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REVIEW

A review of the developments of characteristics of PEI derivatives for gene delivery applications

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ABSTRACT: Redox-active stimuli have gained a great deal of interest as an indicating factor for designing bioresponsive matrices in gene delivery. Hence, a wide range of gene carriers has been designed incorporating the redox-stimuli characteristics. The most important type of gene carriers is the class of redox responsive polymers. Among them, disulfide incorporated redox-responsive polyethyleneimine (PEI) and its derivatives, as a result of their outstanding DNA entrapping characteristics and their intrinsic endosomolytic activity, have attracted considerable attention in recent studies. The review presents the main developments of the characteristics of PEI derivatives and their applications in gene delivery. It is found that despite the uniquely stated characteristics, the noncleavable structure of conventional PEI (high molecular weight PEI: 25k), which makes it a nondegradable material, as well as the frequent inclusion of positively charged amino groups, which reduces its blood circulation period, render conventional PEI a very toxic material for gene-delivery applications. The extremely high cellular toxicity of conventional PEI has restricted its administration for real *in-vivo* physiological media. Recent studies have shown that employing low molecular weight PEI cross-linked by disulfide linkages (SS-PEI) and assembling low molecular weight disulfide linkages PEI (LMW SS-PEI) with bio-detachable anionic groups were two successful approaches for increasing bioavailability of the PEI-based gene carriers, while keeping outstanding cellular transfection.
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INTRODUCTION

Biological media have a redox environment that seriously affects the sustainability of cellular homeostasis as well as the redox potential gradient between extra and intra cellular and/or subcellular organelles. The redox potential gradient between the oxidizing extracellular space and the reducing environment of intracellular compartments has attracted a great deal of interest in studies carried out in the field of gene delivery systems.[1,2] From these studies, it is well known that employing gene carriers that rely on the redox potential gradient results in more selectivity and, hence, lower immune system response. Among the gene carriers, redox-stimuli responsive, or bioreducible, polymers offer particular characteristics, such as excellent DNA condensing ability as well as high structure flexibility.[1–3] The most attractive approach to achieving the redox-response ability of these polymers is integrating disulfide bonds into the backbone or side chains of the polymers. It has been found that incorporating the disulfide bonds in the polymeric structures not only results in more biodegradability, but also limits their cytotoxicity. Moreover, several important features such as site-, timing-, and duration period-specific gene expression are improved as a direct consequence of disulfide incorporation into the polymer-structure of gene carriers.[4,5]

There are several classes of redox-responsive polymers employed in gene delivery applications. Among them, bioreducible polyethylenimine (PEI) derivatives, owing to their outstanding gene encapsulation efficiency and their intrinsic endosomolytic activity, are the main redox responsive polymeric gene carriers. Many studies of PEI-based gene delivery systems have concluded that, despite displaying high gene encapsulation efficiency, their bioapplications were seriously affected by their high cytotoxicity. High toxicity limits the efficiency of transfer *in vitro* and

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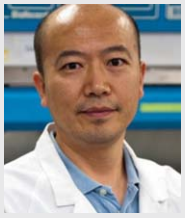

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especially *in vivo*.[6,7] Several approaches have been reported to increase the gene transfection efficiency of PEI-based gene carriers while decreasing their intrinsic high cytotoxicity. However, no comprehensive review has been presented of these studies. This article presents such a review, evaluating the main attempts to improve the characteristics of PEI and its derivatives for gene delivery. First, an overview of redox environment in biological systems is given, followed by a review of the leading approaches that achieve reduced cytotoxicity as well as enhanced transfection efficiency, and which are considered to be two main prerequisites when designing an appropriate gene delivery vehicle.[8]

Redox Environment in Biological Systems and Its Significance in the Designing of Gene Delivery Systems

The biological status of a cell in respect of a wide variety of redox couples, such as glutathione (GSH/GSSG), nicotinamide adenine dinucleotide phosphate (NADPH/NADPþ) and thioredoxin (TRXred/TRXox), is referred to as the redox environment of the biological system. Each of the stated couples has its own specific redox potential. Considering the much higher concentration of GSH in comparison with the other redox couples, GSH is the main effective factor in adjusting redox potential, both in and out cellular environments.[4,5]

