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1. Introduction

Gene therapy holds substantial promise for the treatment of a broad class of overwhelming human diseases such as cancer and AIDS (Verma & Somia, 1997). An essential procedure in gene therapy program involves the delivery of encoded plasmid genes into the patient’s somatic cells so as to express therapeutic proteins. An ideal strategy for successful gene delivery depends on safe and efficient gene delivery vectors (El-Aneed, 2004). Generally, gene delivery vectors are classified into two categories: viral vectors and non-viral vectors. Viral vectors are derived from natural viruses such as adenovirus and retrovirus with eliminated pathogenicity. Because of their unique capability in cell infection, viral vectors are most popular for gene delivery in vitro and in vivo. Unfortunately, clinical practice of viral vectors is seriously hampered by a few inherent issues including random insertion into the host genomes, immunogenicity, gene-carrying capacity limitation, and small-scale production (C.E. Thomas et al., 2003). In the past decades, these safety concerns on viral vectors have led to accelerated advancement in non-viral vectors (S. Li & Huang, 2000). Non-viral vectors such as lipids and polymers take more advantages over conventional viral vectors, including low immunogenicity after repeated administration, easy manufacture, large-scale production and low cost. However, current non-viral systems typically fail to give rise to as efficient gene transfection as powerful viral vectors (Pack et al., 2005). Thus, the availability of highly potent non-viral gene delivery vectors still remains a big challenge.

Among different non-viral vectors, cationic polymers have received much attention because they can be prepared by different polymerization methods and easily modified to introduce different bio-functional groups (Luo & Saltzman, 2000). In the past two decades, a few traditional cationic polymers such as chitosan, polyethylenimine (pEI), poly(L-lysine) (pLL), polyamidoamine (PAMAM) dendrimer (Figure 1) have been studied widely as non-viral vectors for gene delivery (de Smedt et al., 2000). These cationic polymers can self-assemble with negatively-charged genes to form polymer/gene complexes (polyplexes) and induce detectable gene transfection efficiency in vitro. However, these first-generation polymeric gene vectors are not yet applied further for clinical practice, mainly due to low transfection efficiency and/or high cytotoxicity (Anwer et al., 2003; Merdan et al., 2002). In the past few years, extra- and intracellular gene delivery barriers have been identified that may seriously...
hamper efficient gene transfection (Nishikawa & Huang, 2001; Wiethoff & Middaugh, 2003). To overcome these barriers, on-going research works are devoted to molecular design of cationic polymers with multiple properties for circumventing the gene delivery barriers. It has been aware that the structures of polymers play an important role in gene transfection efficiency (Jeong et al., 2007).

A lot of evidences have indicated that biodegradable cationic polymers are new-generation polymeric gene vectors because of their favourable low cytotoxicity profiles (Luten et al., 2008). Particularly, bioreducible cationic polymers, containing the disulfide bond as a bioreducible linker in polymeric main chain or side chain, are of interest. It has been well known that disulfide bond is relatively chemically stable in the extracellular environment, but can be rapidly biodegradable inside the cells due to the presence of a high amount of reducing enzymes and sulphydryl components such as glutathione (Ganta et al., 2008; G. Wu et al., 2004). By intracellular biodegradation, smart disulfide-based cationic polymers are able to efficiently unload genes in the nucleus (Soundara & Oupicky, 2006), thereby giving rise to high levels of gene expression. Meanwhile, the biodegradation also induces relatively low cytotoxicity by avoiding the accumulation of high molecular weight cationic polymer inside cells. These efforts are now actively striving to reach safe and potent polymeric gene vectors.

2. Cationic polymer-mediated gene delivery pathway

DNA is a flexible, negatively-charged biomacromolecule under physiological conditions. It can be electrostatically repelled by negatively-charged cellular membranes and thus fails to
Bioreducible Cationic Polymers for Gene Transfection

efficiently enter the cells. Moreover, naked gene is prone to degradation by enzymes in the cells. Cationic polymers are able to condense gene into nanoscale polyplexes, deliver the genes into the cells and protect DNA from the enzymatic degradation. Therefore, for the availability of safe and potent polymeric gene delivery vectors, it is essential to understand cationic polymer-mediated gene delivery pathway.

