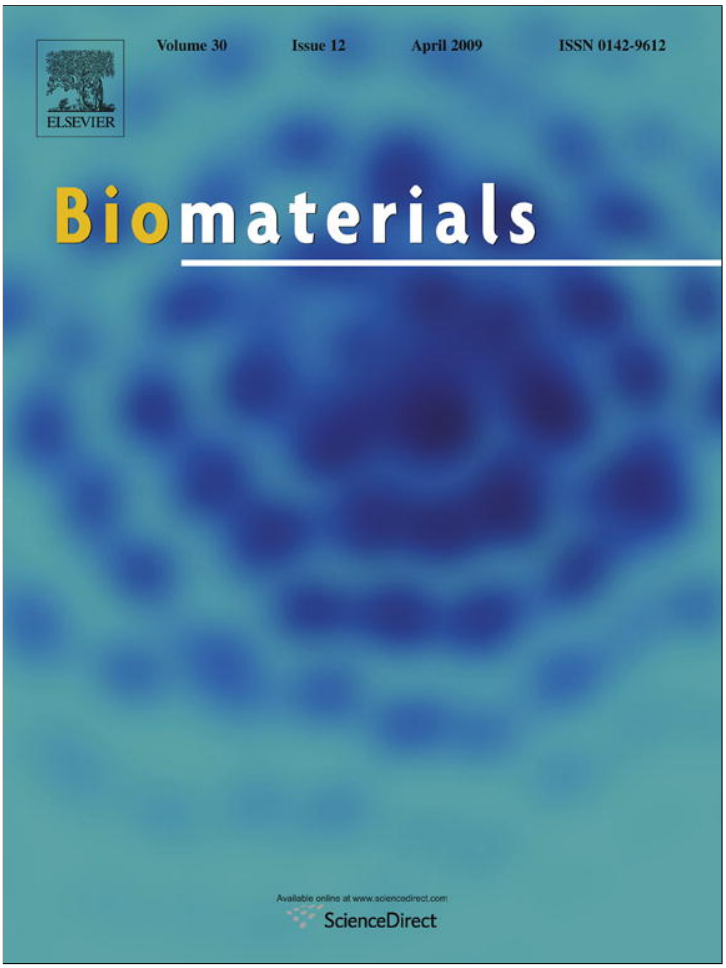

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Review

Reduction-sensitive polymers and bioconjugates for biomedical applications

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Dedicated to Prof. Dr. Jan Feijen on the occasion of his retirement from the University of Twente.

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ABSTRACT

Reduction-sensitive biodegradable polymers and conjugates have emerged as a fascinating class of biomedical materials that can be elegantly applied for intracellular triggered gene and drug delivery. The design rationale of reduction-sensitive polymers and conjugates usually involves incorporation of disulfide linkage(s) in the main chain, at the side chain, or in the cross-linker. Reduction-sensitive polymers and conjugates are characterized by an excellent stability in the circulation and in extracellular fluids, whereas they are prone to rapid degradation under a reductive environment present in intracellular compartments such as the cytoplasm and the cell nucleus. This remarkable feature renders them distinct from their hydrolytically degradable counterparts and extremely intriguing for the controlled cytoplasmic delivery of a variety of bioactive molecules including DNA, siRNA, antisense oligonucleotide (asODN), proteins, drugs, etc. This review presents recent advances in the development of reduction-sensitive biodegradable polymers and conjugates, with particular focus on the up-to-date design and chemistry of various reduction-sensitive delivery systems including liposomes, polymersomes, polymeric micelles, DNA containing nanoparticles, polyion complex micelles, nano- and micro-gels, nanotubes, and multi-layered thin films. It is evident that reduction-sensitive biodegradable polymers and conjugates are highly promising functional biomaterials that have enormous potential in formulating sophisticated drug and gene delivery systems.

1. Introduction

In the past decade, reduction-sensitive biodegradable polymers and conjugates have emerged as a fascinating class of biomedical materials that can be elegantly applied for the development of sophisticated delivery systems for both biotherapeutics (pDNA, siRNA, pharmaceutical proteins and peptides) as well as 'classical' low molecular weight drugs. These materials usually contain characteristic disulfide linkage(s) in the main chain, at the side chain, or in the cross-linker. The disulfide bonds, though sufficiently stable in the circulation and in the extracellular milieu, may be prone to rapid cleavage, at a time scale from minutes to hours, under a reductive environment through thiol–disulfide exchange reactions [1,2]. This quick-response chemical degradation behavior is distinct from common hydrolytically degradable polymers such as aliphatic polyesters and polycarbonates in which the ester and carbonate bonds usually exhibit gradual degradation kinetics inside body with degradation times ranging from days to weeks to months [3–5].

Thiol–disulfide exchange reactions, which are rapid and readily reversible, play an important role in maintaining proper biological functions of living cells, including stabilization of protein structures, enzymatic activity, and redox cycles [6–8]. Glutathione tripeptide (γ-glutamyl-cysteinyl-glycine; GSH) is the most abundant low-molecular-weight biological thiol and GSH/glutathione disulfide (GSSG) is the major redox couple in animal cells [9]. GSH/GSSG is maintained at distinct, non-equilibrium potentials in mitochondria, cytoplasm, nuclei, the secretory pathway and the extracellular space [8]. In body fluids (e.g. blood) and in extracellular matrices and on the cell surface, the proteins are rich in stabilizing disulfides as a result of a relatively high redox potential due to a low concentration of GSH (approximately 2–20 μM). In contrast, inside cells the concentration of GSH is 0.5–10 mM that is kept reduced by NADPH and glutathione reductase, maintaining a highly reducing environment inside cells [9]. The large difference in reducing potential between the intracellular and extracellular milieu may be exploited for triggered intracellular delivery of a variety of bioactive molecules including DNA, siRNA, antisense oligonucleotide (asODN), proteins and low molecular weight drugs [10]. Furthermore, also of particular interest is that tumor tissues are highly reducing and hypoxic compared with normal tissues [11], with at least 4-fold higher concentrations of GSH in the tumor tissues over

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normal tissues [12], rendering the reducible bioconjugates valuable for tumor-specific drug and gene delivery.

This review presents recent advances in the development of reduction-sensitive biodegradable polymers and conjugates, with particular focus on the up-to-date design and chemistry of various reduction-sensitive delivery systems including liposomes, polymersomes, polymeric micelles, DNA containing nanoparticles, polyion complex micelles, nano- and micro-gels, nanotubes, and multi-layered thin films. We are convinced that reduction-sensitive biodegradable polymers and conjugates have become a highly promising functional biomaterials platform that has enormous potential for the design and development of sophisticated drug and gene delivery systems.

2. Reduction-sensitive cationic polymers and networks for intracellular gene delivery

Recently, various reduction-sensitive biodegradable cationic polymers have been designed for gene delivery. Unlike hydrolytically degradable cationic polymers that are rapidly degraded upon exposing to the moistures due to catalysis of amino groups, they are sufficiently stable under physiological conditions (37 °C, pH 7.4) in the circulation as well as in extracellular matrices. In the cytosol, however, due to the high levels of glutathione they may be subject to rapid degradation via thiol–disulfide exchange. The degradation of the gene carrier on the one hand may result in low toxicity by avoiding accumulation of high molecular weight polycations inside cells, and on the other hand may be employed as a means for efficient intracellular release of DNA or siRNA leading to increased transfection efficiency. Therefore, reduction-sensitive gene carriers in principle could meet the conflicting requirements of an ideal nucleic acid delivery system, i.e. high stability in circulation while rapid degradation inside targeted cells.

2.1. Bioreducible polyethylenimine (PEI) polymers and networks

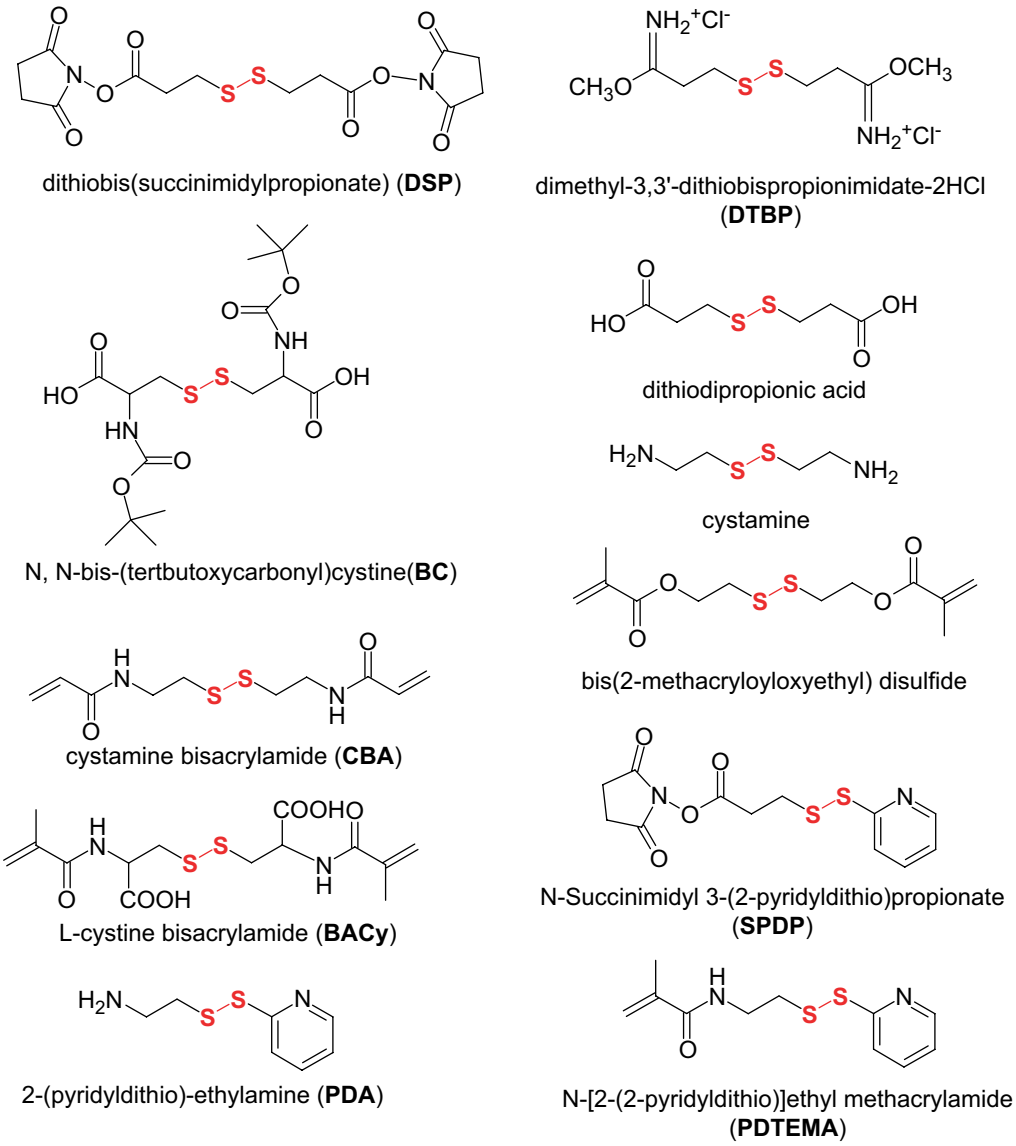
Lee et al. reported that crosslinking of low molecular weight branched PEI (800 Da bPEI) with homobifunctional reducible cross-linking reagents, dithiobis(succinimidylpropionate) (DSP) and dimethyl-3,3′-dithiobispropionimidate-2HCl (DTBP) (for structures see Scheme 1), leads to a significant increase in transfection to Chinese hamster ovary (CHO) cells as compared to the parent 800 Da bPEI, reaching transfection levels close to that of 25 kDa bPEI [13]. The intracellular reduction of transfection complexes by GSH facilitates dissociating of PEI from DNA to enhance the gene expression. In a similar way, Shen et al. cross-linked 1800 Da PEI using DTBP [14]. The crosslinked PEI/DNA complexes prepared at an N/P ratio of 10 revealed a significantly reduced cytotoxicity in comparison with 25 kDa bPEI and were unpacked at 3 mM GSH, though no transfection results were given. Zhuo and coworkers prepared disulfide cross-linked PEIs through two different approaches, i.e. via thiolation of 800 Da bPEI with methylthiirane at different ratios followed by oxidation with DMSO [15] and via Michael addition polymerization between cystamine bisacrylamide (CBA) and 800 Da bPEI [16]. *In vitro* experiments showed that the disulfide-crosslinked PEIs have a lower cytotoxicity and comparable or higher gene transfection efficiency than the 25 kDa bPEI-based systems. Furthermore, fetal bovine serum did not decrease the transfection efficiency. Lee and coworkers have recently reported that the endosomolytic protein listeriolysin O (LLO) from the intracellular pathogen *Listeria monocytogenes* conjugated with bPEI 25 kDa via a reversible disulfide bond (LLO-*ss*-PEI) could further enhance the transfection efficiency of polyplexes of disulfide-crosslinked low molecular weight PEI [17].