GSH, as a very important class of antioxidants, is synthesized from L-glutamate in a reaction catalyzed by gamma-glutamylcysteine synthesize (γ-GCS) and GSH synthesize. The main role of GSH is to regulate the protein structure and function, cell signaling, proliferation and apoptosis. GSH, after being produced, is transported to specific intracellular sections and extracellular environment. Interestingly, there is a considerable difference in GSH concentrations between intracellular and extracellular environments, being 10 μM and $\sim$2.8 μM, respectively. This variation of GSH concentration results in a GSH-induced redox potential gradient between the extra and intracellular environment. Hence, in a redox-responsive gene delivery system, a high degree of stability may be observed in extracellular regions due to the low GSH concentration, while high concentration of GSH in other regions results in rapid release of genes from polyplexes (gene-polymer complexes) induced by cleavage of redox-responsive bonds in the intracellular space (Figure 1).[4,5,9]

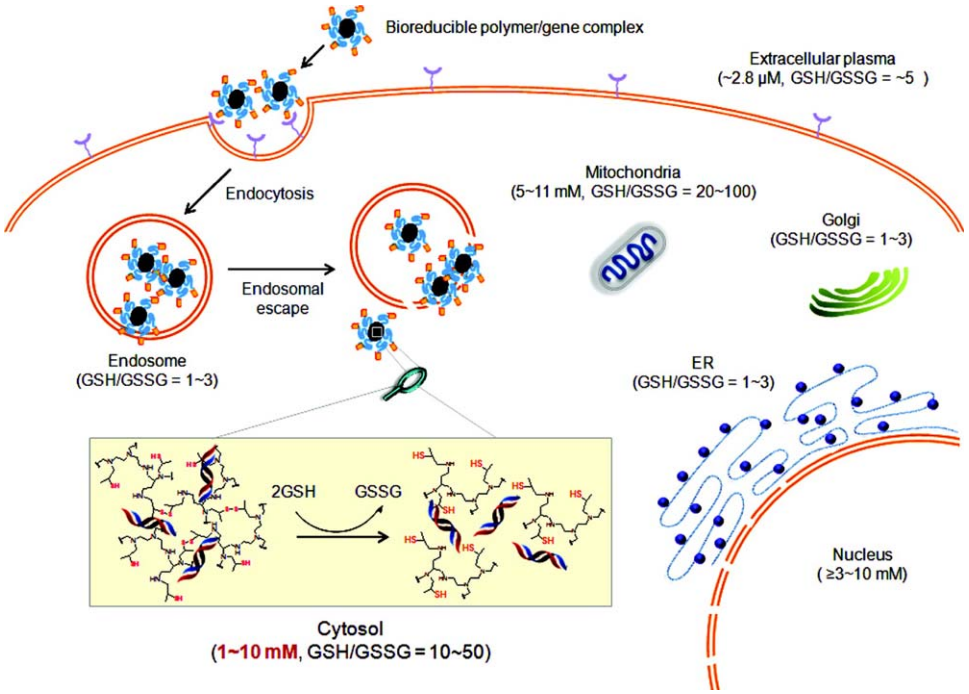

Figure 1. Schematic illustrations of the gene delivery by bioreducible polymeric vector. Bioreducible polymers and DNA form nanosized polyplexes that remain stable during circulation and within the extracellular region. GSH-mediated thiol-disulfide exchange reactions trigger rapid dissociation of polymer under the reductive environment.[4] [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Redox Responsive Polyethylenimine

PEI is a cationic polymer widely used in nonviral transfection of genes owing to its outstanding DNA entrapping characteristic and intrinsic endosomolytic activity. PEI-based homopolymers are classified by considering their molecular weight. Also, the transfection efficacy and level of toxicity of PEI strongly depend on its molecular weight (MW) as well as its structure.[10]

Although high molecular weight PEI (HMW PEI), by forming stable polyplexes, is able to support entrapped genes against degradation by lysosomal enzymes, its noncleavable structure induces greater cytotoxicity. On the other hand, it has been demonstrated that low molecular weight PEI has much less toxicity but very low transfection activity.[5,9]