Fig. 2. Schematic illustration on cationic polymer-mediated gene delivery

A schematic gene delivery mediated by a cationic polymer is illustrated in Figure 2. First, cationic polymers bind DNA via electrostatic self-assembly to form compact polymer/DNA complexes (polyplexes). An excess amount of cationic polymer is normally needed to neutralize negative DNA and cause resulting polyplexes with net positive surface charge. Then, the positive polyplexes can interact with cellular membrane and are internalized into the cells through adsorptive endocytosis or receptor-mediated endocytosis. After they enter cells, the polyplexes normally undergo an undesirable degradation pathway from the early to later endosomes and finally locate in the lysosomes. DNA is easily degraded by enzymes in the acidic endosomes or lysosomes (pH 5–6). In this situation, cationic polymeric vectors can protect DNA from degradation and induce efficient endosomal escape by a mechanism like “proton sponge” effect (Boussif et al., 1995). After endosomal escape, polyplexes stay in the cytoplasm and move towards the nucleus by passive diffusion. At this stage, it is still unclear whether the genes should be unloaded in the cytoplasm. The polyplexes with the particle size below 25 nm may freely diffuse through the nuclear pore in the nuclear membrane (Suh et al., 2003), but the polyplexes with bigger particle size have to undergo a
nuclear translocation process aided by the nuclear pore complex proteins in the nuclear membrane (Gorlich & Kutay, 1999; Ryan & Wente, 2000). When the genes are free from polyplexes in the nucleus, translation and transcription are conducted by gene expression system to produce therapeutic proteins.

3. Non-degradable cationic polymers as non-viral gene delivery vectors

This section reviews typical non-degradable cationic polymers as non-viral vectors for gene delivery. Although these polymer systems normally have low transfection capability in vitro and/or high cytotoxicity, from the studies on these systems, a few fundamentals on gene delivery properties have been well understood, which are valuable in the design of safe and potent polymeric gene delivery vectors.

3.1 Polyethylenimine

Polyethylenimine (PEI) was investigated as a non-viral gene delivery vector in 1995 (Boussif et al., 1995). It is a high charge density polycation, in which every three atom is present with a protonable amino-nitrogen. Linear PEI only has secondary amino group that is almost protonated under physiological conditions. By contrast, branched PEI has not only the primary and secondary amine, but the tertiary amine. As such, only about two-thirds of amino groups in PEI are protonable under physiological conditions. It has been indicated that transfection ability of PEIs depends on their molecule weights, PEI nitrogen/DNA phosphate charge ratios (N/P) and cell types. For 800-Da PEI, it can mediate the delivery of pGL2-Luc gene into NIH 3T3 cells with an optimal gene expression level of $2 \times 10^6$ RLU/mg protein at an N/P of 8/1. However, for 25-kDa PEI, the level is increased to $10^9$ RLU/mg protein at the same N/P ratio. The polyplexes of PEI may transfect different types of cell lines, with the levels of gene expression in the range from $10^5$ (MCR-5 cells) to $10^8$ RLU/mg protein (COS-7 cells).

Currently, high molecular weight PEI (e.g. 25kDa) is regarded as one of the most potent gene transfection agents. This superior gene transfection is explained by so-called “proton sponge” hypothesis (Boussif et al., 1995). In brief, the protonation of PEI in the endosomes induces a massive influx of chloride ions into the endosomes, which triggers the entry of water molecule into the endosome to balance the ion concentration. The entry of massive ions and water thus results in osmotic swelling of the endosome and subsequent membrane disruption. After that, genes are released into the cytoplasm. Buffer capacity (defined as the percentage of amino groups becoming protonated from pH 7.4 to 5.1) is regarded as an important parameter of cationic polymers to determine their ability to mediate endosomal escape, and is correlated with the pKa of protonable nitrogen in the polymers. Thus, cationic polymers containing protonable amino groups of a low pKa (5-7) commonly have good buffer capacity. This may explain why branched PEI can mediate better gene transfection than linear PEI because the former has one-third of protonable tertiary amino groups.