Goepferich and coworkers crosslinked low-molecular-weight linear PEI ($M_w = 2.6$–4.6 kDa) using dithiodipropionic acid or cysteine linkages, which resulted in a branched structure [18]. The *in vitro* transfection experiments using seven different cell lines demonstrated superior transfection efficacies and substantially lower toxicities of reversibly crosslinked linear PEI polymers as compared to commonly used non-viral commercial transfection reagents including SuperFect, Lipofectamine and JetPEI. For instance, the transfection efficacy in HEK cells approximated 70%. In the same study, the authors showed that the intracellular reductive degradation produces mainly non-toxic fragments. The reducible PEIs were subsequently applied for siRNA delivery, wherein siRNA release was shown to play an important role in gene silencing [19]. Moreover, it was demonstrated that the cellular uptake of siRNA was more efficient with increasing branching of the polymer, i.e. lPEI 5 kDa < lPEI cross-linked via disulfide bonds (ssPEI) < branched PEI 25 kDa. The combination of a high branching density and reductively cleavable disulfide bonds within the PEI polymer could be one possible step towards improved siRNA delivery.

Covalently linking low molecular weight PEIs to high molecular weight biodegradable polymers or networks could indeed significantly improve their gene transfer efficiency. The multiple reactive sites (i.e. primary and/or secondary amine groups) of PEIs, however, often give rise to complex coupling reactions and to difficulties in synthesizing polymers with controlled characteristics such as molecular weight, degree of branching, and degradation rate, which are known to have an immediate impact on the transfection activity. It would be highly desirable to develop molecularly well-defined biodegradable PEI-based gene carriers in order to obtain a better control over the gene transfer process. Park et al. designed and synthesized reducible linear PEI, poly(ethylenimine sulfide) (l-PEIS), with M_w ranging from about 10,000 to 20,000 Da, by oxidative polycondensation of bismercapto ethylene imine oligomers [20]. The transfection efficiency increased with increasing amine density of l-PEIS, approaching that of 25 k PEI. In contrast to ExGen 500, l-PEIS displayed low cytotoxicity and was completely degraded inside cells within 3 h, as visualized by fluorescence microscopy using the probe–probe dequenching effect of BODIPY-FL fluorescence dyes.

PEI/DNA complexes were coated with poly[*N*-(2-hydroxypropyl)methacrylamide] (PHPMA) through reducible disulfide bonds between PEI and hydrophilic PHPMA [21]. The resulting PHPMA coated PEI polyplexes were stable to a 250-fold excess of the polyanion poly(aspartic acid) (PAA). As anticipated, complete release of DNA could be accomplished by the addition of 20 mM DTT. The transfection activity of PEI complexes with reversible PHPMA coatings was 40- to 100-fold higher than their non-reversible thioether-linked counterparts. Therefore, hydrophilic polymer coating of PEI/DNA complexes via reducible disulfide bonds represents an attractive approach to simultaneously fulfill the contradictory requirements for extracellular stability and rapid intracellular activation to achieve spontaneous transfection of target cells. A major drawback of PHPMA coatings, either reversible or non-reversible, has been their significant reduction of transfection activity of PEI polyplexes. Transfection activity of these complexes can probably be restored by coupling targeting ligands to their surface.

Kissel et al. prepared reversibly stabilized 25 kDa bPEI/DNA polyplexes via crosslinking with a low molecular weight cross-linking reagent, dithiobis(succinimidyl propionate) (DSP) [22,23]. These disulfide cross-linked polyplexes, as compared to the non-crosslinked 25 kDa bPEI polyplexes, demonstrated enhanced resistance against polyanion exchange and high ionic strength, significantly increased colloidal stability, and reduced interactions with major blood components like albumin and erythrocytes [22]. The *in vitro* transfection experiments showed that polyplexes were

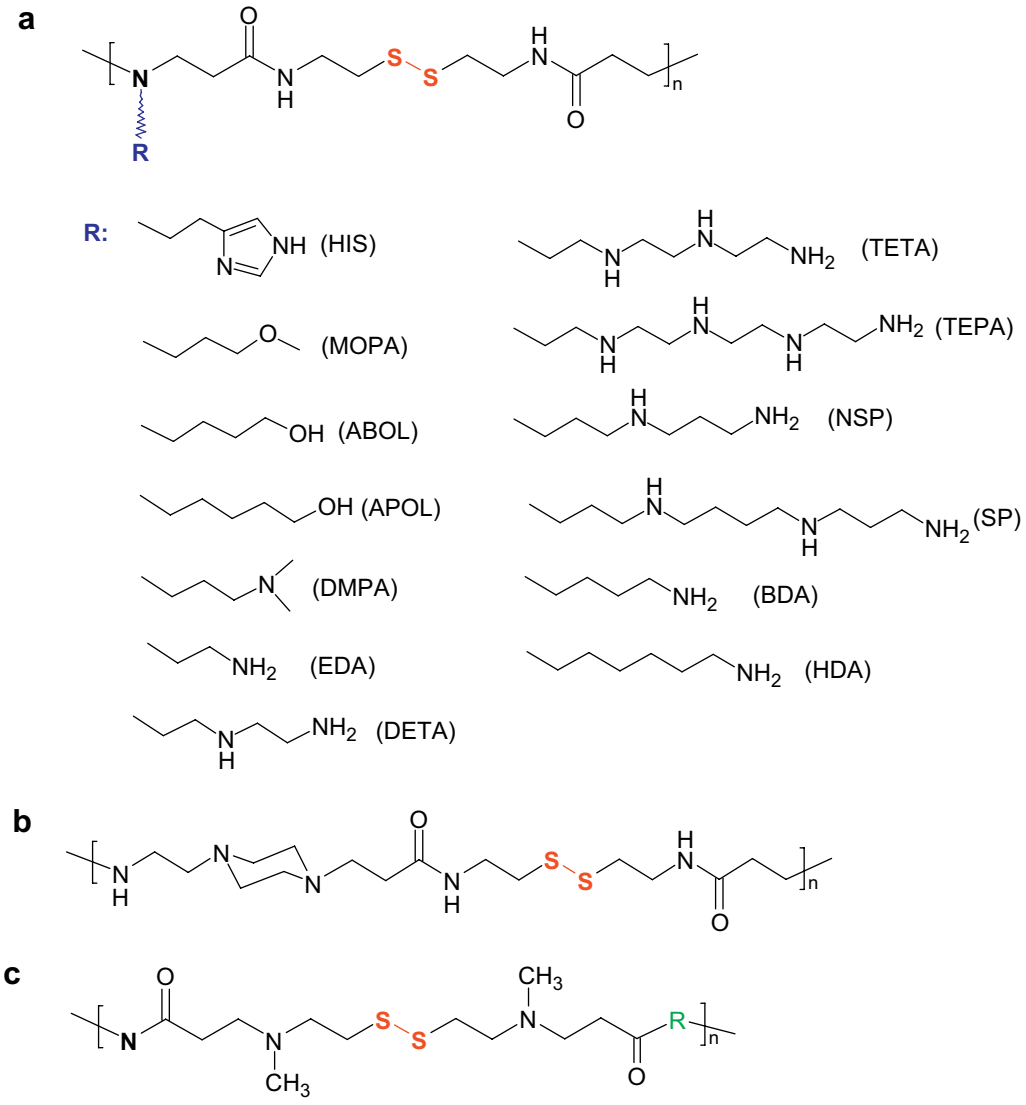


Scheme 1. Chemical structures of disulfide-containing cross-linking agents.

efficiently taken up by cells and DNA release could be triggered by the intracellular redox potential as well as by the addition of 15 mM DTT to the transfection medium. *In vivo* studies in mice revealed elevated blood levels of the surface cross-linked polyplexes, confirming an improved stability in circulation by disulfide cross-linking. Furthermore, these cross-linked polyplexes displayed enhanced liver expression while unwanted lung transfection could be reduced [23]. To further improve the stability of PEI polyplexes in the systemic circulation, a combined strategy of PEGylation and reversible surface crosslinking has been adopted, in which 25 kDa bPEI was modified with one or two high molecular weight PEGs (20 or 30 kDa) to obtain PEG–PEI diblock or PEG–PEI–PEG triblock-type copolymers followed by crosslinking of the polyplexes with DSP [24]. The combined vector modifications displayed a synergistic effect in prolonging circulation times of DNA *in vivo*.

2.2. Bioreducible poly(amido amine)s and poly(β-amino ester)s

Employing Michael-type polyaddition between various primary amines and cystamine bisacrylamide (CBA), we have developed a series of structurally well-defined linear bioreducible poly(amido amine)s with multiple disulfide bonds in their main chain (SS-PAAs) (Scheme 2a) [25]. Five out of seven synthesized polymers could efficiently condense DNA into nano-sized (<200 nm) and positively charged (>+20 mV) polyplexes that are stable under neutral conditions but rapidly destabilized in the presence of 2.5 mM DTT. Interestingly, most SS-PAAs possess higher buffer capacities in the pH range pH 7.4–5.1 than PEI, which may facilitate the endosomal escape of the polyplexes. *In vitro* transfection experiments using COS-7 cells showed that four out of seven SS-PAAs, wherein starting amine monomers are 4-amino-1-butanol, 5-amino-1-pentanol, 3-methoxypropylamine, and histamine, respectively, mediate higher transfection efficiencies than branched PEI. It is interesting to note that poly(β-amino ester)s made of amino alcohols were also shown to give the best transfection among the large library of more than 3000 polymers [26]. Furthermore, under optimal conditions high level of gene expression was also observed in the presence of 5% serum. Remarkably, cytotoxicity assays revealed essentially no or minimal cytotoxicity at SS-PAA concentrations where the highest transfection



Scheme 2. Linear bioreducible poly(amido amine)s (SS-PAAs) synthesized by Michael addition polymerization between cystamine bisacrylamide (CBA) and various amine monomers (a, b) or between *N*,*N*′-dimethylcystamine (DMC) and various bisacrylamide monomers (c).

efficiencies were found. The synthesis of SS-PAAs is very versatile, which allows combination of two distinct amine monomers, e.g. one with a good DNA condensation property and the other with a high buffer capacity, into one SS-PAA polymer chain, resulting in an enhanced transfection activity [27]. Interestingly, Michael addition polymerization of a tri-functional amine monomer, 1-(2-aminoethyl) piperazine (AEP), and equimolar amounts of bis (acrylamide) monomers, including CBA, yielded linear poly(amido amine)s containing secondary and tertiary amine groups in their main chain (Scheme 2b) [28]. These polymers displayed very high buffer capacity in the pH range of 5.1–7.4 (~70% with respect to ~28% of 25 kDa bPEI). Importantly, they also showed good DNA compaction ability giving nano-sized polyplexes. For example, polyplexes of poly(CBA–AEP) displayed a diameter of 108 ± 2 nm at a polymer/DNA weight ratio of 12/1. Further, it was found that incorporation of disulfide linkages in the main chain does not only endow biodegradability facilitating unpacking of DNA inside cells but also results in polyplexes with largely improved biophysical properties, thereby yielding enhanced levels of gene expression along with low toxicity. Hong et al. reported that the topology of reducible poly(amino ester)s, obtained from the Michael addition polymerization of disulfide-based diacrylate and equimolar *N*-methyl ethylenediamine (MEDA), is highly dependent on polymerization temperature [29]. Linear poly(amino ester)s were obtained at a polymerization temperature below 40 °C. However, an elevated temperature yielded hyperbranched poly(amino ester)s, in which the degree of branching (DB) increased with increasing temperature.

In another study, Michael addition reactions between CBA and three different ethylene amine monomers, i.e. ethylenediamine (EDA), diethylenetriamine (DETA), or triethylenetetramine (TETA), yielded reducible poly(amido ethylenimine)s with varying charge density [30]. These polymers could effectively complex pDNA to form nanoparticles with diameters less than 200 nm and positive surface charges of ~32 mV. The *in vitro* transfection using mouse embryonic fibroblast cell (NIH3T3), primary bovine aortic endothelial cell (BAEC), and rat aortic smooth muscle cell (A7R5) lines revealed that polyplexes of these reducible poly(amido ethylenimine)s provoked remarkably high levels of reporter gene expression with nearly 20-fold higher transfection efficiency than 25 kDa bPEI control. Furthermore, the high transfection efficiency was maintained in the presence of 10% serum in the transfection

medium. Confocal microscopy experiments using labelled pDNA confirmed that reducible poly(amido ethylenimine)s aid intracellular release of DNA. Remarkably, it was shown that in both primary rat cardiomyoblasts (H9C2) and rat aortic smooth muscle cells (A7R5), hypoxia-inducible vascular endothelial growth factor (RTP–VEGF) plasmid complexed with reducible poly(amido ethylenimine) produces significantly higher levels of VEGF expression (up to 76-fold) under hypoxic conditions as compared to normoxic conditions [31]. *In vivo* studies using a rabbit myocardial infarct model showed significant VEGF protein expression in the region of the infarct. Reducible poly(amido ethylenimine) could also efficiently condense siRNA to form stable complexes under physiological conditions, which upon the addition of 2.5 mM DTT showed complete release of siRNA [32]. This reducible poly(amido ethylenimine) when formulated with VEGF-directed siRNA, exhibited significantly higher suppression of VEGF in human prostate cancer cells as compared to 25 kDa linear PEI.