Looking for an effective approach to overcome the trade-off between transfection and toxicity resulted in a common strategy to bind low-molecular weight PEI into the HMW PEI with esters, glycosides and disulfides. Among the biodegradable linkages, introduction of disulfide bonds provides an outstanding way to release the DNA into the cytosol via redox reaction in the presence of GSH. Two main strategies have been employed in order to incorporate disulfide bonds into the PEI. The first approach is based on utilizing cross-linkers to supply a linkage with disulfide moieties during the construction of the gene carrier. Another approach is a two-step strategy comprising prethiolation of PEI and then oxidation of thiolated PEIs (PEI-SH) to cross-linked polymers.[4]

Prethiolation can be performed by incorporation of thiol groups into the PEI chain structure. Oxidation of the products gives disulfide cross-linked PEI (PEI-SS). The degree of thiolation (the average number of thiol groups on a PEI molecule) can be controlled by adjusting the ratio of the PEI/thiolating agent. Peng *et al.* employed methylthirrane for the thiolating of LMW branched PEI ($M_w = 800$ Da) (Figure 2). In their studies, they used DMSO as the oxidating agent for synthesizing disulfide cross-linked PEIs from PEI-SH.[11,12]

After the formation of the polyplexes, the diameter of the nanoparticles was below 100 nm. Using HMW PEI resulted in more compact nanoparticles with plasmid DNA. It was shown that a higher degree of thiolation resulted in smaller nanoparticles (Figure 3).[11] Another important approach for thiolating PEI involves employing various kinds of crosslinkers containing disulfide linkages; this is the most common strategy for providing bioreducibility to polymers.[13–15]

Breunig and his colleagues investigated the effects of using PEI cross-linked via disulfide bonds (SS-PEI) compared to PEIs with different degrees of branching of the polymer on the release of siRNA into the cell cytoplasm. The studies revealed that by reducing the transfection efficiency of cells that took up polyplexes, the intracellular amount of siRNA for SS-PEI was similar to, or even higher than, the case of branched PEI.[16]

Click chemistry is a successful approach that has been employed for synthesizing various kinds of disulfide-containing cross-linked PEI.[4] For the first time, disulfide-containing hyperbranched PEI derivatives (PEI-SS-HP) were prepared by click chemistry for nonviral gene delivery. Click reaction was applied between the azide-functional PEIs as core and the monoalkyne-terminated PEIs on the outside, which resulted in PEI-SS-HP. Then, PEI-SS-HP was investigated for nonviral plasmid DNA

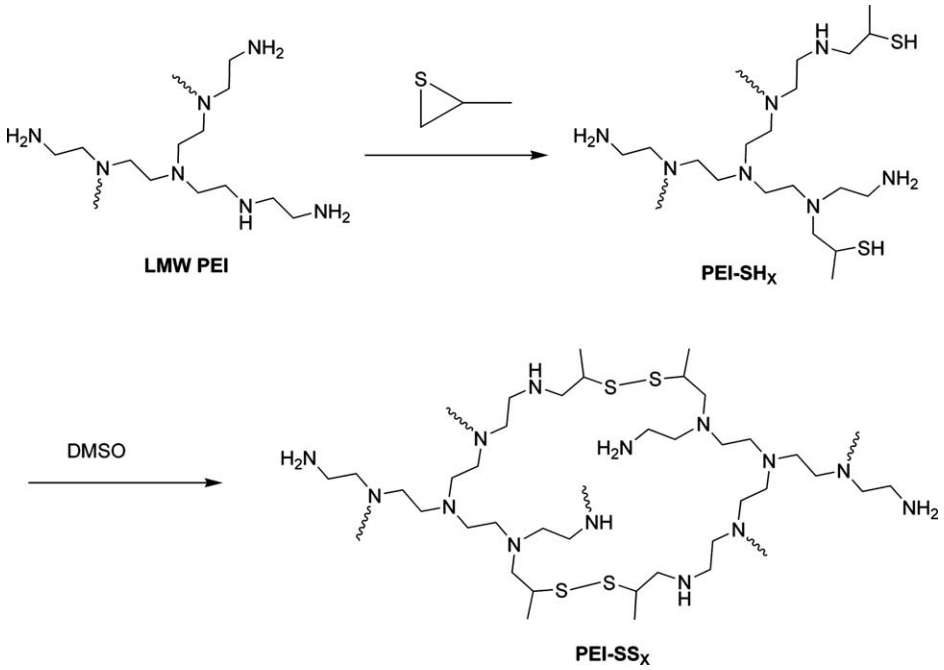

Figure 2. Schematic representation of the preparation of the disulfide-cross-linked LMW PEIs (PEI-SSX).[11]

delivery. The study found that PEI-SS-HP was able to bind plasmid DNA to positively charged nanoparticles.[14]