Also, a lot of investigations on biophysical properties of PEI-based polyplexes have been made to clarify PEI-mediated gene transfection (Sirirat et al., 2003). Dynamic light scattering and zeta-potential meters are typically applied to determine the particle size and zeta-potential of polyplexes. In general, nano-sized polyplexes below 150nm can be found with different molecular weight of PEI in the range of 2-25k at N/P ratios of 1-10. Notably, only
at the N/P ratios above 4/1, the polyplexes with a high surface charge (+10~35 mV) can be obtained. Small particle sizes and positive surface charges are highly desirable for efficient cellular endocytosis, which may be the reason why PEI is potent for highly efficient gene transfection.

An inherent disadvantage of PEI is its high cytotoxicity \textit{in vitro}. Depending on cell line type, the IC$_{50}$ value of PEI is typically below 30 μg/mL. In PEI-mediated transfection process, a two-stage cytotoxicity mechanism is discovered (Godbey et al., 2001; Moghimi et al., 2005). In the first stage, free pEI may destabilize the cellular membrane, inducing necrosis-related cytotoxicity. The removal of free PEI from the polyplexes of PEI indeed can lead to lower cytotoxicity (Boeckle et al., 2004). In the second stage, free PEI that is dissociated from the polyplexes inside the cells can interact with negatively-charged mitochondrial membrane, inducing harmful cellular apoptosis. Thus, the cytotoxicity in this stage could be diminished after cationic polymers are intracellularly degraded into small pieces.

### 3.2 Polyethylenimine derivatives

Low molecular weight PEI (below 2 kDa) normally displays lower cytotoxicity, but inferior transfection capability as compared to high molecular weight counterparts. Klibanov \textit{et al.} modified the primary amines of 2k-Da PEI with dodecyl or hexadecyl iodides (M. Thomas & Klibanov, 2002). The transfection efficiencies of these alkylated 2k-Da PEI are surprising. In the transfection towards COS-7 cells, dodecylated or hexadecylated 2k-Da PEI can induce a high level of gene expression in the presence of serum, that is, 5-fold higher than that of 25k-Da PEI. The cytotoxicity of these alkylated PEI is much lower as compared to 25k-Da PEI (100% vs. 80% cell viability).

The incorporation of poly(ethylene glycol) (PEG) into PEI may yield PEGylated PEI with reduced cytotoxicity (C.-H. Ahn \textit{et al.}, 2002). PEGylated PEI copolymers can be synthesized by coupling activated PEG (2000 Da) with low molecule weight PEI (600, 1200 or 1800 Da). An optimal PEI-PEG copolymer is found that has 87 units of PEI1800 and 100 units of PEG2000. Again, the copolymer can efficiently bind plasmid DNA to form nanoscale polyplexes with positively surface charge (+20~+40 mV) (average diameter 120~150nm) at N/P ratios from 1/1 to 4/1. The transfection efficiency of these polyplexes towards 293T cell is 3-fold higher than that of parent PEI1800. The cytotoxicity is very low with 80% cell viability. It should be noted that PEGylation often leads to reduced transfection efficiency \textit{in vitro}. This is because PEGylated PEI-based polyplexes have low surface charges which impair efficient cellular internalization and also efficient endosomal escape of polyplexes (Mishra \textit{et al.}, 2004). Thus, the molecular weight of PEG and the composition ratio between PEI and PEG must be optimized.

