while application of UV, DTT or α-CD resulted in instantaneous release of calcein.

Lin and coworkers reported an MSN-based controlled intracellular cysteine release system, in which cysteine was tethered to MSN via disulfide bonds (MSN-SS–Cys) [45]. There was no leaching of Cys in PBS solution prior to the addition of reducing agents. However, approximately 99, 90, 70 and 60% of Cys was released from MSN-SS–Cys in 30 min following addition of nicotinamide adenine dinucleotide hydride (NADH), DTT, dihydrolipoic acid (DHLA), and GSH, respectively. Toxicity studies showed that MSN-SS–Cys is approximately 444 times more effective in delivering cysteine into HeLa cells than the conventional N-acetylcysteine (NAC) approach. In comparison, Cys physisorbed to MSN and Cys tethered to MSN via a non-cleavable thioether bond (MSN–Cys) did not show any significant effect on the cell growth inhibition. Brauchle and coworkers studied the intracellular cysteine release behavior of ATTO633-labeled cysteine linked to the inner particle core of MSN via disulfide bridges in HuH7 cells by high-resolution fluorescence microscopy and found that endosomal escape is a limiting factor for the redox-triggered intracellular release of disulfide-bound cysteine from MSN [46]. However, after photochemical rupture of the endosomes by means of a photosensitizer, ATTO633-labeled cysteine was successfully released from MSN into the cytoplasm, indicating that the reducing milieu of the cytoplasm is sufficient to cleave the disulfide bonds.

4. Glutathione-responsive capsules

Hollow capsules are a class of highly versatile vehicles that can be applied for encapsulation and controlled delivery of diverse bioactive molecules including drugs, nucleic acids, peptides, and proteins [47,48]. Usually, hollow capsules are fabricated by deposition of interacting polymers onto a sacrificial colloidal template followed by dissolution of the core [49]. The assembly process allows for engineering of capsules including their composition, size, permeability, colloidal stability and surface functionality.

Caruso and coworkers developed novel reductively degradable capsules based on layer-by-layer (LbL) assembly of thiolated poly (methacrylic acid) (PMA_{SH}) and poly(vinylpyrrolidone) (PVPON) onto silica particles followed by cross-linking of the thiol groups in the PMA_{SH} to form stable disulfide bonds and dissolution of the sacrificial silica core [50,51]. PVPON could be readily removed via disruption of inter-polymer hydrogen bonds in pH 7 buffer, resulting in single component, disulfide cross-linked PMA_{SH} capsules. These capsules were stable in oxidizing conditions but rapidly disassembled in reducing environments similar to those inside living cells, to release the encapsulated cargo. PMA capsules were applied for in vitro and in vivo delivery of proteins and peptides for vaccine applications [52–54]. PMA capsules could efficiently associate with and be internalized by monocytes and dendritic cells (DCs). PMA capsules loaded with KP9 peptide (a model HIV vaccine peptide) stimulated a significant proportion of the KP9-specific T cells to simultaneously express the cytokines interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α). The intravenous vaccination of mice with ovalbumin (OVA) protein- and peptide-loaded PMA_{SH} capsules activated OVA-specific CD4 and CD8 T cells to proliferate in vivo, with at least 6-fold higher



Scheme 6. Schematic illustration of redox-responsive nanogated ensemble based on polymeric network-capped mesoporous silica [43].