Linear reducible poly(amido amine)s containing oligoamines in their side chain were prepared using Michael-type polyaddition of *N*-*tert*-butyloxycarbonyl (*N*-Boc) protected oligoamine monomer to CBA followed by deprotection (Scheme 2a) [33]. In contrast to SS-PAAs derived from single primary amine monomers, they revealed strong DNA binding capability and the formed polyplexes were able to transfect COS-7 cells *in vitro* at low N/P ratios, with transfection efficiencies similar to or even higher than 25 kDa bPEI-based systems. The chemical structure of oligoamine side chains, i.e. number of amino groups as well as length of the alkyl spacer between the amino groups displayed a distinct effect on their buffer capacity, transfection efficiency and toxicity profile. The elongation of the alkyl spacer from ethylene to propylene between the amino units in side chains resulted in significantly lower transfection and increased toxicity. Starting from mono-Boc protected diamine monomers, i.e. *N*-Boc-1,2-diaminoethane, *N*-Boc-1,4-diaminobutane, and *N*-Boc-1,6-diaminohexane, Kim and coworkers prepared a set of linear reducible polymers with tertiary amine groups in main chain along with pendant primary amine groups at the side chain [34]. It is interesting to note that the *in vitro* transfection efficiency increased with increasing side chain spacer length. Polyplexes based on poly(amido amine) with a hexaethylene spacer, poly(CBA–HDA), yielded transfection efficiencies comparable to or significantly higher than 25 kDa bPEI controls depending on the cell lines used. In a following study, poly(CBA–HDA) was shown to tightly condense the prostaglandin E_2 (PGE_2)–Fas siRNA conjugate to nano-sized particles with a diameter of 100–150 nm, wherein PGE_2 is a specific cardiomyocyte targeting ligand and Fas is a key regulator of ischemia-induced apoptosis [35]. The results revealed that PGE_2–Fas siRNA/poly(CBA–HDA) polyplexes were much more efficiently taken up by rat cardiomyocytes (H9C2 cells) compared to the corresponding Fas siRNA polyplexes, supporting PGE_2 receptor-mediated endocytosis. The *in vitro* transfection in H9C2 cells showed that PGE_2–Fas siRNA/poly-(CBA–HDA) polyplexes induced significant Fas gene silencing, resulting in effective inhibition of cardiomyocyte apoptosis. Interestingly, a more recent work demonstrated that poly(CBA–HDA) grafted with arginine while maintained a low cytotoxicity exhibited 9–14 and 20–37 times enhanced transfection efficiency in C2C12 and NIH3T3 cells, respectively, compared to the parent poly(CBA–HDA) [36]. Importantly, the transfection efficiency of arginine-grafted poly(CBA–HDA) was not much reduced by 10% serum. The polyplexes of arginine-grafted poly(CBA–HDA) revealed a cellular uptake pattern similar to those of poly(CBA–HDA), indicating that conjugated arginine residues did not improve cellular penetrating ability. The authors suggested that the enhanced transfection efficiency of arginine-grafted poly(CBA–HDA) may be mediated by other factors such as good nuclear localization ability.

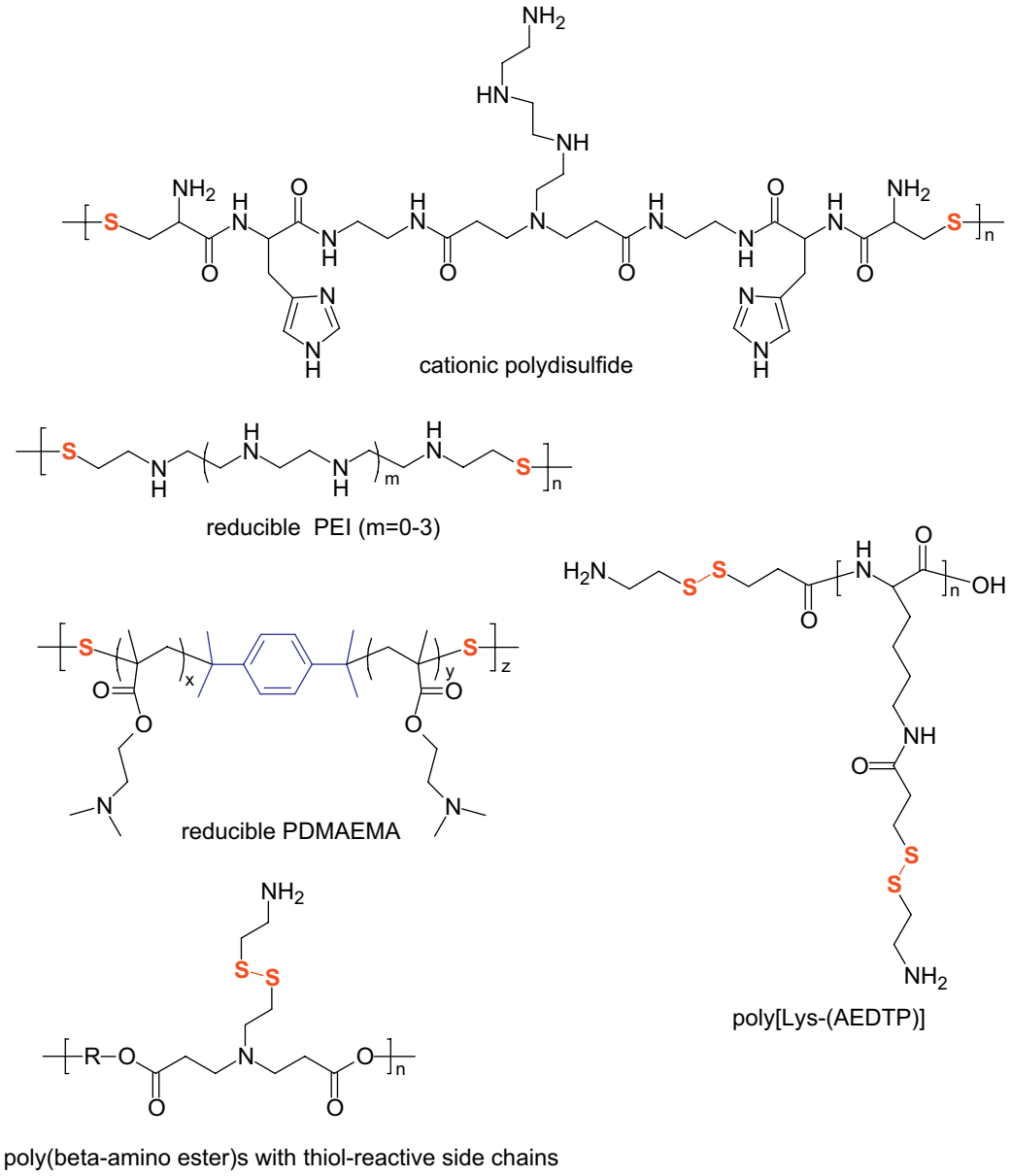
In a different approach, linear bioreducible PAAs containing disulfide linkages in their amino units were synthesized by Michael addition polymerization of *N*,*N*′-dimethylcystamine (DMC) with various bisacrylamide monomers (Scheme 2c) [37]. This synthetic route is complementary to the previously developed route to SS-PAAs in which a disulfide bond is introduced through cystamine bisacrylamide units. Similarly, the DNA complexes were relatively stable in medium mimicking physiological conditions but rapidly disintegrated in the presence of 2.5 mM DTT. The *in vitro* transfection showed up to four times higher transfection efficiencies in COS-7 cells than those of pDMAEMA and PEI.

Lu et al. prepared a cationic polydisulfide with protonatable pendants and multifunctional amino groups, including primary, secondary, tertiary and aromatic groups, through oxidation of the cationic dithiol monomer, which was tailor-made by solid phase chemistry (Scheme 3) [38]. The obtained polydisulfide had an M_w of 6.2 kDa, demonstrated low cytotoxicity and good buffering capacity, and could condense DNA and siRNA into particles of about 150 nm and 300 nm, respectively, at an N/P of 80 or higher. The polydisulfide/DNA complexes were stable in normal physiological environment, while readily dissociated to release DNA in a reductive environment, yielding gene transfection efficiency comparable to or slightly lower than bPEI control. The polydisulfide also resulted in efficient siRNA delivery and effective gene silencing, particularly in the serum-containing medium. In a following study, a multifunctional carrier, 1,4,7-triazanonylimino-bis[*N*-(oleicyl-cysteinyl-histinyl)-1-(aminoethyl)propionamide] (THCO), which possesses hydrophobic groups as well as polymerizable cysteine residues, was designed for the delivery of siRNA [39]. The combination of charge complexation, hydrophobic condensation and reversible polymerization gave rise to stable and compact THCO/siRNA complexes, which revealed siRNA delivery efficiency comparable to that of Transfast™ in serum-free medium. The THCO/siRNA complexes after PEGylation with PEG–Mal resulted in higher transfection efficiency than Transfast™ and DOTAP in the presence of serum.

Langer et al. synthesized poly(β-amino ester)s with thiol-reactive side chains by Michael addition polymerization of 2-(pyridyldithio)-ethylamine (PDA) (Scheme 1) with three different diacrylate monomers [40]. The pyridyldithio groups in the side chains could selectively react with thiol ligands, such as mercaptoethylamine (MEA) and the RGDC peptide. The resulting MEA and RGDC derivatives strongly bind DNA. In particular, the MEA derivative was able to effectively condense DNA to form small-sized polyplexes of around 100 nm in diameter. The PicoGreen exclusion assay revealed that the DNA binding ability of the MEA derivative was substantially reduced in the presence of 10 mM glutathione. It should be noted, however, that complete DNA unbinding was not realized because the polymer still contains tertiary amines in the backbone that likely remained associated with the DNA. The polyplexes of the MEA derivatives exhibited low cellular toxicity and a transfection efficiency comparable to an optimized PEI formulation in human hepatocellular carcinoma cells.

2.3. Biocleavable DMAE–polyrotaxane and PDMAEMA

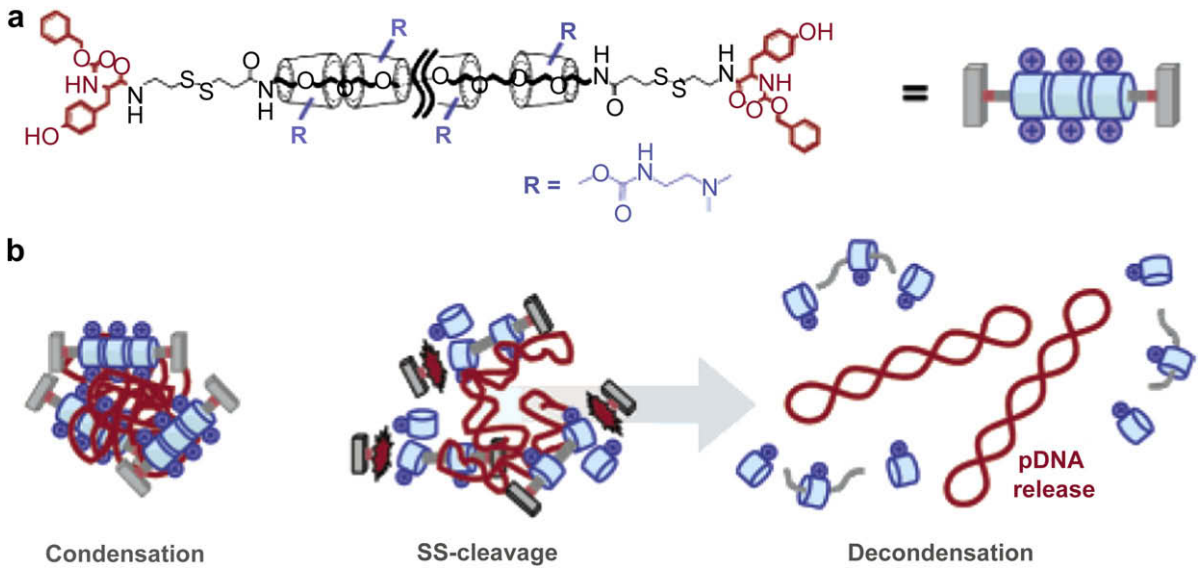
Yui et al. reported on a biocleavable polyrotaxane with a necklace-like structure in which ca. 23–30 cationic dimethylaminoethyl-modified α-CDs (DMAE-α-CDs) were threaded onto a PEG ($M_n = 4000$ Da) chain (Scheme 4) [41,42]. The polyrotaxane was capped with benzyloxycarbonyl tyrosine via disulfide linkages that existed only at both termini of the PEG chain (DMAE-*SS*-PRX). ^{1}H NMR analysis revealed that each polyrotaxane contains ca. 40 DMAE groups. Remarkably, this cationic supramolecular polymer was able to condense pDNA at a low N/P ratio of 0.5 to give



Scheme 3. Examples of reducible cationic polymers.

nanoparticles of ca. 185 nm in diameter and a positive surface chare of +4.8 mV. However, in the presence of 10 mM DTT and a counter polyanion, pDNA was released from the DMAE-*SS*-PRX polyplex due to disulfide cleavage and subsequent dissociation of the non-covalently linked α-CDs from PEG chains. A subsequent study showed that the numbers of α-CD and amino groups of DMAE-*SS*-PRX, which are closely related to the pDNA condensation and release, had a remarkable influence on the transfection activity [43]. Transfection studies demonstrated significantly higher transfection efficiency of biocleavable polyrotaxanes compared to its non-degradable counterpart, though the transfection efficiency was still much lower than 22 kDa LPEI-based formulation.

Oupický et al. recently reported an elegant approach to prepare reducible PDMAEMA polymers [44]. The reversible addition–fragmentation chain transfer (RAFT) polymerization of 2-dimethylaminoethyl methacrylate (DMAEMA) using a bifunctional chain transfer agent, 1,4-bis(2-(thiobenzoylthio)prop-2-yl)benzene (BTBP), yielded α, ω-dithioester functionalized PDMAEMA with a tailored molecular weight (M_n = 3900–5500 Da) and a low polydispersity (PDI = 1.06–1.09). The aminolysis of α, ω-dithioester PDMAEMA afforded α, ω-dithiol-functionalized PDMAEMA, which upon oxidative polycondensation successfully gave a reducible PDMAEMA with a nearly 10-fold increase in molecular weight and with multiple disulfide bonds in the backbone. In comparison with the non-reducible PDMAEMA control, polyplexes based on this reducible PDMAEMA showed a comparable or enhanced transfection activity while exhibiting only minimum toxicity in a panel of cell lines. It should be noted, however, that the molecular weight of these reducible PDMAEMA polymers are relatively low (M_n = 16,700 and 53,000). It has been reported that the transfection efficiency of PDMAEMA is highly dependent on its molecular weight and the optimal M_w is approximately 300,000 [45]. Therefore, the transfection efficiency of reducible PDMAEMA could in principle be further improved by increasing its molecular weight. With the same method, the authors have also prepared high molecular weight reducible linear polystyrene and poly(acrylic



Scheme 4. Chemical structure of biocleavable polyrotaxane (a), image of the polyplex formation and terminal cleavage-triggered decondensation of the polyplex (b) [41].