### 3.3 Poly(L-lysine)

Poly(L-lysine) (PLL) is one of mostly studied cationic polymers for non-viral gene delivery (G.Y. Wu & Wu, 1987). It is a linear polypeptide with L-lysine residues in repeat units. The commonly used PLL as a non-viral gene delivery vector is with the molecular weigh of 25.7 kDa. The transfection efficiency of PLL is much lower than that of PEI since it displays a low buffer capacity, which is not efficient for proton sponge effect. Another disadvantage of PLL is that transfection efficiency of PLL is significantly influenced by serum probably due to the
rapid binding of PLL polyplexes with negatively-charged serum (C.H. Ahn et al., 2004). In PLL-mediated gene transfection against 293T cells, for example, the gene expression level is remarkably reduced by 10 times in the presence of 10% serum. The cytotoxicity profile of PLL is not satisfactory with about 60% cell viability at a tested concentration of 10 μg/mL. Thus, modification of PLL is needed to improve transfection ability and meanwhile decrease cytotoxicity.

Because the low transfection efficiency for PLL is attributed to its poor buffer capacity, Langer et al. introduced imidazole group (pKa ~6.5) into PLL to improve buffer capacity, thereby enhancing transfection efficiency (Putnam et al., 2001). The modified PLL was synthesized by the coupling of amino groups of PLL (34Ka) with 4-imidazoleacetic acid using an EDC/NHS activation. As expected, transfection efficiency of imidazole-modified PLL was increased with increasing amounts of imidazole groups and was much better than that of native PLL. The PLL with the highest imidazole content (86.5%) could mediate the best gene transfection, with gene expression level close to that of polyethylenimine. Low cytotoxicity is another merit for these imidazole-modified PLL (100% cell viability at 30 μg/mL). The PLL with the highest imidazole content could mediate the best gene transfection, with gene expression level close to that of polyethylenimine. Low cytotoxicity is another merit for these imidazole-modified PLL (100% cell viability at 30 μg/mL). The PLL26-co-PEG32 copolymer could mediate almost the same transfection efficiency both in the absence and presence of 10% serum.

3.4 Poly(amido amine) (PAMAM) dendrimer

Poly(amido amine) dendrimers are a family of well-defined cationic polymers (Tomalia et al., 1990). Szoka et al. firstly investigated PAMAM cascade polymers as non-viral gene delivery vectors (Szoka, 1993). These polymers have an ammonia initiator core and different generation (G2-G10) of amido amine repeat units. In the transfection against CV-1 cells, an optimal level of gene expression (1x10^10 RLU/mg protein) was observed for the sixth generation PAMAM (G6, MW 43451) at an N/P ratio of 6/1, but with 64% cell viability. However, 10^8 RLU/mg protein could be obtained for the PAMAM G5 at N/P=3, 6 or 10 with more than 90% cell viability. This high level of gene expression is due to a high buffer capacity of PAMAM since pKa value is 3.9 for internal tertiary amines and 6.9 for terminal amines. Another report showed that PAMAMs with an EDA initiator core can also mediate efficient gene transfection towards different mammalian cells (Kukowska-Latallo et al., 1996). The polyplexes of PAMAM G7 can transfect several types of cells with high gene transfection efficiency (≥1x10^10 RLU/mg protein), which is even better than that of Lipofectamine 2000. The cytotoxicity of these PAMAMs is however terrible. It appeared that the initiator core of PAMAM may influence their transfection efficiency.

In a further study, Szoka, et al. found that when intact PAMAM dendrimers were treated by heating in a solvent such as water or butanol, the resulting dendrimers can surprisingly induce higher transfection efficiency as compared to parent PAMAM (Tang et al., 1993). The efficiency is affected by the generation number of dendrimer and degree of degradation. For example, after the sixth-generation dendrimer initiated with tris(2-aminoethyl)amine (TAEA), termed as 6-TAEA, was degraded in the n-butanol for 43 hours, the resulting
degraded PAMAM, denoted as “fractured” dendrimer, could lead to enhanced transfection efficiency by 3 orders of magnitude compared to that of native PAMAM. This pronounced transfection efficiency is likely due to increased flexibility which results in apparent volume swelling of fractured dendrimer in the endosomes and thus efficient endosomal escape.

