Expert Opinion

The role of the disulfide group in disulfide-based polymeric gene carriers

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Background: An essential prerequisite for successful gene therapy is the development of safe and efficient gene delivery carriers. For this purpose, cationic polymers have been widely studied as non-viral carriers, but they generally suffer from low transfection efficiency and/or high cytotoxicity. To address these problems, disulfide-based cationic polymers have been designed as intelligent gene carriers that are capable of inducing highly efficient gene transfection with low cytotoxicity. *Objective*: The present review discusses the effects of the disulfide linker on the gene delivery properties of cationic polymers in relation to various gene delivery barriers. *Methods*: The literature regarding the gene delivery barriers encountered by polymeric gene delivery is reviewed and discussed in relation to the presence of the disulfide moiety in these gene carriers. *Conclusions*: The presence of disulfide linkages in cationic polymers can in many aspects favorably influence the gene delivery properties, such as increasing DNA binding ability, enabling de-shielding of 'stealth' (PEG) groups, fine-tuning of the buffer capacity for enhanced endosomal escape, improving carrier-unpacking and decreasing cytotoxicity. Therefore, disulfide-based cationic polymers are promising candidates for the next generation of non-viral carriers.

Keywords: bioreducible polymer, cationic polymer, disulfide, gene carrier, gene delivery, gene delivery barrier

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1. Introduction

Gene therapy has shown tremendous promise in curing human diseases like cancer [1-3]. The main process of gene therapy involves the delivery of encoded genes into host somatic cells, producing on-demand therapeutic proteins. For successful gene delivery, the development of safe and efficient gene delivery carriers is an essential requisite [4]. Gene delivery carriers can be divided into two categories: viral carriers and non-viral carriers. Viral gene carriers derived from natural viruses are still popular in clinical gene therapy due to their inherent high transfection capability. However, the clinical application of viral carriers is associated with considerable safety concerns such as high cytotoxicity, insertional mutagenesis and undesirable immune response [5]. Compared to viral carriers, non-viral carriers such as cationic polymers provide better opportunities for low cytotoxicity and low immunogenicity in gene therapy and have additional advantages in facile manufacturing, large-scale production and unlimited gene-carrying capacity [6]. Therefore, non-viral carriers are emerging as attractive alternatives to viral carriers.

Many cationic polymers such as polyethylenimine (pEI) and poly(L-lysine) (pLL) have been and still are being extensively investigated as non-viral carriers for gene delivery *in vitro* and *in vivo* [7-11]. However, the clinical practice of these polymer systems is seriously hampered by their relatively low transfection efficiency as

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compared to viral carriers. This low efficiency can be attributed to their incapability to overcome the multiple extracellular and intracellular barriers on the pathway to gene expression [12,13]. To overcome these barriers, cationic polymers need to possess a multitude of appropriate gene delivery properties, such as good DNA binding ability to form nanosized complexes (polyplexes), protection of DNA from degradation by nucleases, efficient escape of polyplexes from the endosomes and DNA unpacking from the polyplexes in the cytosol or nucleus. Thus, multifunctional cationic polymers displaying various properties in the spatio-temporal domain are needed to meet these requirements and to be capable of inducing high gene transfection [11].

The utilization of different biological stimuli, such as temperature, pH value and redox potential, offers interesting opportunities for the design of multifunctional cationic polymers for non-viral gene delivery [14,15]. This review focuses on the utilization of the bioreducible disulfide bond in polymeric gene carriers. It has been well established that disulfide bonds as redox-sensitive linkages can be cleaved in the presence of high amounts of reducing enzymes like glutathione reductase or thiol-based components such as glutathione. Since the concentration of these reducing species is much higher in the cytoplasm than in plasma (e.g., intracellular vs. extracellular glutathione concentration is 0.5 – 10 mM vs. 2 – 20 μM [16]), the disulfide bond is relatively stable in the extracellular environment, but can be rapidly degraded inside the cells. This feature thus invoked the design of disulfide-based cationic polymers as smart multifunctional gene carriers.

Over the past decade, many disulfide-based polymeric systems have been developed as non-viral carriers for gene delivery *in vitro* and *in vivo*. The presence of disulfide bonds in cationic polymers frequently plays a dominant role in their gene delivery properties. Although there have been appeared recently a few excellent reviews on disulfide-based cationic polymers for non-viral gene delivery [17,18], the primary focus of this review is on understanding the effects of disulfide bonds on gene delivery properties that are important to overcome gene delivery barriers. In this article, first the non-viral gene delivery barriers are briefly described, then the strategies to design disulfide-based polymeric gene carriers are outlined, and finally we discuss the effects of disulfide bonds in cationic polymers on gene delivery properties such as DNA binding ability, protonation of amino groups (buffer capacity), intracellular carrier-unpacking and cytotoxicity.

2. Non-viral gene delivery barriers

As mentioned above, polymeric gene carriers must overcome various hurdles before genes can be efficiently delivered into the targeted cells. Therefore, for the design of efficient polymeric gene carriers, it is essential to understand the molecular processes that are connected with the gene delivery barriers. A schematic illustration of the process of cationic polymer-mediated gene delivery is given in **Figure 1**.

The first step in the transport of genes into cells is that the cationic polymers need to self-assemble by electrostatic interactions with negatively charged genes into nano-sized polymer/gene complexes (polyplexes). In general, polyplexes with positive surface charge are formed when the number of positive charges of the polymer exceeds that of the negative charges in DNA. In order to deliver DNA to distant abnormal organs/tissues, i.v. administration is often performed. After i.v. administration of polyplexes, these nanoparticles have to pass several barriers in the extracellular environment [12]. First, nucleases present in the intravascular or in the intercellular environment can eliminate polynucleotides by enzymatic degradation. Generally, cationic polymers may prevent DNA from such degradation by shielding the DNA in the polyplexes. Second, the physical stability of polyplexes in the physiological milieu forms a great barrier. The high ionic strength due to the presence of salts in the extracellular fluid weakens the electrostatic interaction between cationic polymers and DNA, resulting in destabilization and eventual dissociation of the polyplexes. Third, non-specific interactions between polyplexes and proteins or cellular surfaces are an important barrier. Polyplexes generally have a positively charged surface when they show optimal delivery efficacy, due to absorption to the negatively charged cell membrane and subsequent endocytosis. However, negatively charged blood proteins, such as albumin, can also adsorb to positive polyplexes, leading to the formation of large-sized particles or aggregates. Moreover, polyplexes can interact with negatively charged blood cell membranes and induce aggregates of erythrocytes. Fourth, during circulation in the bloodstream, polyplexes can be accumulated in tissues such as lung and liver or cleaned by phagocytosis.

Polymeric vectors have to overcome not only these extracellular barriers, but also quite a number of intracellular barriers [13]. The cellular membrane that excludes the extracellular environment from the cellular interior is the first barrier. Generally, polyplexes may interact with negatively charged cellular membranes to trigger cellular endocytosis by different mechanisms. After cellular internalization of polyplexes, the endosome–lysosomal degradation pathway is a great barrier to efficient gene delivery. Polyplexes located in the endosomes can undergo degradation during the acidification process from early to late endosomes and finally the fusion with lysosomes. Genes are easily degraded by enzymes present in the acidic endosomes (pH 5 – 7) and lysosomes (pH 4.5) [19]. Therefore, the escape of the polyplexes from the endosomes into the cytosol is required to deliver their cargo to the nucleus. Once in the cytosol, the cytosolic trafficking of polyplexes to the nucleus is the third hurdle to overcome, since the cytoplasm contains many proteins, RNA and organelles that may seriously hamper the diffusion movement of polyplexes. It was reported that the movement of polyplexes to the nucleus along the cytoskeletal network could be mediated by binding of polyplexes with anionic microtubules or molecular motor proteins [20,21]. Fourth, vector unpacking is a barrier to gene delivery. It has been