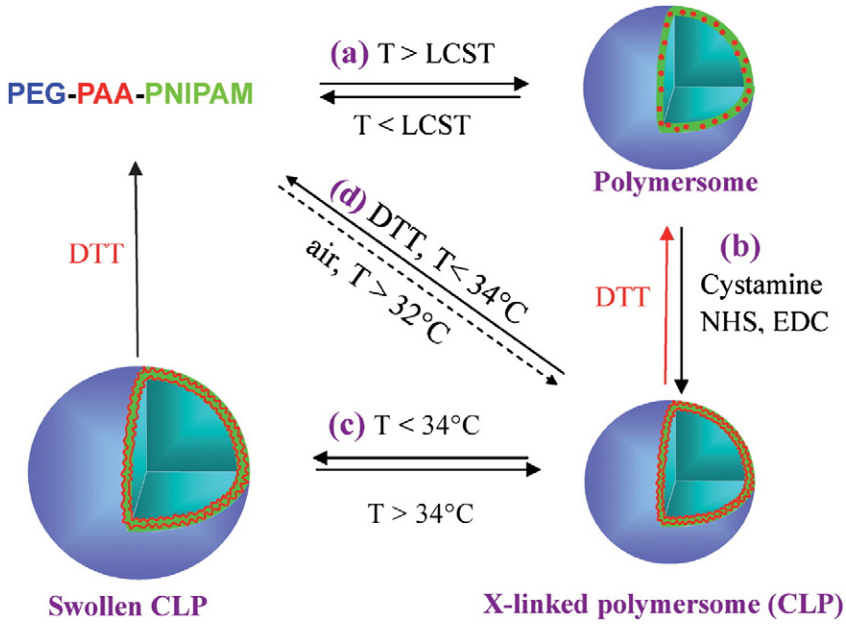
proliferation of OVA-specific CD8 T cells and 70-fold higher proliferation of OVA-specific CD4 T cells compared to the equivalent amount of OVA protein administered alone [54]. These bio-destructible capsules were also investigated for intracellular delivery of two lipophilic anti-cancer drugs, DOX and 5-fluorouracil (5-FU) [55,56]. DOX and 5-FU were loaded into capsules in the form of oleic acid emulsions. In vitro release studies showed that DOX/oleic acid-loaded capsules released minimal amounts of DOX (<5%) in 100 mM PBS at 37 °C in 24 h, while approximately 80% DOX was released in 6 h in the presence of 5 mM GSH. MTT assays revealed that treatment of LIM1215 human colorectal cancer cells with DOX/oleic acid-loaded PMA capsules and 5-FU/oleic acid-loaded capsules resulted in significant cell death (>85%), being more effective than free DOX and 5-FU, respectively. The studies on uptake and intracellular fate of PMA_{SH} capsules showed that the internalized capsules were deformed in endocytic compartments and accumulated in late endosomes and lysosomes [56]. Disulfide-stabilized PMA capsules could also be prepared, up to three polymer layers, via a benign method (oxidation free) by sequential deposition of PMA_{SH} and PMA with activated thiol functions (i.e. 3-carboxy-4-nitrobenzene sulfide and pyridine-2-sulfide) [57].

Kim and coworkers recently reported a novel template-free synthesis approach to reduction-responsive polymer nanocapsules based on self-assembly of amphiphilic cucurbit[6]uril (CB[6]) followed by shell-crosslinking with a disulfide-containing cross-linker [58]. The resulting capsules had an average diameter of ca. 70 nm and a hollow interior, surrounded by an approximately 2.0 nm thickness thin shell. Most nanocapsules had collapsed and aggregated after 30 min treatment with DTT. In vitro release studies showed that encapsulated carboxyfluorescein (CF) was quickly released in response to 100 mM DTT. The capsules decorated with galactose showed efficient internalization into HepG2 cells and rapid intracellular release of CF. Zhang and coworkers reported the preparation of reduction-sensitive hollow polyelectrolyte nanocapsules from cysteamine-conjugated chitosan and dextran sulfate by LbL adsorption on β-cyclodextrin functionalized silica spheres followed by cross-linking thiols and removal of the silica core [59]. In vitro release studies using bovine serum albumin (BSA) as a model protein showed significantly enhanced protein release in response to 10 mM GSH.

5. Glutathione-responsive polymersomes/vesicles

Polymersomes (also referred to as polymeric vesicles) have received enormous attention due to their intriguing aggregation phenomena, cell and virus-mimicking dimensions and functions, as well as tremendous potential applications in medicine, pharmacy, and biotechnology [60,61]. They are particularly interesting for intracellular protein delivery [62]. There are several excellent review papers on polymersomes as well as stimuli sensitive polymersomes [6,63,64]. Here, we present only recent new developments on reduction-responsive polymersomes.