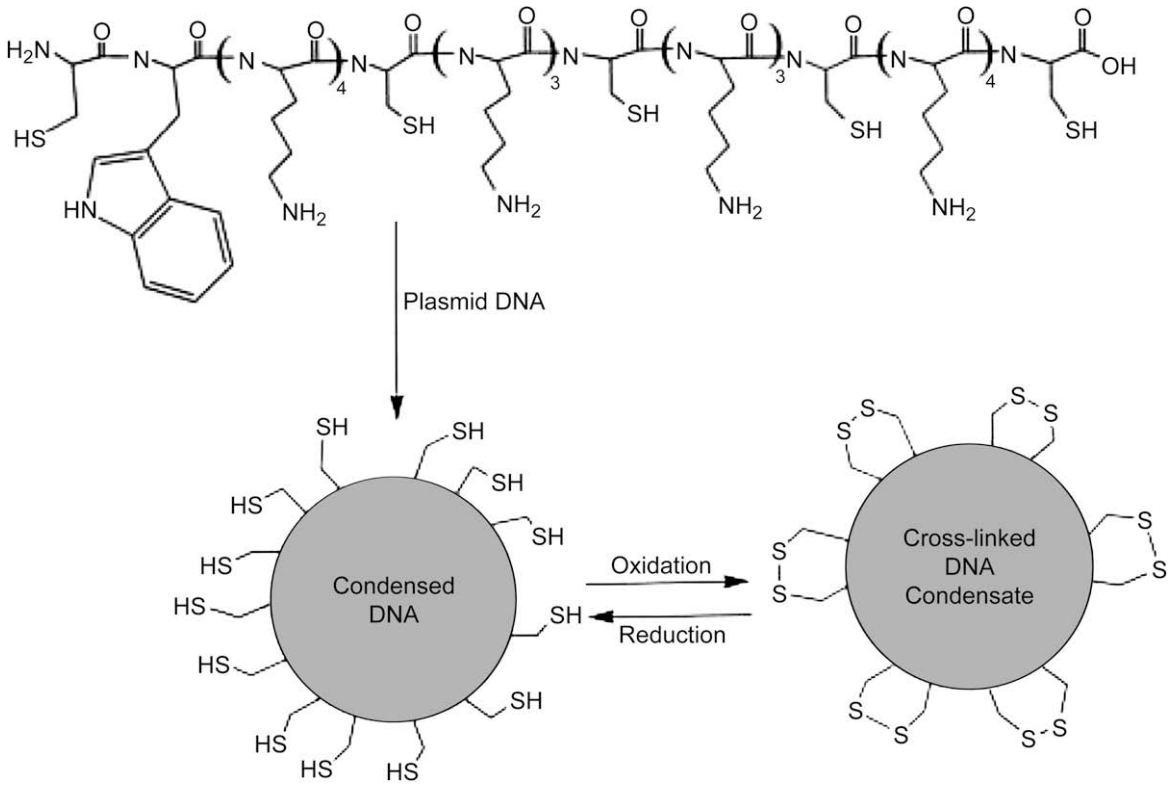
acid) [46] as well as temperature- and redox-responsive multiblock copolymers of poly(*N*-isopropylacrylamide) (PNIPAM) and PDMAEMA [47].

3. Reduction-sensitive polypeptides and proteins

Rice and coworkers developed low molecular weight DNA condensing polypeptides by substituting one to four lysine residues of Cys-Trp-Lys_{18} (CWK_{18}) with cysteine groups (Scheme 5) [48]. These polypeptides could spontaneously oxidize to form inter-peptide disulfide crosslinks after binding to plasmid DNA, resulting in small stabilized DNA condensates. These reversibly cross-linked polypeptide DNA condensates showed a significant enhancement in gene expression, i.e. a 2.5- to 10-fold increase in COS-7 cells and a 4- to 60-fold increase in HepG2 cells as compared to the uncross-linked alkylated CWK_{18} ($AlkCWK_{18}$) DNA condensates. In a following study, it was found that a minimal peptide sequence of four Lys and two terminal Cys residues, i.e. Cys-$(Lys)_4$-Cys, could also yield reversibly stabilized compact DNA condensates that gave high levels of gene expression in HepG2 cells [49]. Moreover, the substitution of Lys residues with His residues for these reversibly cross-linkable polypeptides (e.g. Cys-His-$(Lys)_6$-His-Cys) provided sufficient buffering capacity and yielded enhanced *in vitro* gene expression without aid of chloroquine, an agent that promotes endosomal escape. The thiol cross-linking polypeptides were further derivatized with either an *N*-glycan or PEG [50]. *In vivo* transfection studies in mice showed that ^{125}I-DNA complexed with a mixture of 10 mol% triantennary glycopeptide, 5 mol% PEG–peptide, and 85 mol% backbone peptide resulted in optimal targeting to hepatocytes. However, rapid disulfide bond reduction in liver hepatocytes followed by peptide metabolism limited the metabolic half-life of thiol cross-linked DNA condensates *in vivo*. The half-life of DNA in liver could be extended from 1 to 3 h using a backbone peptide composed of D-amino acids. Midoux et al. designed and synthesized a 3-(2-aminoethyldithio) propionyl-substituted polylysine conjugate (DP = 190), poly[Lys-(AEDTP)], in which each amino group was linked to the polymer backbone via a disulfide bond (Scheme 3) [51]. The results showed that poly[Lys-(AEDTP)] was capable of condensing pDNA into about 100 nm particles at an N/P ratio of 2.8, and the polyplexes were rapidly dissociated in the presence of a reducing agent such as dithiothreitol (DTT) and glutathione. The polyplexes of poly[Lys-(AEDTP)] revealed a significantly enhanced transfection efficiency as compared to those of the parent polylysine (10 and 50-fold more efficient in 293T7 and HepG2 cells, respectively).

Giammona et al. prepared a thiopolycation, PHEA–EDA–SH–CPTA (PESC) in four steps, i.e. functionalizing α, β-poly(*N*-2-hydroxyethyl)-D,L-aspartamide (PHEA) with ethylendiamine (EDA), treating PHEA–EDA with SPDP, reacting with 3-(carboxypropyl)-trimethylammonium chloride (CPTA), and treating with DTT [52]. PESC thiopolycation could efficiently complex with DNA and revealed low cytotoxicity. The polyplexes of PESC mediated notable transfection activity, though the transfection efficiency was 1–2 orders of magnitude lower than 22 kDa lPEI-based systems.

The success of targeted gene delivery *in vivo* demands the polyplexes to be stable in the blood circulation but subject to rapid dissociation inside the cells. Highly water soluble polypeptide DNA condensates were obtained using non-reducible PEG-VS-CWK_{18} and reducible PEG-*SS*-CWK_{18} conjugates [53]. Both PEG–peptides could condense plasmid DNA into small-sized particles with a mean diameter of 80–90 nm and zeta potential of +10 mV. Interestingly, the *in vitro* gene transfer studies using HepG2 cells showed that PEG-VS-CWK_{18} DNA condensates mediated gene expression over two orders of magnitude lower than PEG-*SS*-CWK_{18} and three orders of magnitude lower than $AlkCWK_{18}$ DNA condensates. The partial reduction of disulfide bonds over time resulting in removal of PEG and formation of CWK_{18} DNA condensates in situ was suggested to account for the difference in gene expression between PEG-*SS*-CWK_{18} and PEG-VS-CWK_{18} DNA condensates. Oupický et al. reported a combination of steric and reversible stabilization strategy to address the contradictory requirements [54]. In their work, linear reducible polycations (RPCs) (M_w = 187 kDa or 45 kDa) were prepared by oxidative polycondensation of Cys$(Lys)_{10}$Cys peptide. The polyplexes of RPCs were coated by reacting with a hydrophilic copolymer of *N*-(2-hydroxypropyl) methacrylamide with methacryloylglycylglycine 4-nitrophenyl ester (PHPMA), which resulted in particles with a negative surface charge of −10 mV and suppression of salt-induced aggregation. Polyelectrolyte exchange studies further confirmed that surface coating with PHPMA resulted in resistance to polyelectrolyte exchange reactions with poly-L-aspartic acid (PAA). However, treatment of the coated complexes of RPCs with



Scheme 5. Formation of cross-linked peptide DNA condensates. Peptide DNA condensates are formed instantly through ionic binding of the peptide to the DNA backbone followed by interpeptide oxidization to form disulfide bonds that reversibly stabilize the DNA condensates [48].

about 2.5 mM DTT enabled release of free DNA upon incubation with PAA. The *in vitro* transfection in human retinoblast 911 cells showed over 10 times higher transfection activity of coated RPC/DNA complexes as compared to analogous PHPMA coated polyplexes of non-reducible PLL, validating the concept of triggered intracellular activation of DNA delivery vectors via the intracellular reduction of disulfide bonds. It should be noted, nevertheless, that the general transfection efficiency of the coated RPC polyplexes is low as compared to PEI formulations, which is presumably due to inefficient endocytosis and endosomal escape. The addition of the cationic lipid (*N*-(1-(2,3-dioleoyloxy)propyl)-*N*,*N*,*N*-trimethylammonium chloride) (DOTAP) to RPC polyplexes produced an over 10,000-fold increase in gene expression in HeLa cells, which was 10-fold higher than DOTAP/DNA lipoplexes and over100-fold higher than RPC/DNA polyplexes used in combination with chloroquine [55]. Flow cytometry analysis confirmed that high percentages of cells were transfected by RPC/DNA polyplexes in combination with DOTAP, e.g. 51.5 ± 7.9% for HeLa, 55.2 ± 6.7% for LNCaP, and 66.1 ± 3.7% for PC-3 cells, respectively. The delivery of DNA expressing the ntr gene by RPC/DOTAP sensitized human cancer cells for the prodrug CB1954 led to remarkably high degree of cell killing, achieving IC50 values similar to those previously attained with adenoviral vectors. More recently, Oupický et al. reported that redox-sensitive polyplexes based on reducible RPC with molecular weight ~30 kDa exhibited selectively enhanced activity in MCF-7 mammary carcinoma cells over expressing Bcl-2 compared to the non-reducible polyplexes [56]. Bcl-2 is an anti-apoptotic protein that is overexpressed in multiple human cancers. Bcl-2 overexpression resulted in increased levels of intracellular GSH, which boosted the transfection activity of redox-sensitive polyplexes via facilitating intracellular unpacking of polyplexes. This study indicated that redox-sensitive vectors may be exploited to achieve enhanced selectivity of transfection in Bcl-2 over-expressing tumors *in vivo*.

Histidine-rich RPCs combining pH buffering endosomal escape mechanisms with rapid unpackaging following reduction in the cytoplasm were prepared by oxidative polycondensation of the peptides Cys-His_3-Lys_3-His_3-Cys and Cys-His_6-Lys_3-His_6-Cys, denoted as HIS3 RPC and HIS6 RPC, respectively [57]. Without the additional endosomolytic agent chloroquine, HIS6 RPC mediated efficient cytoplasmic delivery of a broad range of nucleic acids, including plasmid DNA, mRNA and siRNA molecules both in rapidly dividing cells and in primary cultures of post-mitotic cells. It was found that the molecular weight and rate of cleavage of HIS RPCs were important factors in determining transfection activity. The siRNA delivery using HIS6 RPC revealed that unlike DNA transfection efficiency increasing with polymer size, 80 k HIS6 RPC formed more compact siRNA complexes and higher inhibition activity than 162 k HIS6 RPC [58]. Incorporation of a targeting peptide specific for hepatocytes from the Plasmodium falciparum circumsporozoite (CS) protein onto His6 RPCs significantly improved transfection of plasmid DNA and siRNA activity in hepatocytes, but not in most non-liver cells.

Park and coworkers prepared reducible fusogenic peptides (cl-KALA) by crosslinking KALA peptides having two terminal cysteine residues [59]. KALA is an amphipathic, cationic and fusogenic peptide known to effectively destabilize cellular membranes. The results revealed that cl-KALA formed more compact and stable complexes with siRNA than the parent KALA peptide and PEI, most likely due to its increased charge density. In a 10% serum-containing medium, cl-KALA/siRNA–PEG complexes exhibited significantly higher gene inhibition as compared to those of KALA and PEI.

Tat peptides are cationic cell-penetrating peptides frequently used to enhance the cellular uptake of a large variety of molecules

such as drugs and proteins. However, the application of Tat peptides in DNA delivery is limited by the inability to release DNA in endosomes and the instability of Tat/DNA complexes. Wang and Lo incorporated histidine and cysteine residues in the Tat sequence that combines in one single peptide multiple functions critical to efficient gene transfection such as the cell-penetrating property of the Tat peptide, the DNA binding ability of arginine and lysine residues, the endosomolytic effect of histidine residues, and the stabilizing effects of disulfide bonds formed by the cysteine residues [60]. The Tat peptide covalently fused with 10 histidine residues (Tat-10H) revealed 7000-fold improvement in gene transfection efficiency over the original Tat peptide. Further modification with two cysteine residues resulted in a greatly enhanced transfection activity, with the transfection efficiency of C-5H-Tat-5H-C peptide reaching that of 25 kDa bPEI.