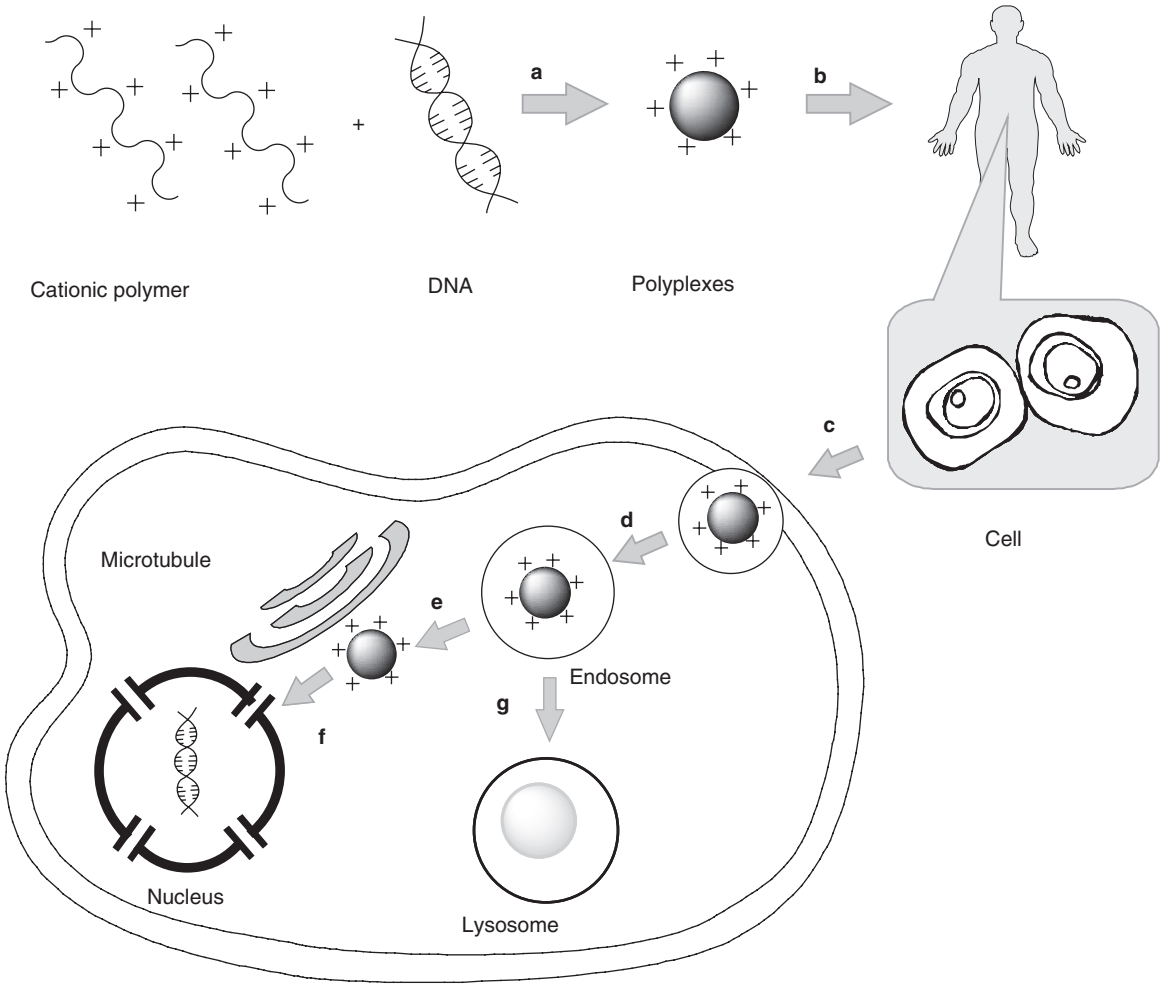


Figure 1. Schematic illustration of cationic polymer-mediated gene delivery. (**a**) Formation of cationic polymer/DNA complexes (polyplexes); (**b**) i.v. administration of polyplexes; (**c**) cellular uptake of polyplexes by endocytosis; (**d**) endosomal pathway of polyplexes; (**e**) endosomal escape of polyplexes; (**f**) polyplexes unpacking and nuclear translocation of DNA; (**g**) degradation of polyplexes in lysosome.

shown that the release of DNA from polyplexes is a limiting step for high levels of gene expression [22]. Although the optimal stage of vector-unpacking is not fully understood and could vary for different polyplexes, it is obvious that genes should be sufficiently released from polyplexes for transgene expression. Finally, the nuclear membrane is an important obstacle for the entry of polyplexes. Polyplexes can enter the nucleus as the nuclear envelope opens during the mitosis of dividing cells [23]. However, in non-dividing cells the transport of polyplexes into the nucleus occurs via an active transport mechanism that is mediated by nuclear pore complexes [24,25].

3. Strategies to design disulfide-based polymeric systems as gene carriers

There are two different conceptual strategies in the design of disulfide-based cationic polymeric systems for gene delivery. The first strategy is to prepare disulfide-containing cationic polymers via chemical synthesis or modifications and to use these polymers in the further preparation of the gene carriers. The second strategy is the formation of disulfide bonds (S-S crosslinks) in appropriately functionalized cationic polymer/DNA polyplexes (post-crosslinking of polyplexes).

3.1 Disulfide-containing cationic polymers as gene carriers

Disulfide-containing cationic polymers refer to those cationic polymers that have the disulfide linkages in the polymeric main chain and/or in the side chains. The different synthetic routes that were developed to prepare such polymers for non-viral gene delivery are summarized in **Figure 2**.

One important route is via the preparation of precursor polymers possessing pyridyldithio residues and the reaction of these residues with appropriate sulfhydryl (thiol) compounds

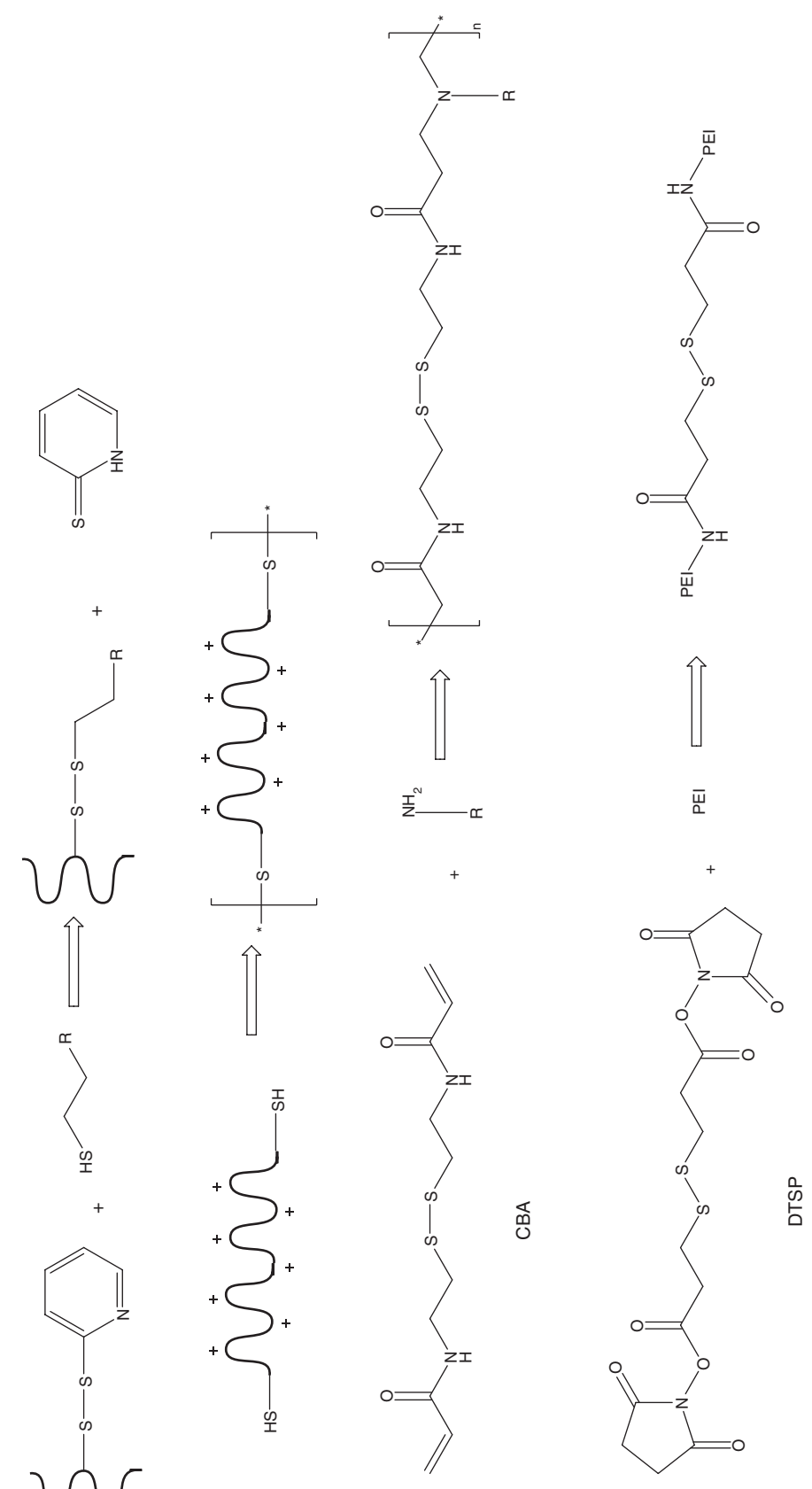


Figure 2. Different synthetic approaches to designing disulfide-containing cationic polymers as gene carriers.