We recently reported the preparation of reversibly crosslinked temperature-responsive nano-sized polymersomes (about 220 nm) from water soluble PEO–b-poly(acrylic acid)–b-PNIPAM (PEO–PAA–PNIPAM) triblock copolymers by raising the solution temperature to above the LCST (e.g. 37 °C) followed by cross-linking at the interface using cystamine via carbodiimide chemistry (Scheme 7) [65]. The vesicular structure was confirmed by CLSM and static light scattering (SLS) measurements. The crosslinked polymersomes, while showing remarkable stability against dilution, organic solvent, high salt conditions and change of temperature in water, were otherwise completely dissociated in 1.5 h in 10 mM DTT media at pH 7.4. The release studies showed that most FITC–dextran (used as a model protein) was retained within the polymersomes after lowering the temperature to below the LCST (e.g. 25 °C). However, fast release of FITC–dextran was achieved upon addition of 10 mM DTT. Mastrobattista and coworkers reported that peptide vesicles could be stabilized by introducing two or three cysteine units into the hydrophobic domain of vesicle forming amphiphilic oligopeptide SA2 (Ac-Ala-Cys-Val-Cys-Leu-(Leu/Cys)-Leu-Trp-Glu-Glu-COOH), allowing the formation of intermolecular disulfide bridges [66]. The in vitro release profiles showed that intermolecular crosslinking of peptides in the vesicles did not affect the calcein release profile. The following studies showed that water-insoluble phthalocyanines (photosensitizer) could be quantitatively loaded into peptide vesicles, which were internalized by cells in their intact form [67]. Incubation of COS-7 cells with phthalocyanine-loaded peptide vesicles in the dark did not result in any cytotoxicity. However, upon illumination, the phthalocyanine-loaded peptide vesicles showed an active photodynamic response towards COS-7 cells, resulting in effective cell killing (IC_{50} = ~2.8 nM phthalocyanine). In contrast, two



Scheme 7. Schematic illustration of reversibly crosslinked temperature-responsive nano-sized polymersomes [65].

controls, free phthalocyanine or empty peptide vesicles, did not show any cytotoxicity.

Kim and coworkers developed a novel reduction-sensitive, robust, and biocompatible vesicle (SSCB[6]VC) from an amphiphilic cucurbit[6] uril (CB[6]) derivative containing disulfide bonds between hexaethylene glycol units and the CB[6] core [68]. Vesicles were prepared with an average diameter of ca. 190 nm by the thin film rehydration method followed by repeated extrusion through a syringe filter. The vesicles were stable in the presence of 3 μM GSH or 15 μM cysteine. However, complete disruption of vesicles occurred in 12 h in response to 5 mM GSH. Notably, these vesicles could be readily decorated with functional moieties such as targeting ligands and imaging probes by using their spermidine conjugates. MTT assays showed that DOX-loaded folate-SSCB[6]VC resulted in a significantly decreased cell viability as compared to free DOX (ca. 28.1% versus 52.7%).