Toxicity of Bioreducible PEI

The cytotoxicity of PEI derivatives as a class of cationic polymers is a major problem for their biological applications.[17–19] Moreover, employing the PEI-based carriers is associated with induced immune response. It has been reported that the positively charged nature of the polyplexes can result in nonspecific interactions with negatively charged serum to form thrombi in the capillary, which carries the risk of perturbing the structure of the plasma membrane to induce high cytotoxicity and excessive immune responses.[20]

PEI has the ability to stimulate a systemic immune response. Some molecular mechanisms are involved in this response. PEI

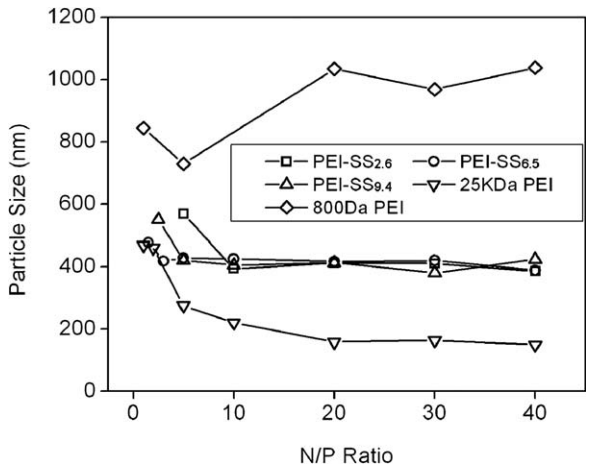

Figure 3. Particle size of the PEI/pDNA polyplexes measured by DLS.[11]

may stimulate the activation of genes with important immunostimulatory functions, but with minor costimulatory signals such as the costimulatory signal during T-cell activation. The immuno-stimulatory action of PEI in the absence of formulated plasmid DNA were related to the elevated levels of the genes involved in the Th1 and Th2 response and the activated adaptive immune response. On the other hand, it has been reported that some genes that are involved in specific cellular responses, including apoptosis, stress responses and oncogenesis, may be activated via employing PEI-DNA complexes. It was found that employing the PEI complexes causes a mixed Th1/Th2 response: activation of both CD8+ and CD4+ T cells, with a significant effect on CD4+; and FasL-mediated antigen-induced cell death. This systemic response is in a good agreement with the theory of immunostimulation by danger genes.[10]

Further with regards to immune system response, a series of studies have been undertaken to determine the different mechanisms involved in PEI administration cellular toxicity. However, the exact mechanisms of PEI toxicity remain unclear. It is found that HMW PEIs lead to increased cytotoxicity compared with LMW PEI. This higher toxicity may result from aggregation of huge clusters of the cationic polymer on the outer cell membrane, which thereby induces necrosis.[21]

Two types of cytotoxicity in PEI-mediated gene delivery may result: immediate and delayed toxicity. Before transfection, free PEIs cause cell death through membrane destabilization. After being internalized, free PEIs may cause delayed toxicity as a result of induced cell apoptosis. In one study, changing the gene expression in the treated cells and the elevated level of some special kinds of genes (specific for oxidative stress, inflammation, and cytotoxicity) were determined as toxicity mechanisms after transfection of the employed HMW PEI. The detected

genes with highly elevated expression levels, just 6 h after exposure, were E2f1, a cell cycle regulating transcription factor, and NfkBia, an important component of the NFκB machinery, involved in many proinflammatory and apoptosis/survival signaling pathways, as well as oxidative stress indicating phase II metabolizing enzyme Ugt1a2.[22]