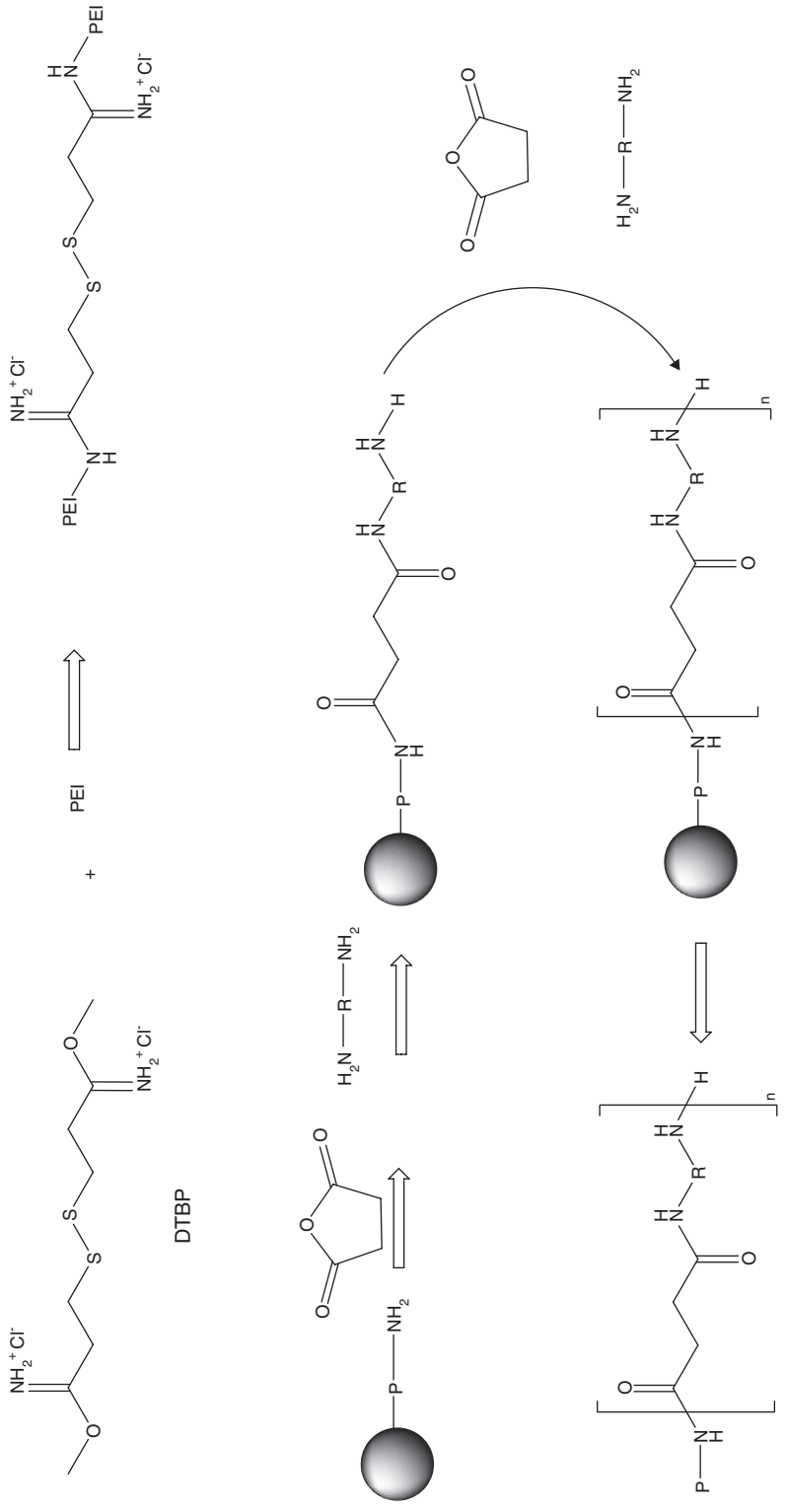


Figure 2. Different synthetic approaches to designing disulfide-containing cationic polymers as gene carriers (continued).

to form new disulfide bonds (**Figure 2a**). The disulfide linkage of the pyridyldithio group is excellently suited for an exchange reaction with thiol compounds since the pyridine-2-thiolate acts as a good leaving group, forming the non-reactive pyridine-2-thione (that is thermodynamically more stable than its pyridine-2-thiol tautomer). The method was used by Midoux *et al.* to prepare pLLs with disulfide linkages in the side chains (Poly[Lys-(AEDTP)]) by modification of the amino groups of pLLs with N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) and subsequent exchanging reaction with mercaptoethylamine [26]. Recently, Langer *et al.* reported the synthesis of poly(amino ester)s with pyridyldithio groups in the side chain by Michael addition polymerization of the 2-(pyridyldithio)-ethylamine to diacrylates. These polymers were further modified with mercaptoethylamine or thiol peptide such as RGDC, giving poly(amino ester)s with disulfide linkages in the side chains [27].

Another route to obtain cationic polymers with disulfide bonds in the polymeric main chain is by polyoxidation of disulfhydryl-based macromonomers containing amino groups (**Figure 2b**). Typical examples are linear disulfide-containing cationic polymers based on pEI [28], pLL [29] and pDMAEMA (**Table 1**) [30]. The synthesis of these polymers requires multiple steps as disulfhydryl-based oligoamines need to be synthesized as starting compounds for the oxidative polymerization. Park *et al.* reported on the synthesis of disulfhydryl-containing oligoamines with different amounts of amino units via multiple-step organic synthesis involving protection and deprotection of amino groups [28]. Oupický *et al.* reported the synthesis of well-defined disulfhydryl-containing pDMAEMA oligomers via reversible addition-fragmentation chain transfer (RAFT) polymerization using a difunc tional chain transfer agent and subsequent aminolysis [30]. Seymour *et al.* prepared disulfhydryl-terminal oligopeptides (Cys-Lys_{10}-Cys) via solid-phase supported organic synthesis [31]. Disulfhydryl-based oligoamines can be oxidized in the presence of dimethyl sulfoxide (DMSO) as the oxidizing agent to yield disulfide-containing polymers with a relatively high molecular weight in the range of 10 – 100 kDa. In the reaction, other disulfhydryl-functional groups, for example nuclear localization sequences comprising two cysteine residues, can be incorporated in the polyoxidation reaction with disulfhydryl-based oligoamines, giving disulfide-containing multiblock copolymers [32]. Since different disulfhydryl amines can be prepared via chemical synthesis, the polyoxidation reaction of disulfhydryl macromonomers allows the generation of new disulfide-containing cationic polymers for non-viral gene delivery.

The most commonly used route to prepare disulfide-containing cationic polymers is by reaction of amine compounds or oligoamines with disulfide-containing reagents, such as cystamine bisacrylamide (CBA) in a Michael-type polyaddition [33-36] (**Figure 2c**), or with dithiobis(succinimidyl propionate) (DTSP) and dithiobispropionimidate (DTBP) [37,38] in a polycondensation reaction (**Figures 2d**, **2e**). These reactions allow for the preparation of linear or branched disulfide-containing cationic polymers with large variation in chemical structures. Lee *et al.* pioneered the synthesis of disulfide-containing branched pEI by chemical crosslinking of low molecular weight pEI with DTSP or DTBP [37,38]. We have synthesized several groups of disulfide-containing poly(amido amine) homopolymers (SS-PAA) and copolymers by Michael addition reaction of CBA to primary amines [36,39], (disulfide-based) secondary diamines [40-42] or oligoamines [43,44]. The structural effects of these polymers on gene delivery properties were systematically investigated [45].

The aforementioned methods usually give polymers with a certain polydispersity in molecular weight and sometimes a less defined structure, depending on the type of reaction and monomers. Although well-defined cationic polymers are not always necessary for non-viral gene delivery, cationic polymers that have a well-defined molecular weight and structure may provide accurate structural information that can be helpful for the investigation of structure–activity relationships. Borner *et al.* [46-48] reported on the synthesis of well-defined disulfide-containing cationic polymers (3 – 10 monomer units) by an alternating condensation coupling reaction of disulfide-containing diamines and diacids via solid-phase supported organic synthesis (**Figure 2f**).

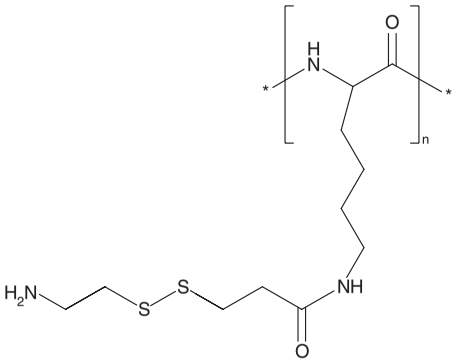
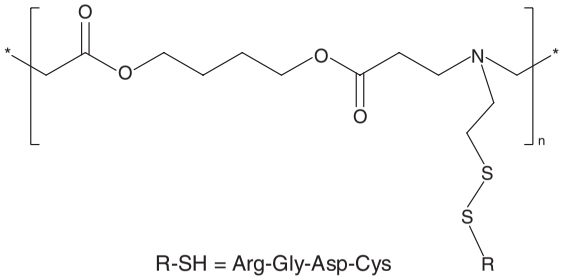
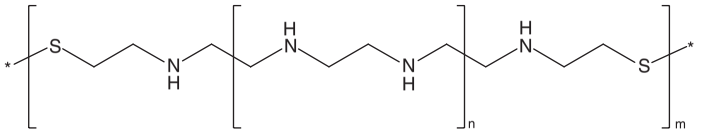
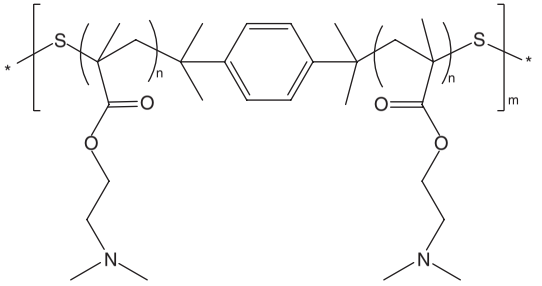
3.2 Disulfide-crosslinked polymer/DNA complexes

The second strategy to obtain disulfide-based gene delivery carriers is post-crosslinking of polymer chains after the complexes between DNA and the polymers have been formed.

In the first post-crosslinking method, the disulfide moieties are introduced by reaction of free amino groups in the polymer/DNA complexes (polyplexes) with a disulfide-containing crosslinking reagent susceptible for reaction with the amino groups, for example a reagent with activated ester groups (**Figure 3a**). Seymour *et al.* prepared crosslinked polyplexes of pLLs by reaction of the crosslinking reagent DTSP with the primary amino groups in the side chains of the polymers [49]. In a similar way, Budker *et al.* prepared crosslinked polyplexes of pLL with DTBP [50]. Since pLLs showed poor gene transfection, Kissel *et al.* selected polyplexes of pEI that were crosslinked with DTSP [51,52].