3.5 Chitosan

Chitosan is a naturally cationic polysaccharide and is degradable by lysozyme in the body. Chitosan is composed of β1→4 linked glucosamine, partly containing N-acetylglucosamine and has an apparent pKa value of 6.5. This indicates that chitosan could be used as non-viral vectors for gene delivery (W.G. Liu & Yao, 2002). Mumper et al. was the first to report chitosan as gene delivery vectors (Mumper et al., July 30-August 4, 1995). The molecular weight of chitosans influences their transfection efficiency. Generally, an optimal molecular weight is in the range of 10-50 kDa for efficient gene transfection. Also, both pH and serum largely influence the transfection efficiency of chitosan. It was shown that the transfection efficiency at pH 6.9 was higher than that at pH 7.6. Moreover, the transfection efficiency was 2 to 3 times higher in the presence of serum than that in the absence of serum. The enhancement with serum may be caused by the cell function raised by the addition of serum since major components in serum like albumin and globulin have little effect on the transfection efficiency of chitosan–based polyplexes (Sato et al., 2002).

Because chitosan is only soluble in acidic solution at pH 1~6, it readily self-aggregates under physiological conditions. A few chemical modifications on chitosan were thus performed to obtain improved solubility. One is to modify chitosan by introduction of a soluble moiety, for example, PEG, and the other is quaternization of the amine groups of chitosan (Thanou et al., 2002). A modification is also made to enhance the thermal stability of DNA by incorporating dodecylate chain into chitosan (F. Li et al., 2002). This modified chitosan displayed enhanced transfection capability with low cytotoxicity. However, the transfection efficiency of chitosan-based derivatives reported so far is normally not superior to that of PEI.

4. Hydrolysable cationic polymers as non-viral gene delivery vectors

This section reviews hydrolysable cationic polymers as non-viral gene delivery vectors. In the past decade, hydrolysable cationic polymers (Figure 3) have been studied as non-viral gene delivery vectors since they display lower toxicity as compared to their non-degradable counterparts. These research works accelerate the availability of safe and efficient non-viral gene delivery vectors.

4.1 Poly(4-hydroxy-L-proline ester) (PHP)

Poly(trans-4-hydroxy-L-proline ester) (PHP) is the first hydrolysable cationic polymers for non-viral gene delivery (Lim et al., 1999). Since hydroxyproline is a component in collagen, gelatin, and other proteins, hydroxyproline-based materials are considered low cytotoxic. PHP ester were synthesized by the polymerization of cbz-protected 4-hydroxy-L-proline to generate poly(4-hydroxy-N-cbz-L-proline), followed by the treatment of formic acid and Pd/C. As expected, PHP is degradable under physiological conditions, but very fast with degradation half time of 2 hours. Moreover, PHP can efficiently bind DNA to form positive polyplexes with average diameter below 200 nm. The polyplexes could transfect CAPE cells.
at a high polymer/DNA ratio of 50/1 (w/w) with transfection efficiency of 1.5 times higher than that of PLL. Importantly, this polymer has very low cytotoxicity compared to 25-kDa PEI. It is worthy pointing out that the transfection of PHP is not influenced by serum, indicating this polymer is biocompatible for non-viral gene delivery.

![Fig. 3. Typical examples of hydrolysable cationic polymers as non-viral vectors](image)

**4.2 Poly[α-(4-aminobutyl)-L-glycolic acid] (PAGA)**

Poly[α-(4-aminobutyl)-L-glycolic acid] (PAGA, 3.3kDa) is an analogue of PLL (Lim et al., 2000). Since the amide bonds in PLL are replaced with ester bonds, PAGA is degradable under physiological conditions. PAGA is rapidly degraded at pH 7.4 and 37°C with the degradation half time of 30 min. The presence of primary amine in the side chain of PAGA renders this polymer highly efficient for gene binding. As such, at a low N/P ratio of 5/1, nanoscale polyplexes from PAGA/DNA (~326nm) can be formed [34]. The transfection efficiency of PAGA is comparable with that of PLL. At an optimal N/P of 40/1, the transfection efficiency of the polyplexes of PAGA is 3-fold higher compared to those of 4-kDa PLL. The cytotoxicity of PAGA is much low in comparison with that of PLL (100% vs. 20% cell viability). Although PAGA is not as efficient as PEI for gene transfection, the study on the PAGA indicates that biodegradable cationic polymers are relatively safer due to low cytotoxicity as compared to non-degradable cationic polymers.