In a later study, two distinct types of cell death, resulting from the employing of either free PEI (which acts within 2 h) or PEI/DNA complexes (which cause death 7–9 h after transfection) were reported for cell death. The first phase occurred immediately, determined by the changing of cell morphology and the destabilizing of the outer membranes, as well as cell detachment. The latest phase was a slower process leading to cell death, associated with cellular processing of PEI/DNA complexes, and is linked to successful transfection of PEI to the nucleus.[19]

Depolarizing mitochondria is another reported route of inducing toxicity after PEI transfection. Potential mechanisms for mitochondrial depolarization are indicated as direct mitochondrial membrane permeabilization via PEI or PEI polyplexes, activation of the mitochondrial permeability transition pores, and interference with mitochondrial membrane proton pumps.[23]

One possible way of overcoming the cytotoxicity is employing degradable disulfide-containing polymers instead of nondegradable polymers.[24] It was reported that disulfide containing PEI, PEI-SS$_X$, disregarding the thiol group content, has a lower cytotoxicity in comparison with HMW PEI (Figure 4).[11,12] The lower toxicity may be attributed to the reduction of disulfide-bonds that increases the disassembly rate for catiomer-nucleic acid complexes.[25] Another reason for the low cytotoxicity of disulfide-containing PEI is the reduced binding affinity of intracellular membranes, nucleic acids and proteins after the sharp drop in molecular weight of polycations as a direct consequence of the rapid intracellular reduction of disulfide bonds.[2]

Similar results have been reported elsewhere for PEI-based gene vectors that were prepared via employing a disulfide carbonate linker. The crosslinked PEIs that are prepared by combining a releasable disulfide carbonate linker with PEI 2k, simply PEI-SS-Cl, demonstrated significantly lower cytotoxicity compared with PEI 25k and Lipofectamine 2000, as two commercially available gene vectors.[26]

Another method to overcome the high level of cytotoxicity of PEI is based on binding PEI with anionic groups. Yeh *et al.* prepared a PEI derivative from binding hyaluronic acid (HA) as a negatively charged functional group to LMW PEI via disulfide bonds. This structure resulted in enhanced DNA transfection efficiency leading to lower cytotoxicity than for HMW PEI.[27]

Copolymerization is another interesting approach for reducing the intrinsic cytotoxicity of PEI derivatives. Copolymers, which can be prepared via grafting or *in-situ* polymerization of PEI via other macromolecules, have been widely used and their cell-cytotoxicity has been assessed. Li *et al.* prepared a ternary copolymer of mPEG-*b*-PLL-*g*-(SS-lPEI) by introducing disulfide bonds to graft low molecular weight linear polyethylenimine (lPEI) to a block copolymer of poly(L-lysine) (PLL) and poly(ethylene glycol) (PEG)for siRNA delivery. The ternary copolymer showed low toxicity as a result of a high degree of degradability.[28] Employing disulfide crosslinked p(HPMA-*co*-PDTEMA)-*b*-PEG polyplexes—where HPMA and PDTEMA represent hydroxypropyl methacrylamide and *N*-[2-(2-pyridyldithio)]ethyl methacrylamide (pHPMA), respectively—resulted in an excellent safety profile (no cytotoxicity was observed even at the highest dose investigated) plus a very low degree of nonspecific uptake.[17]

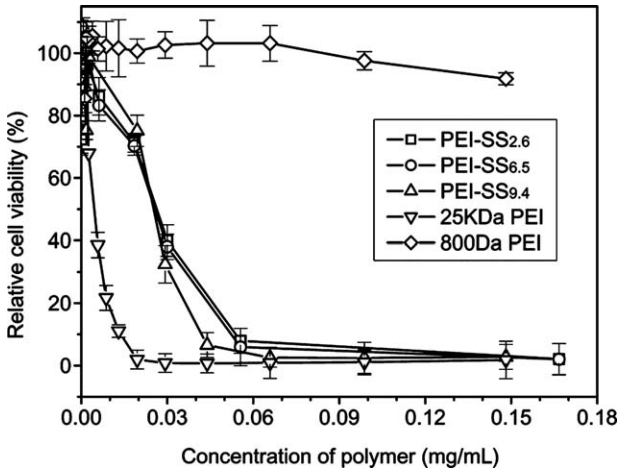

Figure 4. Relative cell viability at 24 h after the addition of PEI-SSX and 25 KDa PEI.[11]