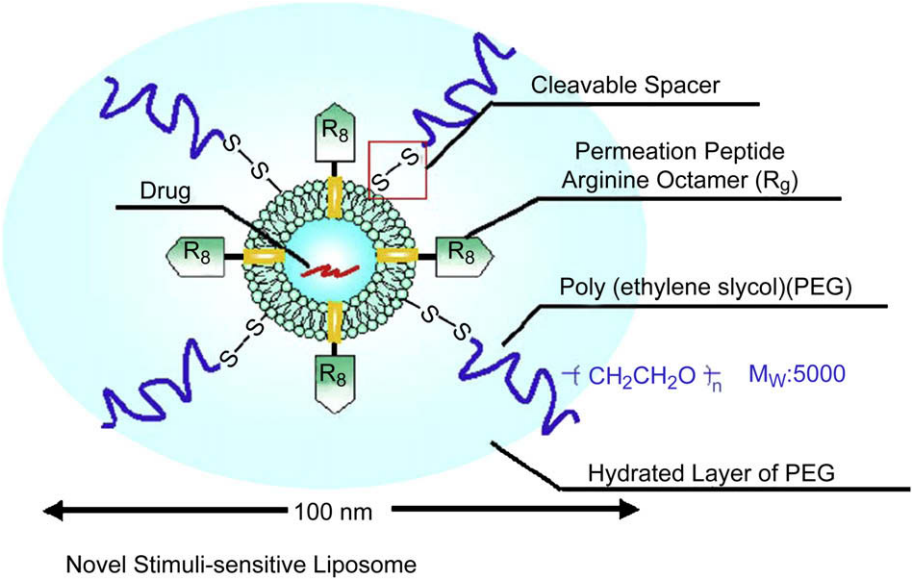
Hubbell and coworkers designed ABC triblock copolymers of PEG, poly(propylene sulfide) (PPS), and a positively charged peptide (PEG–PPS–peptide), wherein selected cationic peptides, TAT peptide domain of HIV (RKKRRQRRR) or an oligolysine (Lys_9), were coupled to the PEG–PPS through a disulfide bond [61]. The resulting triblock copolymers were able to self-assemble with siRNA to form complexes with particle sizes ranging from 171 to 601 nm depending on charge ratio and hydrophobic PPS block length. These ABC triblock copolymers demonstrated enhanced biocompatibility compared to the commercially available cationic liposome LF2000. PEG_{45}-PPS_5-K_9 at higher siRNA doses was able to mediate 90% gene expression down-regulation.

Oupický et al. have prepared a set of multiblock reducible copolypeptides, which are composed of a histidine-rich peptide (HRP, CKHHHKHHHKC, M_r 1431) and a nuclear localization sequence (NLS, CGAGPKKKRKVC, M_r 1274), by random oxidative polycondensation [62]. These reducible copolypeptides had M_w values ranging from ~60,000 to ~200,000, as determined by size exclusion chromatography (SEC), and were able to efficiently condense DNA into polyplexes with sizes and zeta potentials similar to those of 25 k bPEI. They had minimal toxic effects as compared to the PEI control, which was ascribed to a reductive intracellular and plasma membrane degradation, a lower charge density and higher chain rigidity of the reducible copolypeptides. The transfection activity of the copolypeptides in all tested cell lines increased with increasing HRP/NLS ratios as a result of an enhanced buffer capacity. Nevertheless, the NLS sequence appeared to have little effect on the transfection activity of the polyplexes. This apparent lack of NLS effect on the transfection activity might be due to that prior to the nucleus transportation, the NLS peptides have already been dissociated from the polyplexes in the reducing environment of cytosol by the cleavage of the disulfide bonds.

Uludag and coworkers conjugated bisphosphonates (BPs), a class of molecules with an exceptional affinity to bone mineral hydroxyapatite (HA), to fetuin (a model protein) via cleavable disulfide linkages [63]. The fetuin–BP conjugates with 3.3–4.9 BPs/protein exhibited strong (>70%) HA bonding and were stable under aqueous conditions. However, in the presence of physiological thiols such as L-cysteine, DL-homocysteine, and L-glutathione, the conjugates were readily cleaved, resulting in the release of fetuin from the mineral. The disulfide-linked protein–BP conjugates are interesting candidate for effective therapeutic treatment of bone diseases.

4. Reduction-sensitive liposomes and polymersomes

Zalipsky et al. employed the reversible nature of disulfide bonds to develop liposomes with detachable PEG coatings [64,65]. The first generation of detachable liposomes was prepared from dioleoylphosphatidylethanolamine (DOPE) and 3–6 mol% of a poly(ethylene glycol)–phospholipid conjugate with 3,3′-dithiodipropionate (DTP) as the linking moiety (mPEG–DTP–DSPE) (Scheme 6) [64]. DOPE/mPEG–DTP–DSPE liposomes were stable in plasma whereas in the presence of a reducing agent the PEG coating was effectively detached off the liposomal surface, leading to vesicle destabilization and fusion as well as complete release of the entrapped contents. However, a relatively potent thiolytic agent such as DTT was required, and the disulfide cleavage yielded a modified phospholipid, 3-mercaptopropionyl-DSPE. In a subsequent paper by the same authors, an mPEG–DSPE conjugate containing an *o*- or *p*-dithiobenzyl carbamate (DTB–urethane) linkage moiety (mPEG–DTB–DSPE) was designed [65]. Interestingly, these new lipopolymers were cleavable in the presence of cysteine and phosphatidylethanolamine lipid was regenerated in its unmodified form. It was found that small yet clear decomposition of the



Scheme 6. A liposomal carrier possessing membrane-permeable ligands and a detachable coating for the intracellular delivery of drugs and genes [66].

mPEG–DTB–DSPE/DOPE liposomes took place in 1 h at 15 μM cysteine, which is the average plasma concentration. These "stealthed" liposomes, therefore, could in principle circulate in the bloodstream for a sufficient period of time, allowing systemic distribution *in vivo* and/or accumulation in a specific site either passively or through ligand-mediated targeting.

Fujimoto et al. designed a reduction-sensitive liposome which contains a detachable poly(ethylene glycol) (PEG) coating and an octamer of arginine (R8 peptide) as a membrane-permeable ligand (Scheme 6) [66]. Liposomes were prepared from a mixture of dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylethanolamine (DOPE), PEG-S–S-DOPE, and cholesteryl hemisuccinate (CHEMS). The R8 peptides were covalently immobilized onto the CHEMS portion of the liposome surface and were shielded by a thiolytic detachable PEG coating. It was assumed that upon reaching the site of action, some of the PEG-S–S-DOPE chains would be subject to thiolytic cleavage in response to a weak reductive trigger, exposing the R8 peptides and facilitating endocytosis. Further cleavage of the disulfide bonds can be accomplished in the cytosol by the cellular glutathione, which leads to the rupture of the liposomes and subsequent release of the loaded drugs. When L-cysteine was added to a mixture of cells and liposomes that incorporated plasmids encoding the enhanced green fluorescence protein (pEGFP), the expression of EGFP was low, but was observed in almost 100% of the cells.

Ravoo and coworkers have designed and prepared a family of amphiphilic cyclodextrin derivatives in which hydrophobic substituents (e.g. C_8H_{17}, $C_{12}H_{25}$, $C_{16}H_{33}$) were grafted to the primary side of the cyclodextrin molecules via a disulfide linkage and oligo(ethylene glycol) moieties were grafted to the secondary side [67]. These amphiphilic cyclodextrins formed vesicles or nanoparticles in water, which disintegrated upon the addition of DTT resulting in release of a model hydrophobic guest from the nanoparticles.

Balakirev et al. developed reducible polymers of cationic amphiphiles based on lipoic acid, which in the oxidized state condensed DNA into homogeneous spherical particles, but upon reduction swelled into DNA toroids with subsequent release of free DNA [68]. *In vitro* transfection studies showed a several fold increase of transgene expression compared with DOTAP, which could be further enhanced by attachment of a nucleus-targeting peptide to the amphiphile.

Behr and coworkers synthesized a cationic biscysteine detergent (C_{12}CCP) by amide bond formation between dodecanoic acid, cysteinyl-cysteine, and diaminopropane [69]. Interestingly, plasmid DNA was condensed to 32 nm small and neutral particles at an N/P ratio of 1/1 followed by oxidation, which corresponds to the size of monomolecular condensation of plasmid DNA. These monomolecular DNA complexes were more stable towards exchange with extracellular polyanions such as glycosaminoglycans, as compared to those formed with other gene delivery agents. The DNA complexes were dissociated, though slowly, when incubated with an excess amount of phosphatidylserine, a ubiquitous intracellular anionic lipid. Fast release of DNA was observed when exposed to 1 mM DTT, though no release occurred at 10 mM glutathione. The *in vivo* studies in mice showed that 25% of the complexes were still circulating after 30 min in a form not bound to blood cells, which contrasts with 2% for other cationic lipid vectors.

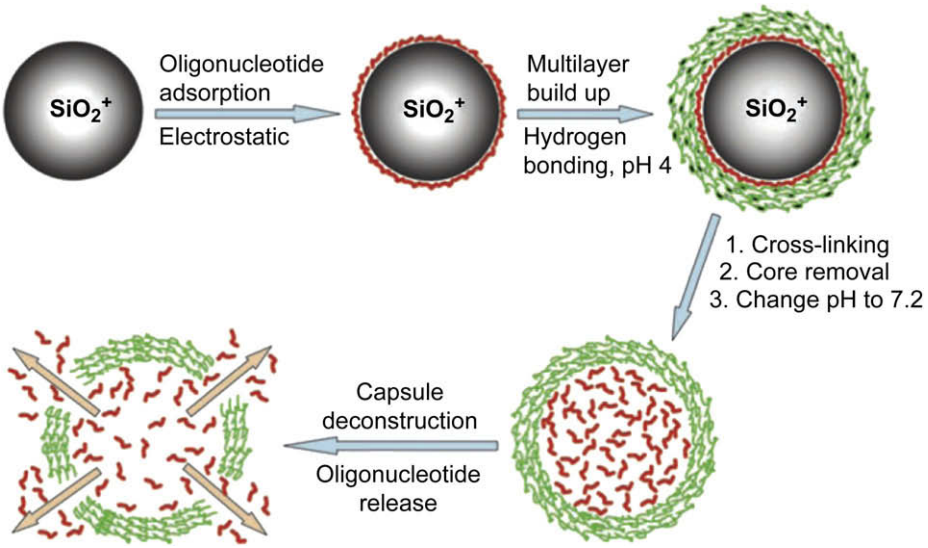
Zuber and coworkers developed an amphiphile consisting of cholesterol linked to carboxy-spermine by cysteine (CholCSper), used in formulation with DOPE to prepare an intracellular protein delivery system [70]. The dimerized lipid formulation was shown to carry efficiently phycoerythrin (a 240 kDa fluorescent model protein) into cells over a long period of time, changing the delivery characteristics from a 'burst' to a prolonged release.

Hubbell and coworkers prepared reduction-sensitive polymersomes based on a block copolymer of PEG and poly(propylene sulfide) (PPS) with an intervening disulfide, PEG_{17}-*SS*-PPS_{30} [71]. The resulting polymersomes were shown to be rapidly disrupted in the presence of intracellular concentrations of cysteine. Cell uptake and release studies using mouse macrophages revealed a fast cellular uptake and disruption of polymersomes in early endosomes, leading to rapid release of encapsulated calcein within the endosome. Interestingly, PEG_{17}-*SS*-PPS_{30} block copolymers after reduction were capable of endosomal disruption, resulting in release of calcein into the cytoplasm, though the exact mechanism of endosomal disruption is not clear.

5. Reduction-sensitive microcapsules

Biodegradable polymeric microcapsules have attracted much attention for a variety of biomedical applications such as drug delivery, gene delivery, and delivery of diagnostics. The microcapsules can be properly engineered with a controlled size, shape, composition, permeability, and functionality. Ideally, as delivery vehicles, capsules should be biocompatible and stable in the circulation, while after localizing at the pathological site, they should be subject to rapid disassembly in response to a particular physical or chemical stimulus and efficiently deliver their encapsulated contents. Based on reversible thiol–disulfide chemistry, Caruso et al. constructed monodisperse, single-component, reversibly cross-linked polymer microcapsules [72,73]. The capsules were composed solely of poly(methacrylic acid) (PMA) held together through biodegradable disulfide linkages. The PMA capsules were prepared by layer-by-layer (LBL) deposition of 18 mol% thiolated poly(methacrylic acid) (PMASH) and poly(vinylpyrrolidone) (PVPON) onto silica particles, followed by controlled oxidation of thiol groups in the PMASH leading to disulfide crosslinking of PMA. After removal of the silica particles and PVPON by altering the solution pH to disrupt hydrogen bonding between PMASH and PVPON (Scheme 7), microcapsules were obtained. These capsules were sufficiently stable at physiological conditions, exhibited reversible swelling in response to changes in external pH, and degraded rapidly in the presence of a reducing agent such as DTT and glutathione. Interestingly, by using amine-functionalized SiO_2 particles (zeta potential of 62 ± 4 mV at pH 4) as templates, DNA with varied sizes and conformations were readily encapsulated within the monodisperse degradable microcapsules [74,75]. These DNA capsules exhibited the following features: (i) attained high loadings of oligonucleotides with more than 10^4 chains per capsule; (ii) showed quantitative incorporation of oligonucleotides from the starting solution with more than 90% of the capsules filled with the DNA; (iii) avoided the use of mechanical forces, such as those typically applied in emulsion encapsulation processes, which may cause DNA degradation; and (iv) had adequate stability at physiological conditions while achieving rapid release of DNA in a reductive environment analogous to that of the cytosol and the cell nucleus. The released DNA maintained its functionality and structural integrity as revealed by DNA hybridization assays and enzymatic reactions. These DNA-loaded polymer microcapsules hold promise as delivery vehicles for gene therapy and diagnostic agents [76].