**4.3 Poly(β-amino ester)s (PAE)**

Poly(β-amino ester)s (PAEs) are a family of degradable cationic polymers that are prepared via Michael-type addition between bisacrylates and amines. Since these reactive monomers are
versatile, a large number of PAEs with different functional groups can be designed (Lynn & Langer, 2000). Due to the presence of multiple ester bonds in polymer main chain, PAEs are degradable and normally possess relatively low cytotoxicity. A particular example is that Langer et al. examined a library containing more than 2000 PAEs for non-viral gene delivery (Anderson et al., 2003). Also, the structure-activity relationships were investigated. The conclusions from the study are: 1) Bisacrylate monomers with strongly hydrophobic residues are almost always present in the 50 best-performing PAEs; 2) Linear, bis(secondary amines) are over represented in the hit structures; 3) Mono- or dialcohol side group in PAE is an important functional entity for efficient gene transfection. One PAE from this library showed transfection ability, with the level of gene expression 5-fold higher than that of 25- kDa PEI against 3T3 cell lines under optimal conditions. The studies on PAEs strongly support the idea that degradable cationic polymers are very promising for safe and efficient gene delivery.

4.4 Polyphosphoester (PPE-EA)
Poly(2-amioethyl propylene phosphate) (PPE-EA) is a degradable cationic polymer which can yield ultimate low-toxic degradation products including α-propylene glycol, phosphate, and ethanolamine. This polymer is synthesized through ring-opening polymerization of 4-methyl-2-oxo-2-hydro-1,3,2-dioxaphospholane, followed by two-step chemical modification (Wang et al., 2001). The transfection of PPE-EA (30 kDa) gives 100-fold higher levels of gene expression at an N/P ratio of 6 as compared to that of 27- kDa PLL at an N/P ratio of 5. Importantly, PPE-EA has low cytotoxicity towards COS-7 cells with more than 80% cell viability up to a tested concentration of 1000 μg/mL. However, this polymer has poor ability of endosomal escape since the presence of chloroquine (100 μM), a reagent known to disrupt endosomal membrane, may lead to remarkably enhanced transfection efficiency.

4.5 Polyphosphazene (PPA)
Polyphosphazenes (PPAs) are cationic polymers derived from poly(dichloro)phosphazene (Luten et al., 2003). They are degradable slowly under physiological conditions with a half-life of more than 10 days, but relatively faster at pH 5 with a half-life of 4-5 days. The PPAs (>100 kDa) can condense genes into nanoscale polyplexes at low polymer/DNA mass ratios of 3~5, with a high positive surface charge (+ 40mV). The transfection of PPA was comparable with that of PEI towards COS-7 cells. The optimal transfection efficiency of PPA was observed at a polymer/DNA mass ratio of 6, however, with a high cytotoxicity profile (~50% cell viability). The pronounced cytotoxicity could be due to high molecular weight and slow degradation profile inside the cells.

5. Bioreducible cationic polymers as non-viral gene delivery vectors
In the design of hydrolysable cationic polymers for non-viral gene delivery, a contradiction has to be found that the polymers are expected to be rapidly degradable intracellularly as one hand, but chemically stable extracellularly as another hand. In order to avoid this issue, disulfide bond as a bioreducible linker has received much attention in recent years. The disulfide bond is chemically stable in the blood plasma, but intracellularly bio-cleavable by reducing enzymes like glutathione reductase and sulfhydryl components like glutathione since the concentration of these reducing species is much higher in the cytoplasm than the
blood plasma (intracellular vs. extracellular glutathione concentration, 0.5-10 mM vs. 2-20 μM) (G. Wu et al., 2004). Thus, this feature makes the disulfide very valuable in the design of biodegradable cationic polymers for triggered gene delivery. Figure 4 shows a schematic illustration on intracellular gene delivery mediated by disulfide-based cationic polymers.