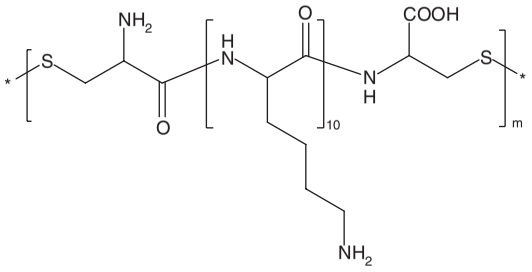
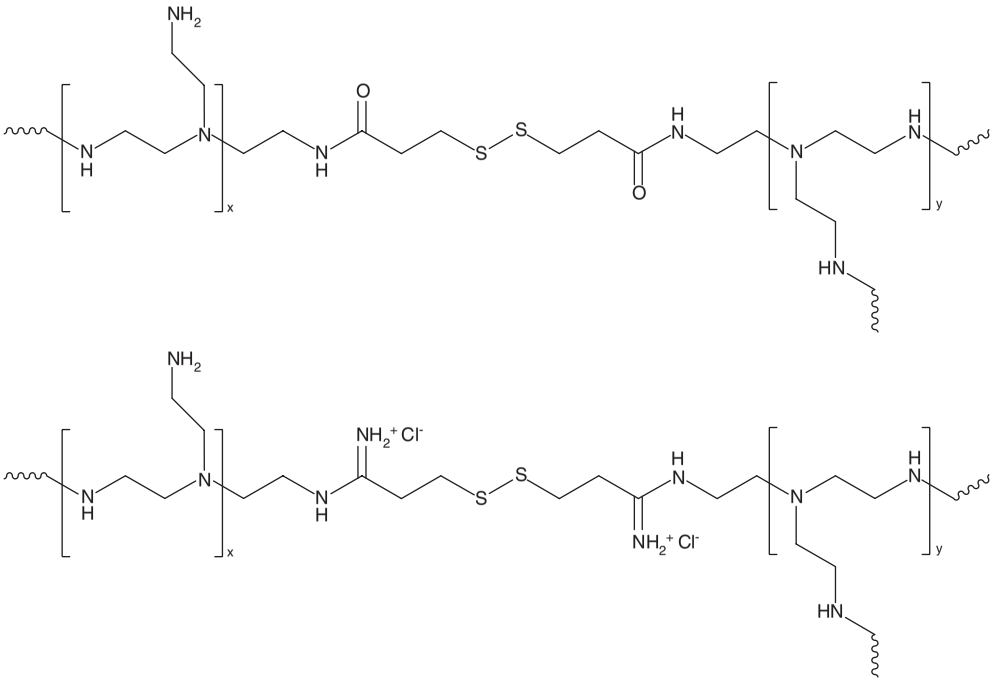
Another post-crosslinking method deals with thiolated cationic polymers (**Figure 3b**, **Table 2**). Polyplexes of thiolated cationic polymers can be crosslinked to form disulfide-based polyplexes by spontaneous oxidation of thiol groups in air to disulfide bonds. In original studies, thiol-based peptides with cysteine residues were first reported by Rice *et al.* and used for the preparation of disulfide-based polyplexes which could mediate non-viral gene delivery *in vitro* and *in vivo* [53-56]. Thiolated pEI and pLL were also synthesized via chemical modifications using 2-iminothiolane (Traut's reagent) or *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) [57-62]. In another route, thiolated pEIs were produced via ring opening reaction of low molecular pEI with methylthiirane [63].

Table 1. Structures of disulfide-containing cationic polymers as non-viral gene carriers.

Polymer name	Structure	Ref.
Poly[Lys-(AEDTP)]		[26]
Poly(β-amino ester)		[27]
Poly(ethylenimine sulfide)		[28]
Reducible poly(2-dimethylaminoethyl methacrylate): rPDMAEMA		[30]

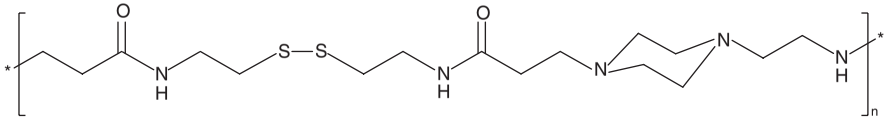
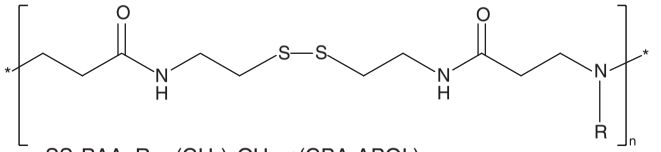
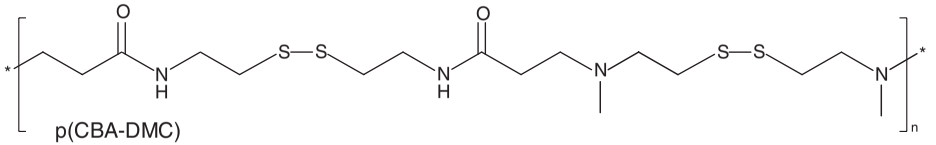
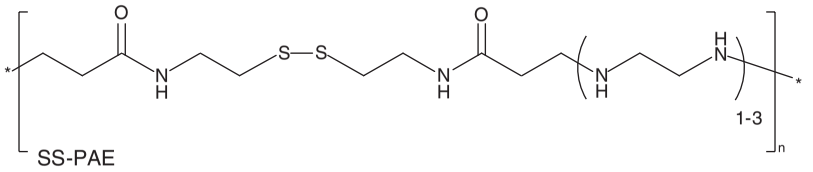
PEI: Polyethylenimine.

Table 1. Structures of disulfide-containing cationic polymers as non-viral gene carriers (continued).

Polymer name	Structure	Ref.
Reducible poly(L-lysine): Cys-Lys$_{10}$-Cys		[29,31]
Reducible PEI		[37,38]

PEI: Polyethylenimine.

Table 1. Structures of disulfide-containing cationic polymers as non-viral gene carriers (continued).

Polymer name	Structure	Ref.
Reducible poly(amido amine): SS-PAA	 p(CBA-AEP)	[41]
	 SS-PAA; R = $(CH_2)_4OH$: p(CBA-ABOL)	[34,36,39,44]
	 p(CBA-DMC)	[40]
	 SS-PAE	[42]

PEI: Polyethylenimine.

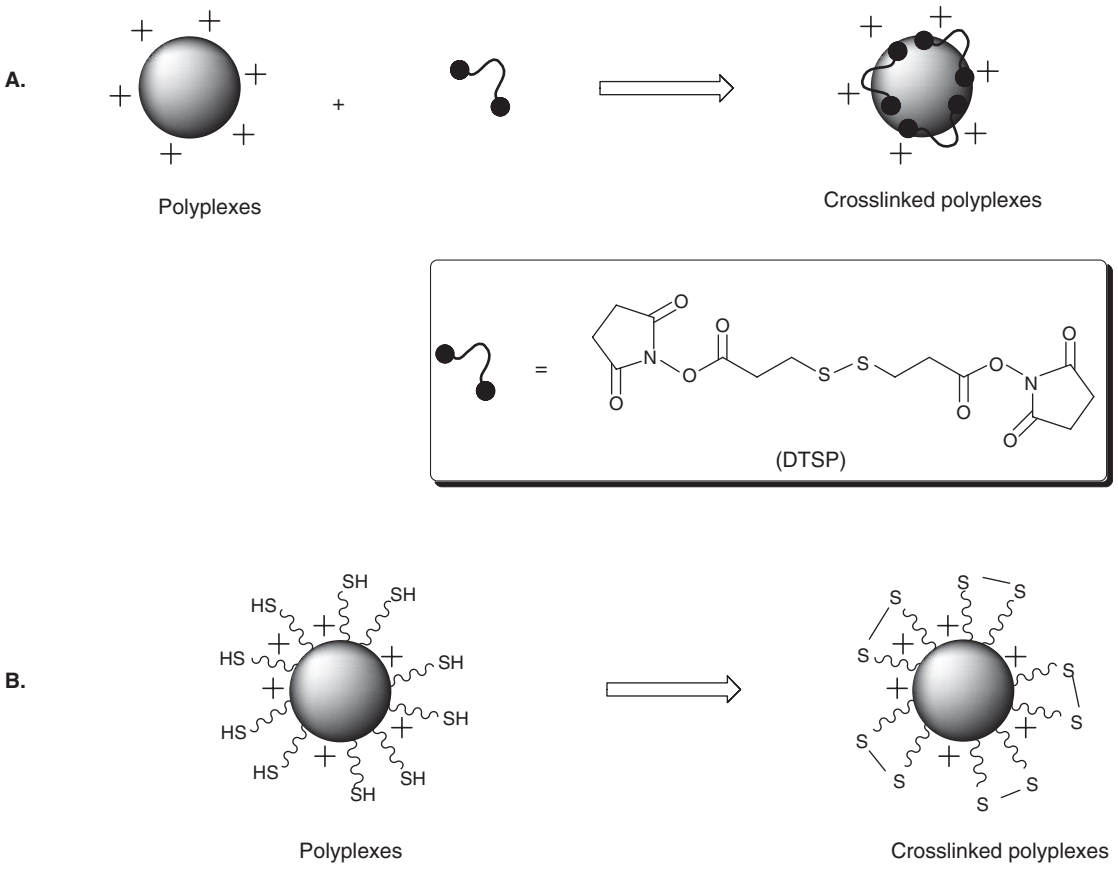


Figure 3. Different approaches to obtaining disulfide-based polyplexes via post-crosslinking.