6. Glutathione-responsive nanogels

Nanogels are biocompatible three-dimensional materials with high water content and sizes ranging from tens of nanometers to submicrons [69,70]. Nanogels can be applied for encapsulation and delivery of various agents including anticancer drugs, proteins, plasmid DNA, and imaging probes [70]. Matyjaszewski and coworkers described the preparation of well-defined reduction-sensitive functional nanogels using inverse mini-emulsion atom transfer radical polymerization (ATRP) and the disulfide–thiol exchange reaction [71,72]. These nanogels could be loaded with various water-soluble biomolecules including anticancer drugs, carbohydrates and proteins [73,74]. For example, DOX was loaded into nanogels with a loading efficiency of 50–70%. DOX-loaded disulfide-crosslinked nanogels (drug loading efficiency approximately 50–70%) were essentially nontoxic, but upon addition of 20 wt.% GSH HeLa cell growth was significantly inhibited. Caruso and coworkers recently reported the synthesis of reduction-sensitive DOX-loaded PEG nanoporous polymer spheres (NPS_{PEG}-DOX) through the following steps: (i) loading and immobilization of alkyne or azide-functionalized PEG into mesoporous template (MSN) via click chemistry, (ii) click crosslinking of PEG and covalent attachment of DOX through degradable linkers containing disulfide bonds, and (iii) dissolution of the MSN templates [75]. These PEG spheres were nontoxic. Under reductive conditions (5 mM GSH), these spheres were disassembled to release DOX over time. Groll and coworkers reported the synthesis of biocompatible and degradable nanogels (average diameter ca. 380 nm) by crosslinking thiol-functionalized star-shaped poly(ethylene oxide-co-propylene oxide) and linear polyglycidol in inverse mini-emulsion via formation of disulfide crosslinkers [76]. These nanogels were degraded following 6 h incubation in 10 mM GSH.

7. Glutathione-responsive dendritic and macromolecular drug conjugates

N-Acetyl-L-cysteine (NAC) is an antioxidant and anti-inflammatory agent with significant potential applications in the treatment of stroke, neuroinflammation and cerebral palsy. However, NAC displays high plasma binding upon IV administration, resulting in low stability and reduced drug efficacy. Kannan and coworkers recently reported the design of macromolecular NAC conjugates based on poly(amido amine) (PAMAM) dendrimers or star PEG for intracellular drug delivery [77–79]. NAC was conjugated to two PAMAM dendrimers, G4-NH_2 and G3.5-COOH, with payloads of 16 and 18 per dendrimer, respectively. In vitro release studies showed that ca. 70% of NAC was released from dendrimer conjugates in 1 h at an intracellular GSH level (10 mM). In contrast, release of NAC was negligible at plasma GSH level (2 μM). The efficacy studies in activated microglial cells (target cells in vivo) using the reactive oxygen species (ROS) assay showed that dendrimer-SS-NAC conjugates afforded an order of magnitude increase in antioxidant activity compared to free drug [78]. Long and coworkers reported that reduction-sensitive GSH–PEG–GSH conjugates were nearly 100% effective at protecting SHSY5Y cells from oxidative stress at 250 μM, whereas reduction-insensitive counterparts did not offer protection [80].

Harth and coworkers designed a modular intracellular peptide delivery system consisting of dendritic molecular transporter molecules and a polymeric scaffold in a size dimension of 5–10 nm [81]. Variable amounts of peptide drugs could be conjugated to the polymeric scaffold via cleavable disulfide bonds. The dendritic molecular transporter molecules were shown to facilitate rapid cellular uptake of nanoparticle-peptide conjugates into 3T3 cells. Sinko and coworkers reported that a new generation peptide core [CH_3CO-(Lys-βAla-βAla)$_X$-Cys-$CONH_2$ (X = 2, 4)] allowed for optimal attachment of multiple PEGs in stoichiometric amounts with low polydispersity via disulfide linkages [82]. Degradation studies showed that treatment of PEG nanocarriers with 3 mM GSH resulted in complete release of the Texas Red-labeled 4-arm monomer from the intact heterodimeric nanocarrier in 7 min at 37 °C. Davis reported the preparation of hetero-bifunctional protein–polymer conjugates via site-specific modification of BSA with a bifunctional RAFT agent terminated with pyridyl disulfide (PDS) groups and subsequent in situ polymerization of oligo(ethylene glycol) acrylate and N-(2-hydroxypropyl) methacrylamide [83]. Notably, only one PDS group had been conjugated to BSA, while the other remained intact and could be utilized to attach thiocholesterol and Rhodamine B to the protein–polymer conjugates via disulfide coupling. Simanek and coworkers reported preparation of second generation triazine dendrimer–paclitaxel conjugates with an ester or disulfide bond [84]. The conjugates were further modified with 2 kDa PEG, to afford dendrimer conjugates with 12 molecules of paclitaxel (26–30 wt.% drug) and 6 to 9 PEG arms (43–54 wt.% PEG). MTT assays using PC-3 cells showed IC50 values in the low nanomolar range with DTT and GSH enhancing the toxicity of reduction-sensitive constructs.