Park and coworkers, using disulfide cross-linked hyaluronic acid (HA) microgels as degradable template core materials, fabricated shell cross-linked hollow microcapsules composed of HA and poly-L-lysine (PLL) (Scheme 8) [77]. These shell cross-linked microcapsules displayed good stability against freeze–thaw cycles and acidic pH conditions. Large amounts of bioactive macromolecular drugs such as proteins, peptides, and genes could readily be encapsulated, which is often a hurdle when using conventional organic and inorganic core materials. Interestingly, the microcapsule wall showed pH-responsive permeability, in which the permeability



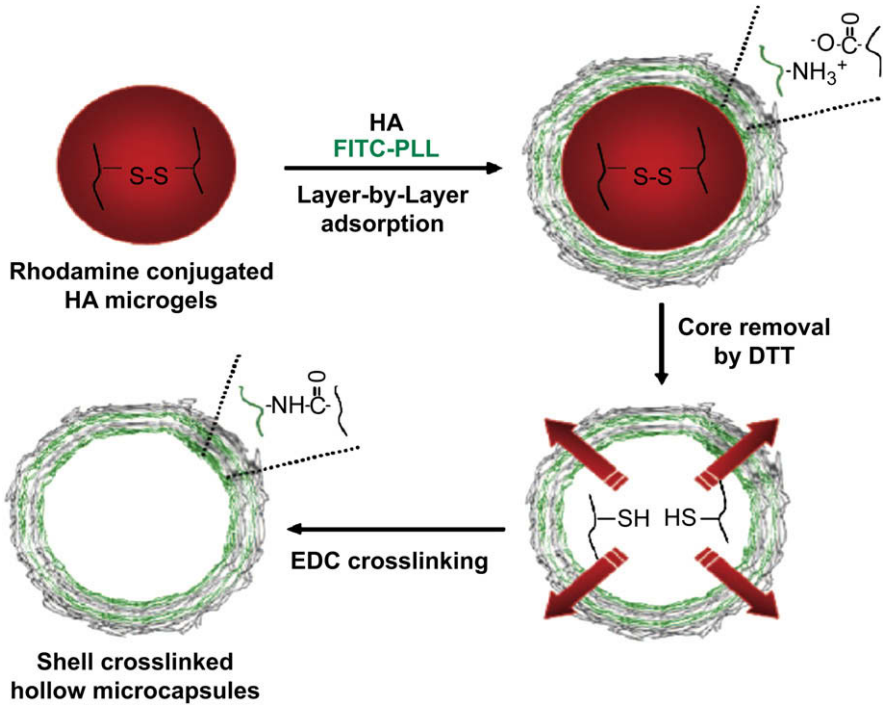
Scheme 7. Schematic representation of the encapsulation of short oligonucleotide sequences within polyelectrolyte capsules [74].

was higher at acidic conditions than at neutral pH. Fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA), a model protein drug, which was conveniently loaded into the microcapsules at low pH, was released in a controlled manner at neutral pH or in the presence of hyaluronidase. These hollow microcapsules, due to their good biocompatibility and biodegradability *in vivo*, are very interesting for the delivery of various macromolecular drugs.

6. Reduction-sensitive multi-layered thin films

Huang et al. reported on the fabrication of reductively degradable thin films through layer-by-layer (LBL) assembly of a plasmid DNA and a reducible linear poly(amido amine) prepared from Michael addition polymerization of 1,4-diaminobutane and CBA [78]. The thickness of the films was accurately controlled by the polymer/DNA bilayer numbers. Remarkably, the films were very stable in PBS, but were peeled off layer-by-layer in the presence of DTT, leading to a sustained release of DNA. The rate of DNA release depended on DTT concentration and time, wherein about 75% of the DNA in the film was released in 216 h in the presence of 1 mM DTT, 85% of the DNA in 24 h in 2.5 mM DTT, and 95% of the DNA in 3 h in the presence of 5 mM DTT.

Oupický et al. reported on the LBL films assembled from plasmid DNA and reducible TAT-based polypeptide (PTAT) with disulfide bonds in the backbone for triggered release of DNA in a reducing microenvironment (Scheme 9) [79]. Similar to the non-reducible



Scheme 8. Schematic illustration for fabrication of cross-linked hollow LBL HA/PLL microcapsules [77].

poly(L-lysine)/DNA films, the thickness of PTAT/DNA films exhibited an exponential growth with respect to PTAT/DNA bilayer numbers. In the presence of DTT, the thickness of the PTAT/DNA film decreased exponentially within the first 5 h followed by a linear decrease of thickness, suggesting that the particulate complexes on the surface were removed first. In contrast, the thickness of the non-reducible poly(L-lysine)/DNA film remained constant throughout the entire 24 h. Bioreducible LBL films of plasmid DNA were also assembled on flexible stainless steel substrate using reducible hyperbranched poly(amido amine) obtained from Michael addition copolymerization of AEP with a mixture of bisacrylamide monomers CBA and *N*,*N*′-methylenebisacrylamide (MBA) (poly(CBA/MBA–AEP)) [80]. The *in vitro* transfection in NIH-3T3 cells and smooth muscle cells using reporter plasmids encoding secreted alkaline phosphatase (SEAP) showed that DNA/poly(CBA/MBA–AEP) films provided higher levels of transgene expression than the control DNA/PEI films. Furthermore, the duration of the transfection in NIH-3T3 cells from the reducible films was significantly longer than that provided by the non-reducible DNA/PEI films (8 vs. 3 days). These results indicated a sustained release of DNA from the reducible films over time, which is likely caused by reduction of disulfide bonds by the mild reducing microenvironment of plasma membrane of cells growing on the LbL films. The *in vivo* transfection in rats showed that the plasma levels of SEAP peaked at ~160 ng SEAP/mL five days post-implantation of a stainless steel substrate coated with the reducible films. These bioreducible thin films capable of sustained release of plasmid DNA from their surface may have great potential in local gene delivery and for tissue engineering applications.

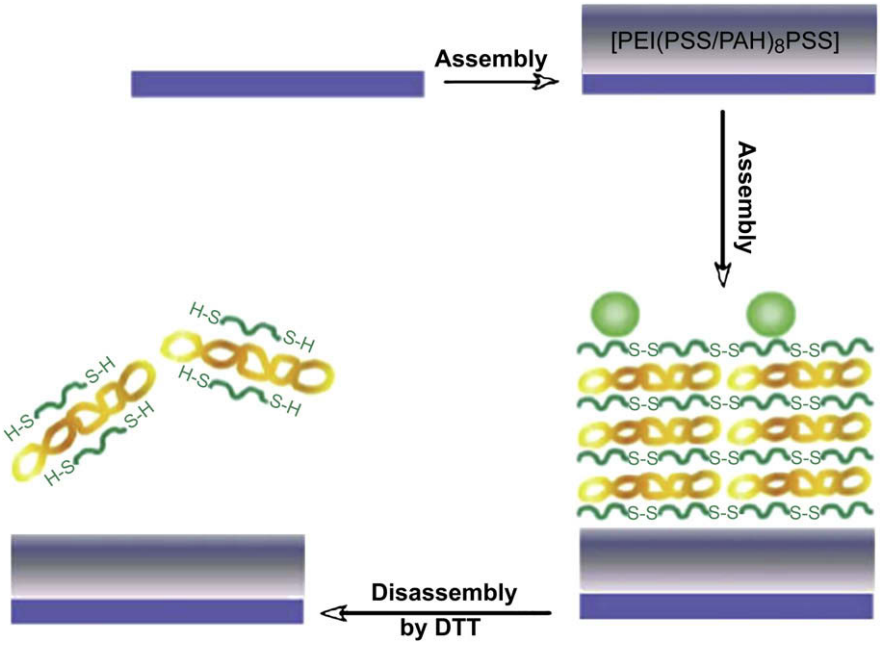
7. Reduction-sensitive polyion complex micelles (PIC micelles)

Kataoka and coworkers designed reversibly core-crosslinked PIC micelles from partially thiolated PEG–PLL block copolymers and poly(α,β-aspartic acid) (PAA) [81]. The crosslinked PIC micelles showed excellent colloidal stability even at 0.5 M NaCl, whereas the addition of 2 mM DTT resulted in rapid dissociation of micelles. The rate of micelle dissociation intimately depended on the DTT concentration. This study provides a first proof of principle for the reversible stabilization of PIC micelles by disulfide crosslinks. In a subsequent study, reversibly core-crosslinked micelles were obtained from thiolated PEG–PLL block copolymer and antisense oligonucleotide (asODN) [82]. As revealed by polyanion exchange experiments, these PIC micelles with a diameter of approximately 40 nm had a high colloidal stability, likely due to the presence of PEG shell and core-crosslinking. Importantly, the ODN in the core cross-linked micelles displayed significantly improved resistance against nuclease degradation compared to the free ODN and non-crosslinked micelle formulation.

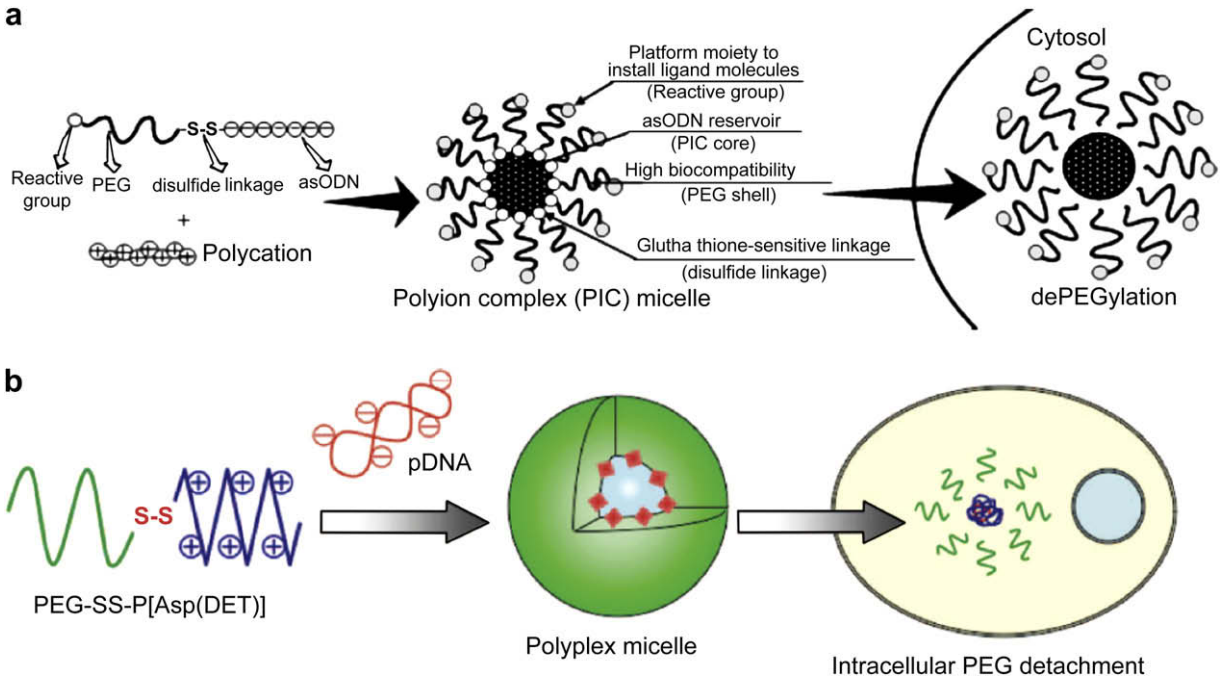
Reducible polyion complex (PIC) micelles based on a poly(ethylene glycol)-*SS*-antisense oligodeoxynucleotide conjugate (PEG-*SS*-asODN) were developed by Kataoka and coworkers for the cytoplasmic delivery of antisense oligodeoxynucleotides (Scheme 10a) [83]. The glutathione-sensitive PIC micelle system using 25 kDa bPEI as the counter polycation facilitated specific intracellular dePEGylation and achieved high levels of antisense effect (48% inhibition) against luciferase gene expression in HuH-7 cells (hepatocytes), which is far more efficient than controls (asODN alone or PEG-*SS*-asODN alone), as well as the non-reducible PIC micelle system. This study suggests that the thiolytic cleavages of disulfide linkage in the cytoplasm may be crucial to induce an efficient release of the active asODN from the PIC core.

Park and coworkers conjugated vascular endothelial growth factor (VEGF) siRNA to PEG via a disulfide linkage (siRNA–PEG), which formed PIC micelles by condensing with PEI or KALA (a cationic fusogenic peptide) [84,85]. The PIC micelles showed greater stability than naked VEGF siRNA against enzymatic degradation. The intact form of VEGF siRNA was released under a reductive condition mimicking the cytosolic environment by cleavage of the disulfide linkage. Remarkably, under an optimized formulation condition, the VEGF siRNA–PEG/PEI PIC micelles effectively silenced VEGF gene expression in prostate carcinoma cells (PC-3) for up to 96.5%. Importantly, it should be noted that high level of VEGF gene silencing was also achieved in the presence of 10% FBS.