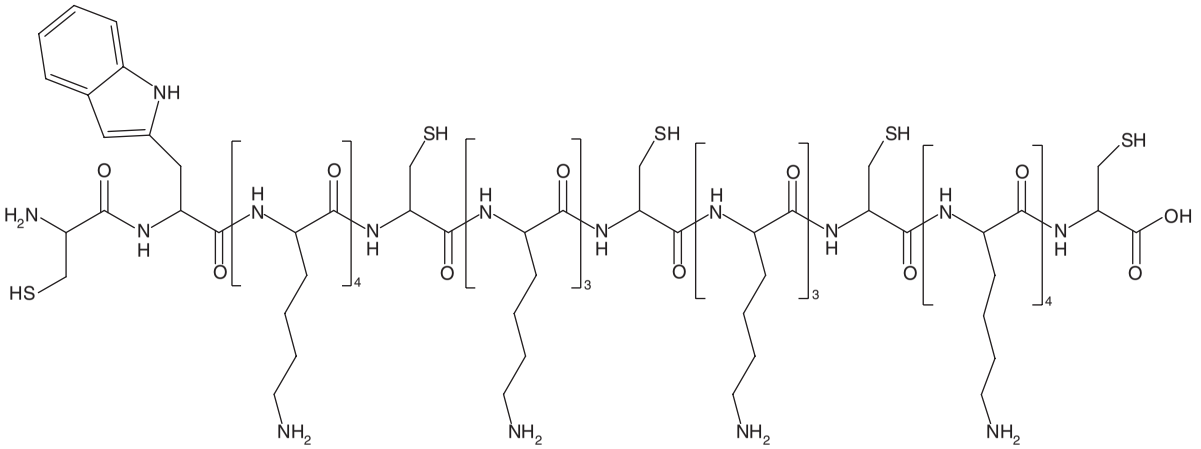
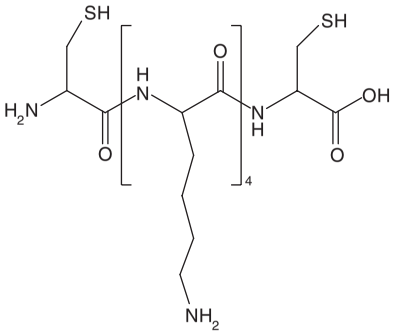
4. Disulfide linkages play an important role in gene delivery properties

4.1 Effect of disulfides on DNA condensation ability of cationic polymers

DNA condensation capability of cationic polymers is an important factor for gene delivery. A high condensation capability of the polymers will induce the formation of nanosized polyplexes that may undergo cellular uptake via endocytosis. The primary driving force in DNA condensation by cationic polymers is electrostatic interaction between the positive charges of the cationic polymer and the negative charges of DNA. Hence, cationic polymers having a high charge density (e.g., pEI) normally reveal strong condensation capacity to DNA. Moreover, there exist secondary driving forces in the polyplexes that may contribute to DNA condensation. These forces are generally weaker than the electrostatic binding and can be derived from the physico-chemical and structural properties of the cationic polymers, for example hydrogen bonding interactions, dipole interactions and hydrophobic interactions.

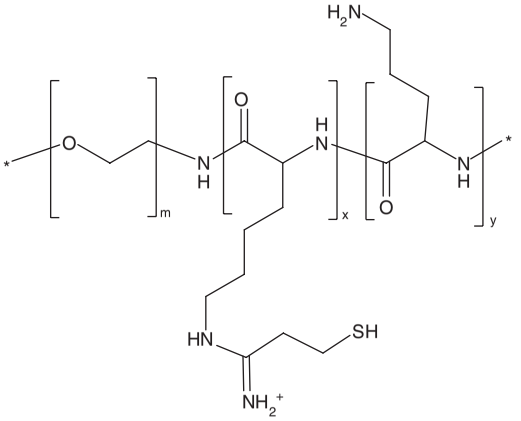
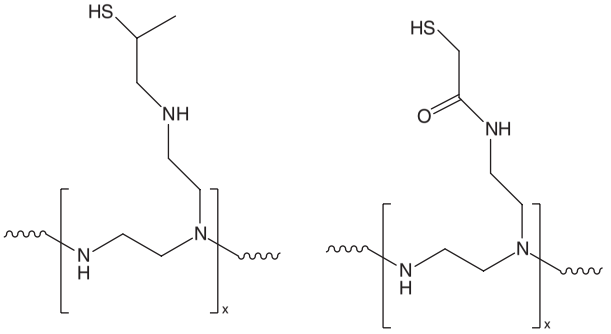
The hydrophilicity/hydrophobicity of a functional group can be evaluated from the octanol–water partition coefficient (log P) of a series of relevant compounds. The fragmentary log P value of the disulfide group is 0.5497 and lower than that of two CH_2 groups (2 × 0.4911) [64]. This indicates that the disulfide group has more hydrophilic character than an ethylene group, which is understandable considering the more electronegative character of the sulfur atoms. The disulfide groups thus can, albeit weakly, contribute to DNA complexation by hydrogen bond acceptation. In addition, sulfur atoms possess a much higher polarizability than methylene groups, which implies that the disulfide moiety may contribute to DNA condensation by interaction with the polarizable aromatic nucleotide bases. Another aspect is that the disulfide bond is considerably longer than the ethylene carbon–carbon bond (0.203 vs. 0.154 nm) and unlike the carbon–carbon bond, it has no further substituents that contribute to the rotational barrier. This implies that disulfide-containing polymeric chains have a higher degree of flexibility as compared to carbon analogs without disulfide linkages. It is thus expected that relatively free chain folding and movement of disulfide-containing cationic polymeric chains can also contribute to the facilitated polymer/DNA interaction and condensation. In a study of DNA-poly(amido amine) condensation parameters, dynamic light scattering measurements

Table 2. Structures of thiol-containing cationic polymers as non-viral gene carriers.

Polymer name	Structure	Ref.
Thiolated poly(L-lysine): Cys-Trp-Lys_4-Cys-Lys_3-Cys-Lys_3-Cys-Lys_4-Cys		[53]
Thiolated poly(L-lysine): Cys-$(Lys)_4$-Cys		[54]

EDA: Ethylenediamine; PHEA: α,β-poly(*N*-2-hydroxyethyl)-D,L-aspartamide.

Table 2. Structures of thiol-containing cationic polymers as non-viral gene carriers (continued).

Polymer name	Structure	Ref.
Thiolated poly(L-lysine): 2-iminothiolane- modified PEG-poly(L-lysine)		[57,58]
Thiolated polyethylenimines: methylthiirane-modified PEI and thioglycolic-modified PEI		[59,63]

EDA: Ethylenediamine; PHEA: α,β-poly(*N*-2-hydroxyethyl)-D,L-aspartamide.

indicated that the disulfide-containing poly(amido amine)s p(CBA-AEP) (**Table 1**) show stronger DNA condensation capability than their analogs lacking the disulfide bonds, and form smaller particles after DNA complexation [41]. Therefore, the presence of disulfide bonds in cationic polymers may promote the DNA condensation by these polymers, which is important for the internalization of polyplexes via endocytosis.

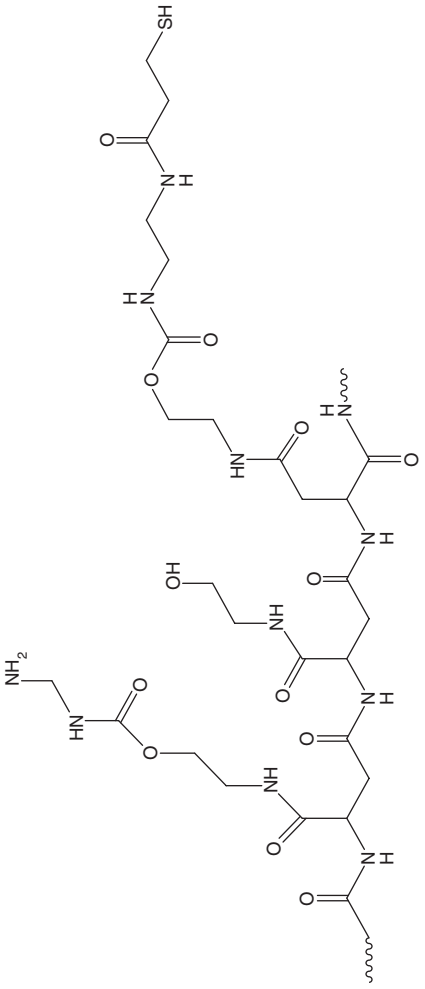
4.2 Effect of disulfides on PEG de-shielding from polyplexes

As mentioned in section 2, blood components in the extracellular environment, such as salts and proteins, can make polyplexes unstable. One important concept in the design of colloid-stable polyplexes involves the surface shielding of positively charged polyplexes. This can be achieved by conjugation of the cationic polymers with biocompatible hydrophilic polymers, such as poly(ethylene glycol) (PEG). The complexation of PEG-conjugated cationic polymers with DNA affords polyplexes with a PEG corona that show improved colloid stability due to reduced interaction with blood components, resulting in prolonged circulation [65,66]. However, compared to positively charged polyplexes, PEGylated polyplexes showed significantly decreased transfection efficiency because their neutral surface compromises efficient cellular association and internalization. Moreover, PEGylated polyplexes also impair the escape of the polyplexes from the acidic endosomes [67,68]. In order to address the PEG dilemma, Kataoka *et al.* designed PEG detachable polyplexes that are sensitive to reducing environment [69]. The disulfide bonds linked between the PEG and the cationic polymers are expected to be cleaved in the endosomes or on the exofacial surface of cells, followed by subsequent endocytosis of the de-PEGylated polyplexes. Moreover, some cell surface-associated proteins such as NADH-oxidase are known to be involved in disulfide–thiol interchange activity, especially in cancerous cells such as HeLa and hepatoma cells [70]. It was suggested that disulfide-linked PEGylated polyplexes have one to three orders of magnitudes higher transfection efficiency than the polyplexes without disulfide linkages. Thus, a redox-sensitive disulfide linker has a favorable effect on the PEG detachment from PEGylated polyplexes, leading to improved transfection.

4.3 Effect of disulfides on protonation of amino groups in cationic polymers

In order to avoid the degradative lysosomal pathway, polyplexes have to be designed to induce efficient endosomal escape. A frequently followed approach is to design cationic polymers which possess functional amino groups (e.g., secondary and tertiary amino groups) that become protonated upon acidification of the endosome after uptake of the polyplexes. The mechanism of endosomal escape that is induced by this type of polymer is frequently explained by the buffer effect in the range of endosomal pH change (pH 7.4 – 5.1). The increasing protonation of the polymer upon decrease of the pH in the endosomes induces an influx of counter ions (Cl^-) and water

Table 2. Structures of thiol-containing cationic polymers as non-viral gene carriers (continued).