8. Glutathione-responsive gene delivery systems

In the past several years, significant effort has been directed to development of reduction-responsive gene delivery systems [13,85]. The dynamic chemical stability of disulfide bonds, i.e. superior stability under the extracellular environments and rapid degradation in the intracellular reducing conditions, could elegantly resolve the contradictory requirements of efficient non-viral gene transfer agents, i.e. excellent binding and protection of nucleic acids in extracellular fluids and efficient release of nucleic acids inside the cells. Notably, vastly different types of reductively degradable non-viral carriers including bioreducible liposomes [86], polypeptides [87], and in particular cationic polymers or networks such as bioreducible linear or branched poly(amido amine) (PAA) [88–90], polyethylenimine (PEI) [91–93] and poly(2-dimethylaminoethyl methacrylate) (PDMAEMA) [94] have been explored for in vitro DNA or small interfering RNA (siRNA) transfection. In addition to bringing about enhanced transfection, bioreducible gene carriers in general show also largely improved toxicity profiles due to decreased charge density upon intracellular cleavage of the disulfide bonds.

The conjugation of nucleic acids including siRNA and antisense oligodeoxynucleotide (asODN) to polymer such as PEG and hyaluronic acid through a disulfide bond represents a second approach to construct GSH-responsive gene delivery systems [95,96]. These nucleic acid conjugates formed more stable complexes with polycations than the parent nucleic acids. The thiolytic cleavage of disulfide linkage in the cytoplasm, however, resulted in efficient intracellular release of active siRNA or asODN. More recently, Park and coworkers reported that multimerized siRNA linked with cleavable disulfide bonds could produce more stable and compact polyelectrolyte complexes with polycations than monomeric siRNA due to substantially increased charge densities and the presence of flexible chemical

linkers in the backbone [97,98]. Interestingly, these reductively cleavable multimerized siRNA showed markedly enhanced gene-silencing efficiencies in vitro and in vivo [97].

9. Concluding remarks

Current studies have shown that GSH-responsive nano-vehicles can uniquely resolve the stability dilemma of drug carriers. Glutathione has been recognized as an ideal and ubiquitous internal stimulus for rapid destabilization of nano-carriers inside cells to dump drugs into the cytosol and cell nucleus. This targeted intracellular drug release approach could significantly enhance drug efficacy, overcome multi-drug resistance (MDR), and/or reduce drug and carrier-associated side effects. The disulfide chemistry is particularly robust and versatile, which facilitates novel designs of diverse multi-functional delivery systems for anti-cancer drugs, anti-oxidants, peptides, proteins, and nucleic acids.

It should be noted, nevertheless, that the exact intracellular fate of reduction-sensitive nano-vehicles remains unclear. There is hardly direct evidence showing that reduction-sensitive nano-vehicles are destructed in the cytosol and/or cell nucleus. Despite controversy, some studies have indicated that disulfide bonds are cleaved in endosomal and lysosomal compartments [11,99,100]. Recently, cell surface thiols were reported to affect cell entry of disulfide-conjugated peptides [101]. The development of GSH-responsive drug delivery systems requires better understanding of the intracellular trafficking and fate of nano-vehicles.

It should further be noted that many of the reported systems are not based on biodegradable and/or biocompatible materials, which are nevertheless the first requirement for most biomedical applications. In the future, more efforts shall be directed to development of novel GSH-sensitive degradable polymers and copolymers including polyesters, polycarbonates, polypeptides, poly(ester amide)s and poly (ester urethane)s. Notably, recently there are interesting reports on the synthesis of reduction-sensitive stepwise cleavable star polymers [102,103], biodegradable polyurethanes derived from L-arabinitol [104], and cascade degradable linear polymers [105]. We are convinced that with rational design GSH-responsive nano-vehicles will eventually be widely applied in targeted cancer therapy.

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