Adjusting surface charge density of the employed catiomers is another approach that significantly affects the cytotoxicity of PEI derivatives.[29] The lower the Zeta potential of polyplexes, the less significant are the interactions with nonspecific negatively charged tissues and biological components; hence, there is a reduced level of toxicity as well as a reduced circulation time.[30–32]

PEGylation is another way of reducing cytotoxicity; it effectively enhances serum stability, circulation time and systemic targeted gene transfer characteristics of polyplexes.[33] Ping *et al.* investigated the cell viability characteristics of different cell types (PC-3, Hep G2, SKOV-3, and HeLa cell lines) using an MTT assay in the presence of PEI (25 kDa) and PEI derivatives. They prepared different complexes from neat PEI based on host-guest structure. The host part (MPC) of the structure was prepared by grafting MC11 peptide (as a targeting factor for FGFR) onto the *b*-cyclodextrin-crosslinked (LMW-PEI) backbone. The guest part (Ad-SS-PEG) was based on PEG attached to adamantyl (Ad) groups by disulfide bonds. Results indicated a much lower cytotoxicity of PEI-*b*-CD, MPC, and MPC/Ad-SS-PEG complexes in comparison with HMW PEI. Employing both cyclodextrin and PEG groups resulted in a lower relative amino (N group) density, and hence the lower cytotoxicity.[30]

In summary, the most successful approaches for overcoming the cytotoxicity of PEI gene carriers have employed LMW PEI with bio-cleavable linkages, introducing redox-responsive disulfide bonds, PEGylation and binding with anionic groups and bioavailable material.

Gene Transfection Efficiency of Bioreducible PEI

The gene transfection efficiency of bioreducible PEI has been widely investigated in previous studies. In series of polymer-protein interactions, such as an interaction with anionic cell-