Polymer name	Structure	Ref.
PHEA-EDA-SH		[62]

EDA: Ethylenediamine; PHEA: α,β-poly(*N*-2-hydroxyethyl)-D,L-aspartamide.

(osmotic pressure) resulting in membrane rupture of the endosomes ('proton sponge effect') [71]. However, conformational changes that occur on protonation and increased membrane interaction of the polymers may also contribute to the endosomal escape [72,73]. Typical cationic polymers that are considered to take advantage of the 'proton sponge effect' include polyethylenimine and polyamidoamine dendrimers [74].

In bioreducible poly(amido amine)s prepared by Michael addition of the bisacrylamides to the disulfide-containing secondary diamine *N,N′*-dimethylcystamine [40], the tertiary amino groups in, for example, p(CBA-DMC) (**Table 1**) have apparent pK_a values of 6.6 – 7.3, much lower than that of low-molecular tertiary amines (pK_a ~ 9 – 10). Thus, these polymers showed high buffer capacities in the pH range pH 5.1 – 7.4. The relatively low apparent pK_a of these polymers can be attributed to the inductive electron-withdrawing effect of the disulfide group at the β-position to the amine groups in the polymers. Transfection studies indicated that these polymers are capable to induce high levels of gene expression in COS-7 cells as compared to pEI. Thus, in the design of cationic polymers with a high buffer capacity, the presence of disulfide bond in the β-position to protonable amino groups has a positive effect on the buffer capacity of these amino groups. This is important for the endosomal escape of the polyplexes and may lead to more efficient gene transfection.

4.4 Effect of disulfides on intracellular DNA release

It has been demonstrated that intracellular carrier unpacking to release DNA is possibly an important barrier for efficient gene delivery [22]. A few approaches have been reported in the design of polymeric carriers aiming at effective gene release inside the cells, thereby inducing improved gene transfection [18]. Among these approaches, the use of disulfides in cationic polymers may eventually lead to smart gene carriers that trigger controlled carrier-unpacking for efficient gene delivery.

Although many disulfide-based cationic polymers were designed for triggered gene delivery, for many of them it is not clear whether their genes are unloaded in the cytoplasm or in the nucleus. Since DNA release is associated with the cleavage of disulfide bonds in the polymers and the subsequent dissociation of the polyplexes, the release can be deduced by understanding the degradation profile of the disulfide-based polymers in the cells. Supporting evidence that disulfide-based cationic polymers are degraded inside the cytosol, thereby releasing DNA, have recently been obtained by fluorescence-labeling studies. We have studied the intracellular distributions of the fluorescence-labeled poly(amido amine) containing multiple disulfide linkages in the polymeric main chain (p(CBA-ABOL), **Table 1**)) and its disulfide-free analog p(BAP-ABOL). For the p(CBA-ABOL), a homogeneously dispersed fluorescence was observed both in the cytoplasm and in the nucleus, whereas for the p(BAP-ABOL) many micro-sized aggregated clumps were located in the perinuclear space. The results may serve as an indication that the disulfide-containing poly(amido amine) is degraded relatively quickly in the cytosol by reductive cleavage of the disulfide bonds, resulting in a diffuse distribution of polymer fragments inside the cells. In contrast, the slow, or absence of, degradation of p(BAP-ABOL) may result in the formation of aggregates. Transfection experiments in COS-7 cells showed that the polyplexes of p(CBA-ABOL) induce about 20-fold higher transfection efficiency than those of p(BAP-ABOL). Fluorescence labeling of DNA provides similar results. Kim *et al.* and our group showed that disulfide-containing poly(amido ethyleneamine) (SS-PAE, see **Table 1**) and its analog pEI, lacking the disulfide groups, mediate different intracellular distributions of DNA [42]. A dispersed DNA fluorescence was observed in the cytoplasm after 24 h gene transfection with SS-PAE, where for pEI many micro-sized aggregated clumps of DNA fluorescence were located inside the cells. In another study, Park *et al.* investigated the degradation profiles of disulfide-containing pEIs by fluorescence microscopy using the probe–probe dequenching effect of BODIPY-FL fluorescent dye. This study indicated that full degradation of the polymers inside the cells occurs within 4 h [28]. A number of other studies on the fate of disulfides in the extra- and intracellular environment have been performed [75,76] and some of these have been outlined in a recent review paper [17]. It appears that disulfide degradation mainly proceeds in the cytosol and in the nucleus. However, depending on the type of cells and the polymer constructs, the degradation can also occur in the micro environment of the cell surface [70,77], the endosomes and the lysosomes [78,79]. Further studies are thus needed to understand the factors governing the degree of degradation at specific locations.

Gel electrophoresis has also been used as a tool to study gene delivery factors that are triggered by disulfide-based cationic polymers. We have examined gene delivery from polyplexes of poly(amido amine) copolymers p($HMBA_x$/CBA_y-AEP) containing various amounts of disulfide linkages, determined by the ratios x/y of the HMBA and CBA monomeric units, respectively. Gel electrophoresis experiments showed that a limited amount of disulfide linkages in the repeating bisacrylamide units ($y \geq 60\%$) of the copolymers is necessary to afford sufficient DNA release in a reductive environment. Transfection experiments in COS-7 revealed that the disulfide-containing poly(amido amine) p(CBA-AEP) (**Table 1**) induced threefold higher transfection efficiency than its analog without the disulfide p(HMBA-AEP) [41].

Taken together, these experiments suggest that disulfide-based cationic polymers efficiently release the genes inside the cells via the cleavage of disulfides, thereby yielding higher transfection efficiencies.

4.5 Effect of disulfides on cytotoxicity

Although cationic polymers like pEI exhibit relatively effective gene transfection, their therapeutic application is

seriously hampered by their high cytotoxicity. Further studies on cytotoxicity of cationic polymers such as pEI and pLL showed that various structural properties of these polymers affect their cytotoxicity, including molecular weight [80], charge density [81], amine type [82,83], topological structure and conformational flexibility [81]. The mechanisms of cytotoxicity caused by cationic polymers are not fully understood. For pEI-mediated transfection, a two-stage cytotoxicity has been proposed [84,85]. In the first stage, free pEI may destabilize the cellular membrane, inducing necrosis-related cytotoxicity. The purification of polyplexes from free pEI indeed leads to lower cytotoxicity [86]. In the second stage, free pEI dissociated from the polyplexes inside the cells may interact with the negatively charged mitochondrial membrane, leading to cellular apoptosis. Thus, the cytotoxicity in this stage could be diminished when cationic polymers are intracellularly degraded into small pieces. Since disulfide bonds are intracellularly degradable, incorporation of disulfide linkages between relatively short cationic oligomer or polymer chains may lead to low cytotoxicity in gene delivery.

Many studies suggest that disulfide-based cationic polymers indeed have much lower cytotoxicities than their non-degradable analogs [87]. We established that disulfide-based poly(amido amine)s have lower cytotoxicity profiles than their analogs lacking the disulfide [36,41]. Park *et al.* showed that disulfide-containing pEIs have significantly lower cytotoxicity than 25 kDa linear pEI against Caco-2 cells (> 100 μg/ml vs. 20 μg/ml) [28]. However, structural properties of the polymer can also dominate the cytotoxicity, as was demonstrated by Davis *et al.* for β-cyclodextrin-containing cationic polymers. Introduction of disulfide bonds did not result in a decrease in cytotoxicity, and it was shown that the cytotoxicity was dependent on different types of amino groups present in these polymers [88].

5. Conclusions

Cationic polymers are promising candidates as non-viral vectors for gene delivery. At present, various extra- and intracellular barriers have been identified that limit efficient gene transfection. Development of multifunctional smart cationic polymers that are able to overcome these barriers is certainly the future direction of gene delivery. The design of cationic polymers that incorporate bioreducible disulfide moieties in their chains offers many opportunities to arrive at smart gene carriers.

The presence of disulfide linkages in cationic polymers favorably influences the gene delivery properties of the polymers by enhanced DNA binding ability, triggered de-shielding of 'stealth' groups, increased buffer capacity and facilitated DNA release. Moreover, the facile intracellular degradation generally leads to a decreased cytotoxicity. These positive effects thus result in enhanced transfection efficiency. The results summarized in this review strongly suggest that multifunctionalized disulfide-based cationic polymers will be the next generation in non-viral gene delivery systems.

6. Expert opinion

During the last few decades, there has been reported a great variation of polymers that show more or less efficient gene delivery properties. However, although polymeric gene delivery vehicles have distinct advances over viral gene vectors with respect to immunogenicity, mutagenicity and ease of production, their transfection efficiency remains far behind. Therefore, improvement of transfection efficiency of polymeric gene vectors by overcoming efficiently all hurdles in the pathway of targeted delivery to expression is the main direction of future research. This implies that polymeric vectors must have virus-like properties, which can only be attained if the polymers are able to fulfill a multitude of functions in the spatio-temporal domain. Highly efficient, virus-like gene transfection cannot be expected from simple polymeric structures; instead multifunctional polymers are needed that respond to various biological stimuli in time and place. To this end, the use of stimulus-sensitive linkers in cationic polymers may provide interesting opportunities to increase transfection and to contribute to the understanding of the non-viral delivery process.

Although also other stimuli-sensitive linkers, such as acid-labile linkers [89,90], have been investigated, the use of the redox-sensitive disulfide linker is the most straightforward approach for intracellular disassembly of polyplexes, because there exists a large difference in redox potential between the extracellular environment and the intracellular environment. Degradation profiles of acid-liable linkers (e.g., esters) are not so easily controlled and are dependent on subtle structural properties. For instance, amino groups in poly(amino ester)s can promote the hydrolysis rate of acid-liable esters under physiological conditions [91,92], leading to undesired degradation of the polymer in the extracellular stage. In contrast, the disulfide linker is chemically stable under physiological conditions, but is prone to fast degradation in the intracellular reducing environment. This property is particularly on-demand for an ideal gene delivery carrier that is stable in the extracellular environment, but is degraded inside the cell to release DNA. Therefore, the presence of disulfide moieties in cationic polymers is expected to be one of the factors contributing to the ideal gene delivery carrier in the near feature.