Reducible hyaluronic acid (HA)–antisense oligodeoxynucleotide (asODN) conjugates (HA-*SS*-asODN) were designed by Park and



Scheme 9. Assembly and disassembly of reductively degradable PTAT/DNA LbL films [79].



Scheme 10. Schematic illustration of glutathione-sensitive PIC micelle formation and intracellular dePEGylation [83,88].

coworkers to increase the negative charge density of asODN for the efficient complexation with polycations [86]. Dynamic light scattering (DLS) analysis demonstrated that the HA–asODN conjugates formed significantly more stable and compact complexes with protamine (~200 nm in diameter) than free asODN (~800 nm in diameter). Polyanion exchange studies revealed that in order to destabilize the protamine complexes HA–asODN required a 5-fold higher heparin concentration than the free ODN based systems. The protamine/HA–ODN complexes formulated at a weight ratio of 1.5 and 6 showed a far greater inhibition efficiency of GFP expression than the protamine/ODN complexes, which was comparable to that of the cytotoxic PEI/ODN complexes. HA and protamine are naturally occurring biocompatible materials, and the current formulation based on a cleavable conjugation strategy of ODN to HA could be potentially applied as safe and effective non-viral carriers for ODN and siRNA nucleic acid therapeutics.

PEO-*b*-PAA block copolymers with a single disulfide linkage between two blocks were prepared using cystamine as building block for the solid phase supported synthesis of a monodisperse, sequence-defined PAA block [87]. The synthesized cationic block copolymer, PEO_{60}-Lys-(Suc-Cya-Lys_2)-(Suc-Damp-Lys_2)$_4$, could condense plasmid DNA into nano-sized particles at an N/P ratio of 10/1. The reductive removal of the sterically stabilizing PEO resulted in more compact polyplexes with a higher positive net charge.

Kataoka and coworkers prepared a block catiomer, PEG-*SS*-P[Asp(DET)], which contained biocleavable disulfide linkage between PEG and polycation segment, to trigger PEG detachment, as well as a cationic segment based on polyaspartamide with a flanking *N*-(2-aminoethyl)-2-aminoethyl group (DET) to condense DNA and facilitate endosomal escape (Scheme 10b) [88]. PEG-*SS*-P[Asp(DET)] formed stable micelles with pDNA, due to the formation of hydrophilic PEG palisades. However, the PIC micelles underwent aggregation after exposure to 10 mM DTT, indicating PEG detachment from the micelles through disulfide cleavage. Interestingly, PEG-*SS*-P[Asp(DET)] micelles showed 1–3 orders of magnitude higher gene transfection efficiency and a more rapid onset of gene expression than PEG-P[Asp(DET)] micelles without disulfide linkages. CLSM images indicated that this transfection behavior of the PEG-*SS*-P[Asp(DET)] micelle could be explained by effective endosomal escape due to the PEG detachment in the endosome.

8. Reduction-sensitive nano/micro-gels and nanoparticles

Park and coworkers developed disulfide-crosslinked hyaluronic acid (HA) nanogels physically encapsulating green fluorescence protein (GFP) siRNA for tumor-targeted intracellular delivery of siRNA [89]. These HA nanogels with a size distribution from 200 to 500 nm and with siRNA loading efficiency of approximately 50% were prepared from thiol-derivatized HA via an inverse water-in-oil emulsion method followed by self-crosslinking through the formation of disulfide linkages. HA nanogels effectively protected the encapsulated siRNA from enzymatic degradation. The entrapped siRNA was released completely within 1 h in a medium with 10 mM GSH. The HA/siRNA nanogels were readily taken up by HCT-116 cells, which possess HA-specific CD44 receptors on their surface, but not by CD44 deficient NIH-3T3 cells. Gene silencing studies showed that HA nanogels encapsulating GFP siRNA exerted a significant level of GFP gene inhibition in HCT-116 cells, in both 10% serum ($62.1 \pm 6.1\%$ inhibition efficiency) and non-serum ($68.5 \pm 10.6\%$ inhibition efficiency) conditions. Stable PEG/DNA nanogels were prepared by using a thiol-functionalized six-arm branched PEG for DNA solubilization in DMSO followed by oxidative cross-linking [90]. In the presence of a reducing agent, intact plasmid DNA was released from the nanogels. The PEG/DNA nanogels exhibited appreciable gene transfection efficiency. Very recently, reducible heparin nanogels with an average diameter of 249 ± 27 nm in aqueous solution were obtained by forming nanocomplexes between thiolated heparin and PEG in DMSO followed by intermolecular disulfide crosslinking between thiolated heparin molecules by ultrasonication [91]. Under reductive environments such as in the intracellular cytosol, these heparin nanogels rapidly disintegrated and released free heparin molecules, which

significantly inhibited proliferation of mouse melanoma cells by inducing caspase-mediated apoptotic cell death.

Robust biodegradable nanogels cross-linked with disulfide linkages were prepared by inverse miniemulsion atom transfer radical polymerization (ATRP) of oligo(ethylene oxide) monomethylether methacrylate (OEOMA) and disulfide-functionalized dimethacrylate (DMA) cross-linker (Scheme 11) [92,93]. These nanogels were non-toxic to cells in the concentration range tested. Degradation studies showed that over 90% of the nanogels were degraded within 3 h in the presence of 20% glutathione in water. The biodegradation of nanogels could be employed to trigger release of encapsulated molecules, such as water soluble rhodamine 6G (R6G) and doxorubicin (Dox) [92], and rhodamine B isothiocyanate–dextran (RITC–Dx) [93]. It was further demonstrated that hydroxyl-functionalized nanogels could be prepared by introducing 2-hydroxyethyl acrylate (HEA) during inverse miniemulsion ATRP, which could subsequently be derivatized with biotin to form bioconjugates with avidin [92].

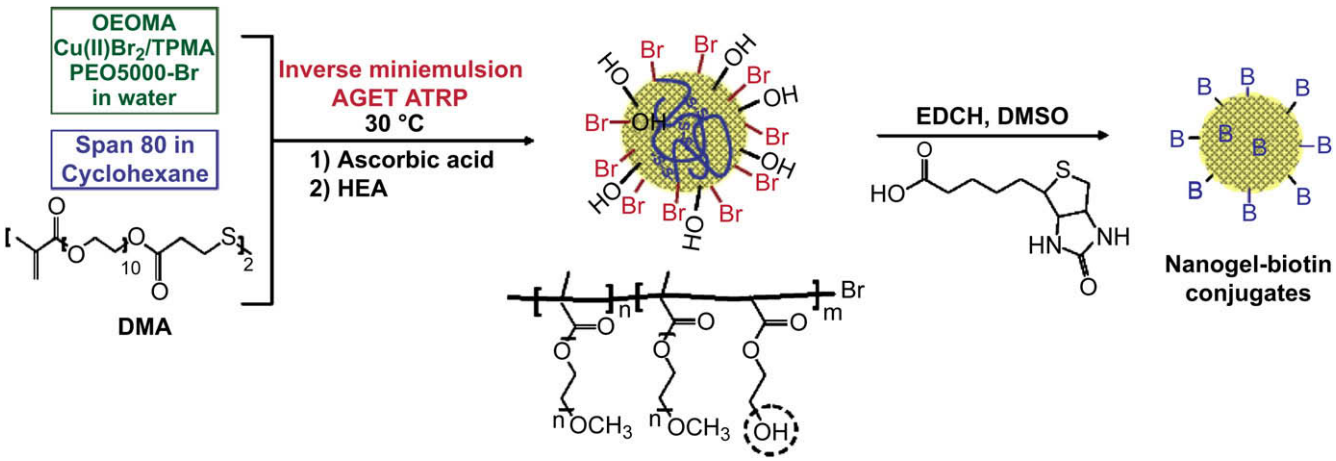
Winnik et al. prepared dual stimuli-responsive nanogels based on polysaccharides lightly grafted with thiol-terminated poly(*N*-isopropylacrylamide) (PNIPAM), which was synthesized via RAFT polymerization of NIPAM using 2-(1-isobutyl)-sulfanylthiocarbonylsulfanyl-2-methyl propionyl acid chloride conjugated pullulan followed by aminolysis [94]. Increasing the solution temperature of PNIPAM-graft-pullulan from 25 to 50 °C triggered the formation of 20–25 nm particles with a narrow size distribution. Subsequent purging with air resulted in cross-linking of PNIPAM chain ends through disulfide bond formation. The resulting nano network could be relaxed either upon cooling to room temperature or upon treatment with a reducing agent.

Moore and coworkers developed chymotrypsin and reduction responsive polyacrylamide microscopic hydrogels using a tetrapeptide sequence, Ac-Cys-Tyr-Lys-Cys-NH_2 (CYKC), as a cross-linker [95]. These microscopic hydrogels dissolved when exposed to a solution of *R*-chymotrypsin, while control hydrogels cross-linked with the tetrapeptide, Ac-Cys-Ser-Lys-Cys-NH_2 (CSKC), were not affected by *R*-chymotrypsin. Both the CYKC and CSKC cross-linked hydrogels were eroded in the presence of the disulfide reducing agent tris(2-carboxyethyl) phosphine (TCEP). Galaev et al. prepared biodegradable macroporous PHEMA cryogels by combining two crosslinkers, PEG diacrylate and a disulfide-containing water soluble crosslinker, *N*,*N*′-bis(methacryloyl)-L-cystine (MAS-S) [96]. The cryogels disintegrated into small pieces when subjected to reductive agents like DTT. The degradation time was shown to be controlled by both the content of S–S bonds and DTT concentration.

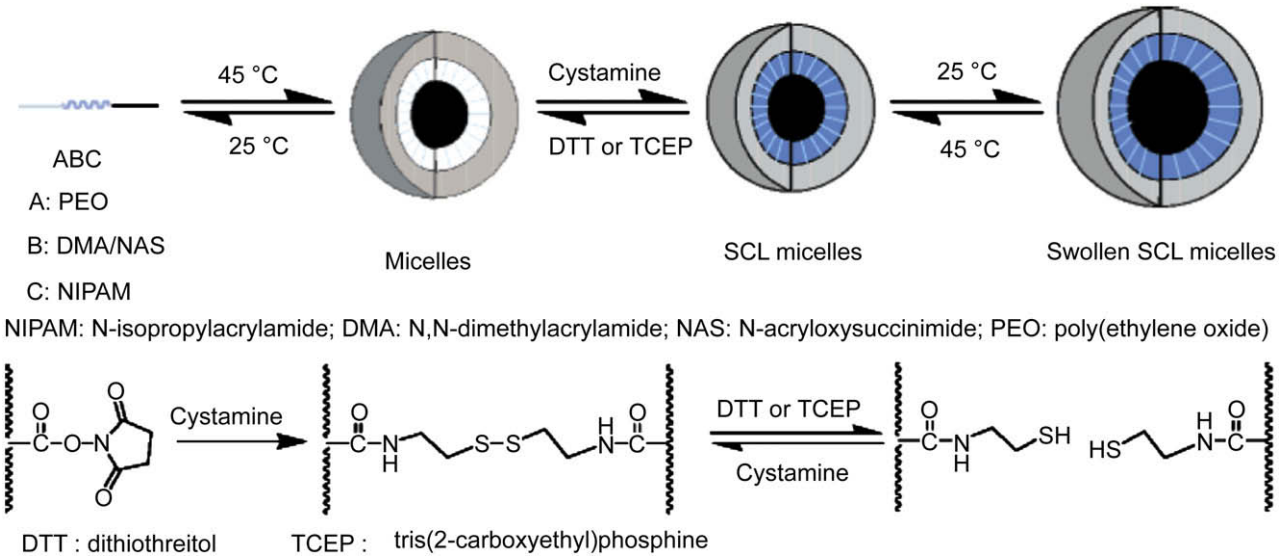
Amiji and coworkers synthesized thiolated gelatin by covalent modification of the primary amino groups of Type B gelatin using 2-iminothiolane (Traut's reagent) [97]. The thiolated gelatins prepared with 20 mg and 40 mg of 2-iminothiolane (SHGel-20 and SHGel-40) per gram of gelatin had comparable cell viability profile to that of the unmodified gelatin. *In vitro* release studies of fluorescein isothiocyanate (FITC)-labeled dextran (mol. wt. 70 kDa) showed enhanced release by about 40% of the loading in the presence of GSH. The thiolated gelatin nanoparticles (SHGel-20) carrying plasmid DNA encoding for enhanced green fluorescent protein (EGFP-N1) were highly efficient in transfecting NIH-3T3 murine fibroblast cells and showed sustained expression of GFP for up to 96 h. In a subsequent study, PEG-modified thiolated gelatin (PEG–SHGel) nanoparticles were developed as a long-circulating passively targeted delivery system that rapidly responded to intracellular GSH to enhance DNA release and transfection [98]. Bernkop-Schnurch et al. prepared chitosan-thiobutylamidine by treating chitosan with 2-iminothiolane, which formed nano-sized coacervates with pDNA [99]. These thiolated chitosan–DNA particles obtained after oxidation were rather stable, but were prone to dissociation in a reducing environment as found intracellularly, releasing about 50% of pDNA within 3 h. Transfection studies with Caco2 cells showed an improved expression of transgene as compared to chitosan based systems.