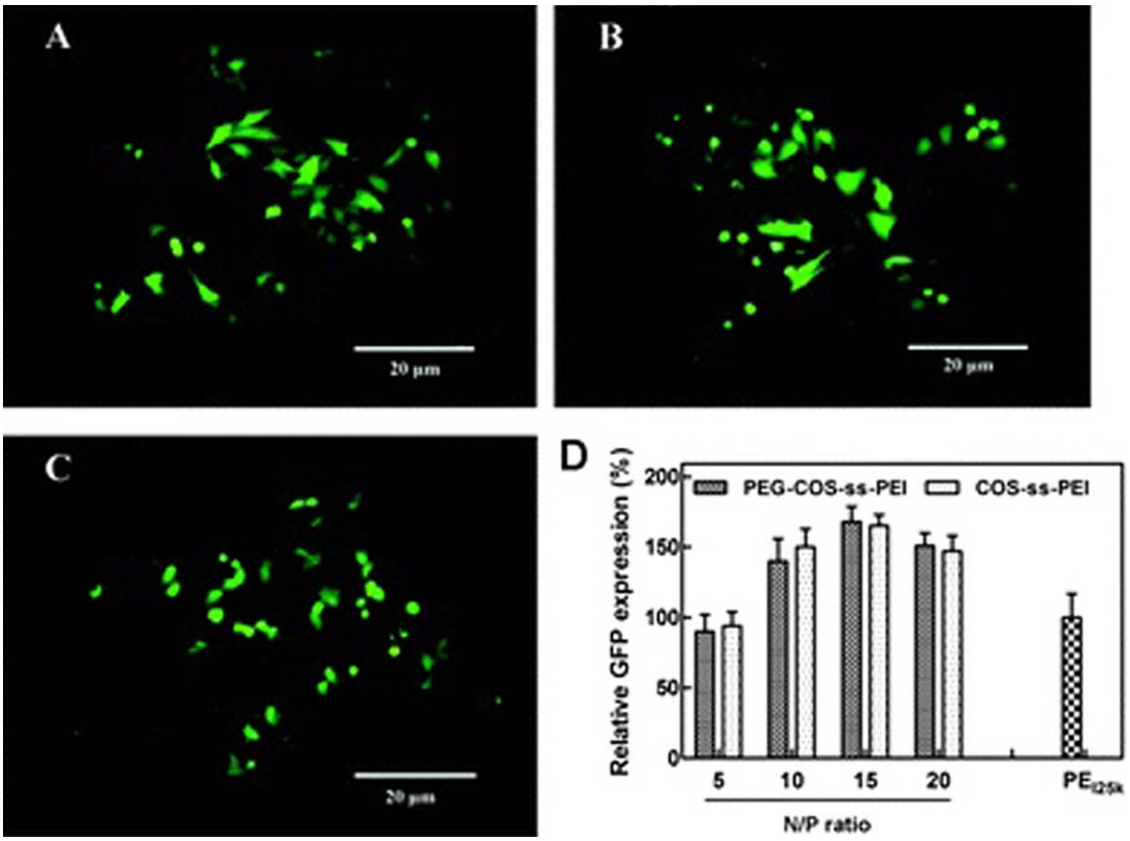

Figure 5. Transfection efficiency of the COS-SS-PEI based polyplexes: (A) the representative fluorescence image of the cells exposed to PEG-ss-COS-ss-PEI–DNA complex at the N/P ratio of 15/1, (B) the representative fluorescence image of the cells exposed to COS-ss-PEI–DNA complex at the N/P ratio of 15/1, (C) the representative fluorescence image of the cells exposed to the PEI25k–DNA complex at the N/P ratio of 10/1 as the control, and (D) the relative GFP expression efficiency of polyplexes ($n = 3$).[5] [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

surface proteoglycans of adherent cells, presumably the transmembrane protein syndecans are involved in the cell entry and release, transfection, of PEI polyplexes. According to the nature of the interactions, the cationic surface charge is required for efficient cell transfection.[34] Previous investigations showed that conventional HMW PEI has a much greater degree of gene transfection efficiency than LMW PEI.[9,17] Decreasing the molecular weight of PEI may be associated with a lower ability to form small complexes with genes, which subsequently results in less cell transfection.[35]

Some researchers have accredited high transfection efficiency of PEI molecules to the so-called "proton sponge" effect. The inherently protonation of the PEI chains, due to their amine groups, inside the endolysosomes causes a series of consequences including an influx of counter (chloride) ions, increasing the osmotic pressure inside the endocytic vesicle, bursting the endocytic vesicle and ultimately releasing the polyplexes. Hence, free short PEI chains have less effective transfection because they are too short to shield the membrane proteins. Long free cationic chains can more significantly increase the transfection of the polyplexes, presumably due to their ability to disrupt the anionic cell membrane via electrostatic interaction.[36]

A more recent study, undertaken by Yue *et al.*, stated another hypothesis. Free cationic PEI chains, not involved in PEI/gene complexes, with a proper length (~15–20 nm) embedded inside the anionic cell membrane via electrostatic interaction cause more efficient transfection due to their inherent ability to: (1) destabilize/weaken the endosome membrane ("proton sponge" effect) and promote the escape of the polyplexes entrapped inside and (2) act as a hindrance to the fusion between the endosomes and lysosome. The free cationic chain end(s) may effectively shield those "signaling" anionic proteins embedded on the inner surface of the cell membrane (i.e., the outer surface of the endosomes) and thereupon prevent the development of endolysosomes.[37]

Different approaches have been employed for increasing the gene transfection efficiency of PEI derivatives. First, it has been reported that employing thiolated PEI may result in higher gene transfection efficiency. This enhanced efficiency usually results from increasing thiol concentration in PEI structure. Moreover, crosslinking PEI with redox responsive disulfide bonds results in even higher gene transfection efficiency in comparison with thiolated PEI derivatives. The cross-linking procedure increases the effective molecular weight of the positively charged macromolecules and, hence, the transfection efficiency. Meanwhile, introducing these bio-cleavable bonds into the backbone of the cationers is associated with simplified intracellular breakdown and thus the minimizing of cytotoxicity.[9,17]

Peng *et al.* reported that employing PEI-SS resulted in greater gene transfection efficiency, which in a maximized state led to nearly 10 times higher expression in comparison with the optimal value for 25 kDa HMW PEI. They reported that reducing the degree of thiolation resulted in less compact polyplexes with poor transfection efficiency, which showed that the transfection efficiency trend was in good agreement with the results of particle size measurement. Furthermore, they observed that polymers with very low or very high degrees of thiolation formed uncompact polyplexes and had very poor transfection efficiency.[11]