Fig. 4. A conceptual illustration of DNA binding and subsequent intracellular release: (a) formation of the polyplexes of bioreducible cationic polymers, which are relatively stable in the extracellular environment, (b) intracellular cleavage of disulfide linkages in the polymer of the polyplex, and (c) intracellular DNA release from the degraded polymer.

This section reviews current progress in disulfide-based cationic polymers as non-viral gene delivery vectors. The topics are focused on the synthesis of bioreducible cationic polymers, unique biophysical properties of the polyplexes based on the polymers.

5.1 Preparation of bioreducible cationic polymers as non-viral gene vectors

Bioreducible cationic polymers can be designed and synthesized that contain disulfide bond either in polymer main chain or side chain. In earlier studies, the disulfide was introduced in the polymer side chain to conceptually confirm the role of the disulfide in gene delivery. A typical synthesis route is the preparation of cationic polymers with pyridyldithio residue, which is then modified with suitable thiol compounds via an exchange reaction (Figure 5a). By this method, the pLL containing disulfide linkages in the polymer side chains (termed as poly[Lys-(AEDTP)]) was prepared through chemical modification of the primary amines in pLL with N-succinimidyl-3-(2-pyridyldithio)propionate, followed by an exchange reaction with mercaptoethylamine (Pichon et al., 2002). The polyplexes of poly[Lys-(AEDTP)] can transfect HeLa cells with a level of gene expression 10-fold higher than that of parent pLL. This thus implies that disulfide linker plays a pivotal role in improved gene transfection. In another work, PAEs with pyridyldithio groups in the polymer side chains were synthesized via Michael-type addition reaction between diacrylates and 2-(pyridyldithio)-ethylamine. These polymers were further modified with mercaptoethylamine or thiol peptide such as RGD, yielding the PAEs with disulfide linkers in the side chains (SS-PAEs) (Zugates et al., 2006). The polyplexes of SS-PAEs could transfect HCC cells with the efficiency comparable to that of 25-kDa PEI.
Alternatively, one route to generate bioreducible cationic polymers is the polyoxidation of dithiol-based monomers having amino groups (Figure 5b). Typical examples are disulfide-containing cationic polymers based on pEI, pLL and pDMAEMA (SS-PEI, SS-PLL and SS-PDMAEMA, respectively, in Figure 5). In general, the preparation of these dithiol-based oligoamines is time-consuming and these compounds cannot be stored for long term due to oxidation of thiol groups by air. As typical examples, Park et al. reported on the synthesis of dithiol-containing oligoamines via organic synthesis involving protection and deprotection of amino groups (Lee et al., 2007). Oupický et al. described the preparation of well-defined dithiol-based PDMAEMA oligomers via reversible addition-fragmentation chain transfer polymerization (You et al., 2007). Seymour et al. produced dithiol-based oligopeptides (Cys-Lys10-Cys) via solid-phase organic synthesis (Oupicky et al., 2002). These dithiol-based oligoamines can be oxidized by DMSO as an oxidant agent to yield disulfide-containing cationic polymers. Also, different dithiobearing groups, e.g. nuclear localization sequences comprising two cysteine residues, can be incorporated in the oxidation reaction, giving rise to disulfide-containing copolymers with multiple functionalities (Read et al., 2005).

A simple approach for the availability of disulfide-based cationic polymers is the chemical coupling of amine compounds with disulfide-containing reagents, such as cystamine bisacrylamide (CBA) in a Michael addition reaction (Lin et al., 2006, 2007a; Lin et al., 2007b; Lin et al., 2008; Lin & Engbersen, 2008) (Figure 5c), and dithiobis(succinimidyl propionate) (DTSP) or dithiobispropionimidate (DTBP) in a polycondensation reaction (Figures 5d&5e). These reactions can generate linear or branched disulfide-containing cationic polymers with different molecular structures (Figure 6). Lee et al. firstly prepared disulfide-containing branched pEI by the crosslinking of low molecular weight PEI with DTSP or DTBP (Gosselin...
et al., 2001; Gosselin et al., 2002). Recently, disulfide-containing poly(amido amine) (SS-PAA) (co)polymers were synthesized through Michael-type addition reaction of CBA to primary amines, secondary diamines or PEI oligoamines. The structural effects of these SS-PAA on gene delivery properties were systematically investigated. It was shown that the SS-PAA with the hydroxybutyl or hydroxypentyl side groups led to higher transfection efficiencies and lower cytotoxicity in COS-7 cells than 25-kDa branched PEI. Herein, we summarize those typical bioreducible cationic polymers in Table 1 and their performance in gene transfection efficiency against different cell lines.