9. Reduction-sensitive micelles for controlled drug delivery

McCormick and coworkers using a RAFT polymerization approach have prepared a thermosensitive triblock copolymer PEO-*b*-P(DMA-stat-NAS)-*b*-PNIPAM, which molecularly dissolved in water at room temperature but transformed into spherical micelles with hydrodynamic diameters of 38 nm at 45 °C (Scheme 12) [100]. The shell of the micelles contained reactive *N*,*N*-acryloxysuccinimide (NAS) units, which after cross-linking with cystamine yielded reversibly shell-crosslinked (SCL) micelles. The SCL micelles could swell but did not dissociate when the solution temperature was lowered again to room temperature, indicating stabilization of the micellar structure. Furthermore, these micelles were destabilized using either DTT or tris(2-carboxyethyl)-phosphine (TCEP) and re-crosslinked using cystamine as a thiol-exchange compound. These SCL micelles were applied for sustained release of dipyridamole, in which the rate of drug release could be readily controlled by the concentration of DTT and temperature. In a similar way, Liu and coworkers synthesized a thermoresponsive double hydrophilic block copolymer, PEO-*b*-P(NIPAM-*co*-NAS),



Scheme 11. Biodegradable nanogels cross-linked with disulfide linkages [92].



Scheme 12. Schematic illustration of the formation of reversible shell cross-linked (SCL) micelles from PEO-*b*-(DMA-*s*-NAS)-*b*-NIPAM triblock copolymers [100].

which molecularly dissolved in aqueous solution at 25 °C but self-assembled into micelles at an elevated temperature of 40 °C [101]. The P(NIPAM-*co*-NAS) core was crosslinked using cystamine, yielding reversible core-crosslinked (CCL) micelles that could be de-cross-linked by DTT. The CCL micelles exhibited thermoresponsive swelling/deswelling behavior.

Thayumanavan and coworkers reported an elegant design of redox-responsive micelle systems on the basis of a surfactant molecule containing a disulfide linkage between the hydrophilic head group and the hydrophobic tail [102]. Lipophilic guest molecules such as Nile red could be easily loaded into the micelles. Interestingly, micelles disassembly kinetics in response to reducing agent DTT could be fine-tuned by mixing a redox-responsive surfactant with a suitable non-responsive co-surfactant at varying ratios, which offers a valuable means of gaining control over the release rate of encapsulated guest molecules.

Leroux and coworkers designed PEG-*b*-PDMAEMA asymmetric block copolymers exposing a thiol group at the end of the PEG chain to produce thiol-decorated micelles [103]. Anionic polymerization of ethylene oxide using *tert*-butyl mercaptan and potassium naphthalene yielded t-Bu-*S*-PEG-OH, which was subsequently converted into ATRP macro-initiator to bring about living radical polymerization of DMAEMA. The *tert*-butyl group was removed using trifluoroacetic acid in DMSO. These functional micelles displayed improved mucoadhesion through the formation of disulfide bonds with mucin and resulted in reduction-responsive micellar networks under oxidative (air) conditions through the formation of inter-micellar disulfide bonds.

10. Reduction-sensitive nanotube bioconjugates

Dai and coworkers conjugated various biological molecules, including oligonucleotides and siRNA, to phospholipid–PEG functionalized single-walled carbon nanotube (SWNT) via cleavable disulfide linkage (Scheme 13) [104]. Due to the presence of a PEG linker, these SWNT conjugates form highly stable suspensions in aqueous solutions including physiological buffers. The SWNT carriers mediated efficient delivery and release of DNA and siRNA inside cells. Gene silencing experiments using HeLa cells displayed a two-fold higher silencing efficiency compared to lipofectamine at the same siRNA concentration. This is ascribed to a high surface area of SWNT for efficient siRNA cargo loading, high intracellular transporting ability of SWNT, and high degree of endosome/lysosome escape owing to the disulfide approach.

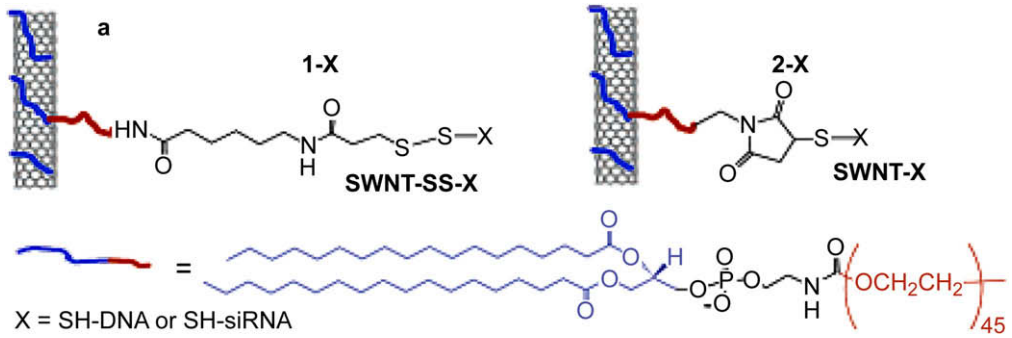
You and coworkers have prepared dual-responsive polymer–carbon nanotube conjugates by disulfide exchange reaction between thiol ended poly(*N*-isopropylacrylamide) (PNIPAAm) and pyridyldithio functionalized carbon nanotube [105]. In a follow-up study, bovine serum albumin (BSA), a model protein, was immobilized onto multi-walled carbon nanotube (MWNT) via a disulfide linkage, wherein the release of BSA from the surface of MWNT could be controlled by the concentration of glutathione [106].

11. Reducible polymeric carriers for medical imaging

Macromolecular gadolinium Gd(III) complex-based magnetic resonance imaging (MRI) contrast agents are superior to their low-molecular-weight counterparts in terms of cardiovascular and tumor imaging, due to their prolonged blood circulation and their preferential tumor accumulation. The clinical application of macromolecular gadolinium (Gd) complexes has been limited, nevertheless, by the slow excretion of Gd(III) complexes and consequent tissue accumulation of toxic Gd ions. In the past several years, the group of Lu has developed as a class of biodegradable macromolecular Gd(III) complexes by either incorporating disulfide bonds in the backbones of macromolecular Gd(III) complexes [107–111] or by conjugating Gd(III) chelates to biocompatible polymers via a disulfide bond [112,113]. Preliminary studies in different animal models revealed that the biodegradable macromolecular agents provide superior contrast enhancement in the vasculature and tumor tissue and can be rapidly excreted from the body as low molecular weight contrast agents.

12. Reduction-responsive drug conjugates

Gabizon et al. developed a lipid-based prodrug of mitomycin C (MMC) in which the drug is attached to 1,2-distearoyl glycerol lipid via a cleavable dithiobenzyl linker [114]. The lipophilic MMC prodrug (MLP) was incorporated with nearly 100% efficiency in cholesterol-free pegylated liposomes with hydrogenated phosphatidylcholine as the main component. The pegylated liposomal MLP revealed drastically reduced *in vitro* and *in vivo* cytotoxicity as



Scheme 13. Two schemes of SWNT functionalization by thiolated biological molecule X with (1-X) and without (2-X) disulfide bond respectively. Both DNA and RNA cargos contain a thiol functional group and a six-carbon long spacer at the 5′ end of the DNA or RNA [104].

compared to free MMC. Importantly, its activity increased to nearly comparable levels to those of free MMC in the presence of reducing agents like cysteine or *N*-acetylcysteine. *In vivo* studies using three tumor models demonstrated that the therapeutic index and absolute antitumor efficacy of the pegylated liposomal MLP were superior to that of free MMC.

Kopeček and coworkers conjugated the photosensitizer mesochlorin e6 (Mce6) to a water soluble *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer via a disulfide bond, which showed a time-dependent release of Mce6 and concomitant increase in the photodynamic efficacy when exposing to DTT [115]. *In vitro* studies using SKOV-3 human ovarian carcinoma cells demonstrated a faster release kinetics and a higher cytotoxicity of disulfide-linked HPMA copolymer–Mce6 conjugates as compared to the corresponding conjugate with a proteolytically cleavable GFLG spacer.

13. Miscellaneous reduction-sensitive polymers

Park et al. reported a facile synthesis of reducible poly(ethylene oxide), termed as poly(ethylene oxide sulfide) (PEOS), by oxidative polymerization of bismercapto ethylene oxide oligomers in dimethyl sulfoxide [116]. The water solubility of PEOS polymers depends on the length of ethylene oxide segment between the disulfide bonds: polymer with a short ethylene oxide segment (number of ethylene oxide repeating units $\leq$4) was insoluble in water whereas polymers with a long ethylene oxide segment (number of ethylene oxide repeating units = 11–36) were water soluble. PEOS was stable in a medium mimicking the extracellular environment but was completely degraded in 2 h in 5 mM glutathione buffer. These reducible PEO might be interesting for many drug delivery applications.

Hoffman and coworkers designed "smart" terpolymers, poly(alkyl acrylic acid-*co*-butyl acrylate-*co*-pyridyl disulfide acrylate), which exhibit pH-dependent membrane-disruptive properties and meanwhile allow reversible conjugation of thiol-containing biomolecular drugs such as oligopeptides and 5′-thiol-terminated asODN [117,118]. These terpolymers, while displayed high hemolytic activity at low pH, showed no cell toxicity at physiological pH, even at high concentrations. Enhanced cytoplasmic delivery of FITC–ODN was observed in macrophage-like cells. The pH sensitivity and hemolytic activity of these terpolymers could be tuned by controlling the length of the alkyl group substituted on the pH-sensitive monomer and by the incorporation of a hydrophobic butyl acrylate monomer [118].

Ferruti and coworkers firstly reported cationic or amphoteric linear poly(amido amine)s (PAAs) containing multiple disulfide linkages in their main chain, synthesized by Michael-type polyaddition of 2-methylpiperazine to cystamine bisacrylamide (BACy1) and L-cystine bisacrylamide (BACy2) [119], or L-cystine to 1,4-bis(acryloyl)piperazine (BP) and 2,2-bis(acrylamido)acetic acid (BAC) [120], respectively. Degradation experiments revealed that these disulfide-containing PAAs, though having a similar hydrolytic degradation rate as that of conventional PAAs, degraded rapidly in the presence of a reducing agent such as 2-mercaptoethanol. Linear soluble PAAs bearing reactive 2-ethenyldithiopyridine as side substituents were obtained by preparation of cross-linked PAA networks using cystamine as a tetra-functional crosslinking agent followed by disulfide–disulfide exchange reaction with 2,2′-dithiodipyridine [121]. The dithiopyridyl side groups could easily undergo exchange reactions with thiol-containing biologically active peptides such as reduced L-glutathione, to yield soluble PAA–peptide conjugates which are linked by reversible disulfide bonds. In a similar way, poly(1-vinylpyrrolidin-2-one) (PVP) with pendant 2-ethenyldithiopyridine moieties has also been synthesized [122].

Using bis[2-(2-bromoisobutyryloxy)ethyl] disulfide as an initiator, linear well-defined degradable polymethacrylates with one internal disulfide link were prepared by atom transfer radical polymerization (ATRP) [123]. The reduction of the disulfide bond with tributylphosphine yielded polymers of half the molecular weight of the starting materials. Copolymerization of methyl methacrylate with a disulfide-containing dimethacrylate monomer, bis(2-methacryloyloxyethyl) disulfide, under similar reaction conditions yielded degradable gels, which upon reduction with tributylphosphine gave rise to soluble, low molecular weight linear PMMA fragments containing thiol groups at the chain end and along the backbone, originating from the disulfide difunctional initiator and monomer, respectively [123]. In a following study, multisegmented degradable polystyrenes were obtained via atom transfer radical cross coupling of α, ω-dihalogenated polymers prepared by ATRP with disulfide dinitroxides [124].

DeSimone et al. recently developed reductively labile PRINT (Particle Replication in Nonwetting Templates) particles by incorporating 30 wt% disulfide-containing cross-linker cystamine bisacrylamide (CBA) [125]. Interestingly, the PRINT particles loaded with 2 wt% DOX were shown to release DOX in response to a reducing environment and were found to be highly proficient at killing HeLa cells *in vitro*. The authors suggested that reduction of the disulfide bonds leads to a decrease in the mesh density of the particle creating pores to allow diffusion of DOX.

14. Conclusion and perspectives

The past decade has witnessed remarkable advancement of reduction-sensitive biodegradable polymers and conjugates for the triggered delivery of a variety of bioactive molecules including

DNA, siRNA, antisense oligonucleotide (asODN), proteins, anti-cancer drugs, etc. This class of materials meet the conflicting requirements of an ideal delivery system, i.e. high stability in circulation while rapid degradation in tumor tissues and/or inside targeted cells, achieving markedly improved therapeutic effect and minimal carrier-related *in vitro* and *in vivo* cytotoxicity. Reduction-sensitive nano-sized delivery systems are in particular suited for the efficient delivery of cytosolically active nucleic acid drugs like siRNA.

It should be noted, however, that reduction-sensitive delivery systems are still at its infancy. For successful clinical applications, bioresponsive multifunctional nano-carriers that combine reduction-sensitivity, biocompatibility, long circulation time, cellular and/or intracellular targetability are desired. Before going clinics with these systems, extensive preclinical evaluations are required. In these preclinical studies attention should be given to the pharmacokinetics of the drug loaded polymers/particles, their efficacy and their biocompatibility/toxicity in particular after repeated administration. Also mechanistic studies are required to investigate where and how exactly inside the cells the reduction takes place as well as on the intracellular destabilization kinetics. It is also not clear how the micro-environment of the disulfide bond influences the kinetics of reductive degradation. The mechanistic studies will undoubtedly help the further rational design of even more advanced delivery systems.

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Appendix

Figures with essential colour discrimination. The majority of the schemes in this Review Paper may be difficult to interpret in black and white. The full colour schemes can be found in the on-line version, at doi:10.1016/j.biomaterials.2009.01.026.

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