It should be noted that inside the cell the reductive cleavage of the disulfide linker is regulated by reductive agents such as glutathione, but that the levels of intracellular reducing potential may be different in different types of cells, due to different capacities for glutathione synthesis to maintain the reductive environment [93]. Thus, disulfide degradation profiles can by influenced by the glutathione levels. Some studies suggest that an optimal glutathione level inside cells is needed to result in the highest transfection [17]. Therefore, further

examinations with disulfide-based cationic polymers on gene transfection *in vitro* and *in vivo* are necessary to fully understand the effects of disulfide cleavage.

In this review, we have demonstrated that the presence of disulfide bonds can play an important role in the gene delivery process. However, it should be stressed that additional functional groups certainly need to be incorporated into disulfide-based cationic polymers to further enhance the transfection efficiency. Moreover, these different functionalities have to be present at an optimal degree and ratio to arrive at virus-mimicking polymeric carriers that are capable of effectively overcoming the various gene delivery barriers and lead to highly efficient gene transfection.

Declaration of interest

The authors state no conflict of interest and have received no payment in the preparation of this manuscript.

Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. El-Aneed A. An overview of current delivery systems in cancer gene therapy. J Control Release 2004;94:1-14
2. Guinn BA, Mulherkar R. International progress in cancer gene therapy. Cancer Gene Ther 2008;15:765-75
3. Chen YC, Huang L. Tumor-targeted delivery of siRNA by non-viral vector: safe and effective cancer therapy. Expert Opin Drug Deliv 2008;5:1301-11
4. Verma IM, Somia N. Gene therapy – promises, problems and prospects. Nature 1997;389:239-42
5. Thomas CE, Ehrhardt A, Kay MA. Progress and problems with the use of viral vectors for gene therapy. Nat Rev Genet 2003;4:346-58
6. Li S, Huang L. Non-viral gene therapy: promises and challenges. Gene Ther 2000;7:31-4
7. Pack DW, Hoffman AS, Pun S, Stayton PS. Design and development of polymers for gene delivery. Nat Rev Drug Discov 2005;4:589-93

•• **Good review about gene delivery principles and cationic polymers as gene carriers.**

8. Han S, Mahato RI, Sung YK, Kim SW. Development of biomaterials for gene therapy. Mol Ther 2000;2:302-17
9. Merdan T, Kopecek J, Kissel T. Prospects for cationic polymers in gene and oligonucleotide therapy against cancer. Adv Drug Deliv Rev 2002;54:715-58
10. Anwer K, Rhee BG, Mendiratta SK. Recent progress in polymeric gene delivery systems. Crit Rev Ther Drug Carr Syst 2003;20:249-93
11. Schaffert D, Wagner E. Gene therapy progress and prospects: synthetic polymer-based systems. Gene Ther 2008;15:1131-8
12. Wiethoff CM, Middaugh CR. Barriers to nonviral gene delivery. J Pharm Sci 2003;92:203-17
13. Nishikawa M, Huang L. Nonviral vectors in the new millennium: delivery barriers in gene transfer. Nucleic Acid Res 2001;12:861-70
14. Ganta S, Devalapally H, Shahiwala A, Amiji M. A review of stimuli-responsive nanocarriers for drug and gene delivery. J Control Release 2008;126:187-204
15. Nagasaki T, Shinkai S. The concept of molecular machinery is useful for design of stimuli-responsive gene delivery systems in the mammalian cell. J Incl Phenom Macrocycl Chem 2007;58:205-19
16. Wu G, Fang YZ, Yang S, et al. Glutathione metabolism and its implications for health. J Nutr 2004;134:489-92
17. Manickam DS, Oupický D. Polyplex gene delivery modulated by redox potential gradients. J Drug Target 2006;14:519-26
18. Wong SY, Pelet JM, Putnam D. Polymer systems for gene delivery – past, present, and future. Prog Polym Sci 2007;32:799-837

•• **Good review about recent progress in cationic polymers as gene carriers.**

19. Rybak SL, Murphy RF. Primary cell cultures form murine kidney and heart differ in endosomal pH. J Cell Physiol 1998;176:216-22
20. Vaughan EE, Dean DA. Intracellular trafficking of plasmids during transfection is mediated by microtubules. Mol Ther 2006;13:422-8
21. Suh J, Wirtz D, Hanes J. Efficient active transport of gene nanocarriers to the cell nucleus. Proc Natl Acad Sci USA 2003;100:3878-82
22. Schaffer DV, Fidelman NA, Dan N, Lauffenbuger DA. Vector unpacking as a potential barrier for receptor mediated polyplex gene delivery. Biotechnol Bioeng 2000;67:598-606
23. Bruner S, Sauer T, Carotta S, et al. Cell cyclo dependence of gene transfer by lipoplex, polyplex and recombinant adenovirus. Gene Ther 2000;7:401-7
24. Ryan KJ, Wente SR. The nuclear pore complex: a protein machine bridging the nucleus and cytoplasm. Curr Opin Cell Biol 2000;12:361-71
25. Gorlich D, Kutay U. Transport between the cell nucleus and the cytoplasm. Nat Rev Mol Cel Biol 1999;15:607-60
26. Pichon C, Lecam E, Guerin B, et al. Poly [Lys-(AEDTP)]: a cationic polymer that allows dissociation of pDNA/cationic polymer complexes in a reductive medium and enhances polyfection. Bioconjug Chem 2002;13:76-82
27. Zugates GT, Anderson DG, Little SR, et al. Synthesis of poly(beta-amino ester)s with thiolreactive side chains for DNA delivery. J Am Chem Soc 2006;128:12726-34
28. Lee Y, Mo H, Koo H, et al. Visualization of the degradation of a disulfide polymer, linear poly(ethylenimine sulfide), for gene delivery. Bioconjug Chem 2007;18:13-18

• **Interesting article in which degradation of disulfide-containing cationic polymers was studied by fluorescence microscopy using the probe–probe dequenching effect of BODIPY-FL fluorescence dye.**

29. Oupický D, Parker AL, Seymour LW. Laterally stabilized complexes of DNA with linear reducible polycations: strategy for triggered intracellular activation of DNA delivery vectors. J Am Chem Soc 2002;124:8-9
30. You YZ, Manickam DS, Zhou QH, Oupický D.

Reducible poly(2-dimethylaminoethyl methacrylate): synthesis, cytotoxicity, and gene delivery activity. J Control Release 2007;122:217-25

• **New method on the synthesis of disulfhydryl oligoamines via living polymerization reaction for preparation of disulfide-containing cationic polymers for gene delivery.**

31. Read ML, Singh S, Ahmed Z, et al. A versatile reducible polycation-based system for efficient delivery of a broad range of nucleic acids. Nucleic Acid Res 2005;33:e86

32. Manickam DS, Oupický D. Multiblock reducible copolypeptides containing histidine-rich and nuclear localization sequences for gene delivery. Bioconjug Chem 2006;17:1395-403

• **Interesting article about reducible cationic polymers containing nuclear localization sequences for nucleus-targeting gene delivery.**

33. Sun YX, Zeng X, Meng QF, et al. The influence of RGD addition on the gene transfer characteristics of disulfide-containing polyethyleneimine/DNA complexes. Biomaterials 2008;29:4356-65

34. Ou M, Wang XL, Xu RZ, et al. Novel biodegradable poly(disulfide amine)s for gene delivery with high efficiency and low cytotoxicity. Bioconjug Chem 2008;19:626-33

35. Christensen LV, Chang CW, Yockman JW, et al. Reducible poly(amido ethylenediamine) for hypoxia-inducible VEGF delivery. J Control Release 2007;118:254-61

36. Lin C, Zhong ZY, Lok MC, et al. Novel bioreducible poly(amido amine)s for highly efficient gene delivery. Bioconjug Chem 2007;18:138-45

37. Gosselin MA, Guo W, Lee RJ. Efficient gene transfer using reversibly cross-linked low molecular weight polyethylenimine. Bioconjug Chem 2001;12:989-94

38. Gosselin MA, Guo W, Lee RJ. Incorporation of reversibly cross-linked polyplexes into LPDII vectors for gene delivery. Bioconjug Chem 2002;13:1044-53

39. Lin C, Zhong ZY, Lok MC, et al. Random and block copolymers of bioreducible poly(amido amine)s with high- and low-basicity amino groups: Study of DNA condensation and buffer capacity on gene transfection. J Control Release 2007;123:67-75

40. Piest M, Lin C, Mateos-Timoneda MA, et al. Novel poly(amido amine)s with bioreducible disulfide linkages in their diamino-units: structure effects and in vitro gene transfer properties. J Control Release 2008;130:38-45

41. Lin C, Zhong ZY, Lok MC, et al. Linear poly(amido amine)s with secondary and tertiary amino groups and variable amounts of disulfide linkages: synthesis and in vitro gene transfer properties. J Control Release 2006;116:130-7

42. Christensen LV, Chang CW, Kim WJ, et al. Reducible poly(amido ethylenimine)s designed for triggered intracellular gene delivery. Bioconjug Chem 2006;17:1233-40

43. Mateos-Timoneda MA, Lok MC, Hennink WE, et al. Poly(amido amine)s as gene delivery vectors: effects of quaternary nicotinamide moieties in the side chains. ChemMedChem 2008;3:478-86

44. Lin C, Blaauboer C-J, Timoneda MM, et al. Bioreducible poly(amido amine)s with oligoamine side chains: synthesis, characterization, and structural effect on gene delivery. J Control Release 2008;126:166-74