Jia *et al.* also reported that the particle size of the polyplexes is an effective factor for gene transfection efficiency. In the study, the transfection efficiencies of chitosan-disulfide linked PEI copolymer (COS-SS-PEI), chitosan-SS-PEI-SS-Polyethylene glycole (COS-SS-PEI-PEG) and HMW PEI were compared. They reported that COS-SS-PEI had enhanced transfection efficiency comparable to 25 kDa PEI standards (Figure 5).[5] These findings can be explained by considering the fact that the polymer only partially shielded DNA polyplexes.[38–40]

Introducing disulfide bonds increases the gene transfection efficiency of PEGylated PEI. PEGylation usually results in reduction of transfection efficiency of polycations, as it can neutralize the surface charge of resulted polyplexes. As employing PEG groups is a highly suitable approach for decreasing toxicity of catiomers, the disruptive effect of PEG on transfection efficiency may be compensated via incorporation of disulfide bonds between cationic polymer and PEG. Ping *et al.* reported that PEG-catiomer delivery systems with redox-cleavable PEG-catiomer linkages have more effective endosomal escape, gene expression and efficient transfection in comparison with PEG-undetachable polyplexes. They found that MPC had the same level of transfection efficiency when compared with PEI-*b*-CD. Employing MC11 peptide group led to decreasing transfection activity as a direct result of increased particle size and decreased zeta potential. They reported that incorporation of nonremovable PEG modifier (Ad-PEG) decreased transfection activity of MPC polyplexes, while all the MPC/ Ad-SS-PEG polyplexes had comparable or even higher transfection efficiency than for HMW PEI polyplexes in the FGFR-positive cell lines. Hence, the detachment of PEG from the polyplexes may facilitate endosomal escape, and as expected, higher transfection efficiency can be achieved.[30]

Another approach to enhancing cellular transfection of polyplexes is the binding of a single chain monoclonal antibody to the carrier. Li *et al.* prepared a copolymer of poly(ethylene glycol) and poly-(L–lysine) grafted to PEI through a reducible disulfide linkage for siRNA delivery.[28] They found that conjugating Herceptin as a single chain monoclonal antibody to the carrier for the Her2/neu receptor significantly increased the transfection efficiency of the copolymer/siRNA polyplex for Skov-3, a human ovarian cancer cell line.[28]

In summary, several approaches have been employed to increase gene transfection efficiency of PEI derivatives for gene delivery applications. In addition to thiolating and disulfide cross-linking, attaching a monoclonal antibody, as well as peptide groups, and increasing molecular weight by means of bio-detachable linkages, are the main successful routes for the purpose without any severe disruptive effects on the bioavailability of PEI-based gene carriers.

CONCLUSION

This article has reviewed previous research into bio-reducible PEI derivatives as a novel class of gene carriers that have high gene encapsulation. It is found that despite the high gene encapsulation efficiency of the HMW PEI based nanostructure, these derivatives suffer from a high immune system response along with a high level of cellular toxicity; this is mainly due to their cationic nature and noncleavable molecular structure. Introducing bio-cleavable disulfide bonds into the PEI structure, in addition to binding anionic groups, are two main approaches that have been employed in order to overcome the intrinsic toxicity of the PEI based gene carriers, while keeping the molecular weight required for gene delivery applications. Moreover, PEGylation and the addition of bioavailable materials (e.g., chitosan and beta-cyclodextrin) in the structure of the PEI based nanoacarriers have resulted in increased blood circulation time and less cytotoxicity, respectively.

In addition to reducing the cytotoxicity, raising gene transfection efficiency is another challenge that has attracted strong interest from researchers. Among different approaches that have been carried out for this purpose, employing bio-cleavable linkages as well as attaching a single chain monoclonal antibody can be considered as two main successful approaches to enhancing cellular transfection of the polyplexes while minimizing their cytotoxicity. This review has focused on ways of achieving favorable characteristics of bioreducible PEI- based gene carriers as a typical class of redox-responsive gene delivery systems. However, in future, in order to obtain even more effective gene delivery systems, the characteristics of these gene carriers still need to be improved for real physiological conditions that have not only redox characteristics but also the simultaneous presence of other stimuli.

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