45. Lin C, Engbersen JFJ. Effects of chemical functionalities in poly(amido amine)s for non-viral gene transfection. J Control Release 2008;132:267-72

46. Hartmann L, Haefele S, Peschka SR, et al. Tailor-made poly(amidoamine)s for controlled complexation and condensation of DNA. Chem Eur J 2008;14:2025-33

47. Hartmann L, Haefele S, Peschka-Suess R, et al. Sequence positioning of disulfide linkages to program the degradation of monodisperse poly(amidoamines). Macromolecules 2007;40:7771-6

48. Hartmann L, Krause E, Antonietti M, Borner HG. Solid-phase supported polymer synthesis of sequence-defined, multifunctional poly(amidoamines). Biomacromolecules 2006;7:1239-44

• **Interesting article about the synthesis of well-defined poly(amido amine)s via solid-phase organic synthesis.**

49. Oupický D, Carlisle RC, Seymour LW. Triggered intracellular activation of disulfide crosslinked polyelectrolyte gene delivery complexes with extended systemic circulation in vivo. Gene Ther 2001;8:713-24

50. Trubetskoy VS, Loomis A, Slattum PM, et al. Caged DNA does not aggregate in high ionic strength solutions. Bioconjug Chem 1999;10:624-628

51. Neu M, Sitterberg J, Bakowsky U, Kissel T. Stabilized nanocarriers for plasmids based upon cross-linked poly(ethylene imine). Biomacromolecules 2006;7:3428-38

52. Neu M, Germershaus O, Mao S, et al. Crosslinked nanocarriers based upon poly(ethylene imine) for systemic plasmid delivery: in vitro characterization and in vivo studies in mice. J Control Release 2007;118:370-80

53. McKenzie DL, Kwok KY, Rice KG. A potent new class of reductively activated peptide gene delivery agents. J Biol Chem 2000;275:9970-9977

54. McKenzie DL, Smiley E, Kwok KY, Rice KG. Low molecular weight disulfide cross-linking peptides as nonviral gene delivery carriers. Bioconjug Chem 2000;11:901-9

55. Chen CP, Kim JS, Liu DJ, et al. Synthetic PEGylated glycoproteins and their utility in gene delivery. Bioconjug Chem 2007;18:371-8

56. Kwok KY, Park Y, Yang YS, et al. In vivo gene transfer using sulfhydryl cross-linked PEG-peptide/glycopeptide DNA co-condensates. J Pharm Sci 2003;92:1174-85

57. Miyata K, Kakizawa Y, Nishiyama N, et al. Block catiomer polyplexes with regulated densities of charge and disulfide cross-linking directed to enhance gene expression. J Am Chem Soc 2004;126:2355-61

58. Kakizawa Y, Harada A, Kataoka K. Environment-sensitive stabilization of core-shell structured polyion complex micelle by reversible cross-linking of the core through disulfide bond. J Am Chem Soc 1999;121:11247-8

59. Wang YX, Zhu Y, Hu QL, Shen JC. Construction of caged polyplexes with a reversible intracellular unpacking property to improve stability and transfection. Acta Biomater 2008;4:1235-43

60. Choi S, Lee KD. Enhanced gene delivery using disulfide-crosslinked low molecular weight polyethylenimine with listeriolysin o-polyethylenimine disulfide conjugate. J Control Release 2008;131:70-6

61. Neu M, Germershaus O, Mao S, et al. Crosslinked nanocarriers based upon poly(ethylene imine) for systemic plasmid delivery: In vitro characterization and in vivo studies in mice. J Control Release 2007;118:370-80

62. Cavallaro G, Campisi M, Licciardi M, et al. Reversibly stable thiopolyplexes for intracellular delivery of genes. J Control Release 2006;115:322-34

63. Peng Q, Zhong ZL, Zhuo RX. Disulfide cross-linked polyethylenimines (PEI) prepared via thiolation of low molecular weight PEI as highly efficient gene vectors. Bioconjug Chem 2008;19:499-506

64. Meylan WM, Howard PH. Estimating log P with atom/fragments and water solubility with log P. Perspect Drug Discov Des 2000;19:67-84

65. Ogris M, Brunner S, Schuller S, et al. Pegylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. Gene Ther 1999;6:595-605

66. Verbaan FJ, Oussoren C, Snel CJ, et al. Steric stabilization of poly(2-(dimethylamino)ethyl methacrylate)-based polyplexes mediates prolonged circulation and tumor targeting in mice. J Gene Med 2004;6:64-75

67. Walker GF, Fella C, Pelisek J, et al. Toward synthetic viruses: endosomal pH-triggered deshielding of targeted polyplexes greatly enhances gene transfer in vitro and in vivo. Mol Ther 2005;11:418-25

68. Knorr V, Allmendinger L, Walker GF, et al. An acetal-based PEGylation reagent for pH-sensitive shielding of DNA polyplexes. Bioconjug Chem 2007;18:1218-25

69. Takae S, Miyata K, Oba M, et al. PEG-detachable polyplex micelles based on disulfide-linked block catiomers as bioresponsive nonviral gene vectors. J Am Chem Soc 2008;130:6001-9

• **Interesting article about PEG de-shielding from polyplexes via disulfide cleavage.**

70. Morre DJ, Morre DM. Cell surface NADH oxidases (ECTO-NOX proteins) with roles in cancer, cellular time-keeping, growth, aging and neurodegenerative diseases. Free Radical Res 2003;37:795-808

71. Boussif O, Lezoualch F, Zanta MA, et al. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc Natl Acad Sci USA 1995;92:7297-301

72. Richardson SCW, Pattrick NG, Man YKS, et al. Poly(amido amine)s as potential nonviral vectors: ability to form interpolyelectrolyte complexes and to mediate transfection in vitro. Biomacromolecules 2001;2:1023-8

73. Griffiths PC, Paul A, Khayat Z, et al. Understanding the mechanism of action of poly(amidoamine)s as endosomolytic polymers: correlation of physicochemical and biological properties. Biomacromolecules 2004;5:1422-7

74. Haensler J, Szoka FJ. Polyamidoamine cascade polymers mediate efficient transfection of cells in culture. Bioconjug Chem 1993;4:372-9

75. Breunig M, Lungwitz U, Liebl R, Goepferich A. Breaking up the correlation between efficacy and toxicity for nonviral gene delivery. Proc Natl Acad Sci USA 2007;104:14454-9

76. Yang J, Chen H, Vlahov IR, et al. Evaluation of disulfide reduction during receptor-mediated endocytosis by using FRET imaging. Proc Natl Acad Sci USA 2006;103:13872-7

77. Blacklock J, You Y-Z, Zhou Q-H, et al. Gene delivery in vitro and in vivo from bioreducible multilayered polyelectrolyte films of plasmid DNA. Biomaterials 2009;30:939-50

78. Sahaf B, Heydari K, Herzenberg LA, Herzenberg LA. The extracellular microenvironment plays a key role in regulating the redox status of cell surface proteins in HIV-infected subjects. Arch Biochem Biophys 2005;434:26-32

79. Donoghue N, Yam PTW, Jiang XM, Hogg PJ. Presence of closely spaced protein thiols on the surface of mammalian cells. Protein Sci 2000;9:2436-45

80. Fischer D, Bieber T, Li YX, et al. A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity. Pharm Res 1999;16:1273-9

81. Fischer D, Li Y, Ahlemeyerc B, et al. In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. Biomaterials 2003;24:1121-31

82. Hill IRC, Garnett MC, Bignotti F, Davis SS. In vitro cytotoxicity of poly(amidoamine)s: relevance to DNA delivery. Biochim Biophys Acta 1999;19:161-74

83. Ferruti P, Knobloch S, Ranucci E, et al. A novel modification of poly(L-lysine) leading to a soluble cationic polymer with reduced toxicity and with potential as a transfection agent. Macromol Chem Phys 1998;199:2565-75

84. Moghimi SM, Symonds P, Murray JC, et al. A two-stage poly(ethylenimine)-mediated cytotoxicity: implications for gene transfer/therapy. Mol Ther 2005;11:990-5

85. Godbey WT, Wu KK, Mikos AG. Poly(ethylenimine)-mediated gene delivery affects endothelial cell function and viability. Biomaterials 2001;22:471-80

86. Boeckle S, von Gersdorff K, van der Piepen S, et al. Purification of polyethylenimine polyplexes highlights the role of free polycations in gene transfer. J Gene Med 2004;6:1102-11

87. Luten J, van Nostruin CF, De Smedt SC, Hennink WE. Biodegradable polymers as non-viral carriers for plasmid DNA delivery. J Control Release 2008;126:97-110

• **Good review about recent progress in biodegradable cationic polymers as gene carriers.**

88. Hwang SJ, Bellocq NC, Davis ME. Effects of structure of β-cyclodextrin-containing polymers on gene delivery. Bioconjug Chem 2001;12:280-90

89. Guo X, Szoka FC. Chemical approaches to triggerable lipid vesicles for drug and gene delivery. Acc Chem Res 2003;36:335-41

90. Knorr V, Russ V, Allmendinger L, et al. Acetal linked oligoethylenimines for use as pH-sensitive gene carriers. Bioconjug Chem 2008;19:1625-34

91. Zhong ZY, Song Y, Engbersen JFJ, et al. A versatile family of degradable non-viral gene carriers based on hyperbranched poly(ester amine)s. J Control Release 2005;109:317-29

92. Lynn DM, Langer R. Degradable poly(beta-amino esters): synthesis, characterization, and self-assembly with plasmid DNA. J Am Chem Soc 2000;122:10761-8

93. Wang W, Ballatori N. Endogenous glutathione conjugates: occurrence and biological functions. Pharmacol Rev 1998;50:335-56

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