Fig. 6. Typical examples of bioreducible cationic polymers as non-viral gene delivery vectors

5.2 Intracellular fate of disulfide-based polymeric gene vectors

It is assumed that higher efficient transfection induced by disulfide-based cationic polymers is at least partly due to intracellular degradation via the cleavage of the disulfides in the reducing intracellular environment. In order to obtain experimental evidences, polyplexes of fluorescently labelled P(CBA-ABOL) containing disulfide bonds and P(BAPABOL) lacking disulfide bonds (Figure 7) were used for gene transfection against COS-7 cells at the same polymer/DNA mass ratio of 12/1. Dynamic light scattering and zeta-potential measurement showed that polyplexes of P(CBA-ABOL) and p(BAP-ABOL) had comparable average particle size and surface charge (128 nm vs. 82 nm; +20.2 mV vs. +19.2 mV), allowing good comparison of the transfection activity of both types of polyplexes. The intracellular distributions of the two polymers, labelled by a Rhodamine dye, are clearly different under fluorescence microscopy (Figure 7). For
P(CBA-ABOL), a homogeneous dispersed fluorescence was observed both in the cytoplasm and the nucleus. By contrast, for the p(BAP-ABOL) lacking the disulfide linkages, many micro-sized aggregated clumps were found in the perinuclear space and only a few weak fluorescence was observed in the nucleus. These results may serve as an indication that P(CBA-ABOL) is intracellularly degradable faster by reducing cleavage of the disulfide bonds, resulting in a diffuse distribution of fluorescently labeled polymer fragments inside the cells. Slow degradation of p(BAP-ABOL) may, however, contributes to the formation of the aggregation.

![Figure 7](image)

**Fig. 7.** Intracellular distribution of the polyplexes from bioreducible P(CBA-ABOL) (left) and non-degradable P(BAP-ABOL) lacking disulfide bonds (right), observed under confocal laser scanning microscopy. The polymers are shown in green and the nucleus in red.

<table>
<thead>
<tr>
<th>Disulfide-based polymers</th>
<th>Plasmid</th>
<th>Cell line/animal</th>
<th>Transfection</th>
<th>Ref.</th>
</tr>
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<tbody>
<tr>
<td>DTSP or DTBP-crosslinked PEI</td>
<td>pCMV-Luc</td>
<td>CHO</td>
<td>Comparable to 25-kDa PEI</td>
<td>(Gosselin et al., 2001)</td>
</tr>
<tr>
<td>PEGylated PEI crosslinked with DTSP</td>
<td>pCMV-Luc</td>
<td>Mice</td>
<td>enhanced plasmid blood levels up to 60 min.</td>
<td>(Neu et al., 2007a)</td>
</tr>
<tr>
<td>Crosslinked PEI with DSP</td>
<td>pCMV-Luc, 32P-labeled plasmid</td>
<td>NIH-3T3/Balb/c mouse</td>
<td>3-fold higher than 25-kDa PEI, considerable gene expression in the liver and lung</td>
<td>(Neu et al., 2007b)</td>
</tr>
<tr>
<td>Disulfide-based polymers</td>
<td>Plasmid</td>
<td>Cell line/animal</td>
<td>Transfection</td>
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<td>Linear SS-PEI</td>
<td>pCMV-Luc</td>
<td>HepG2, HeLa</td>
<td>comparable to ExGen 500 (25kDa l-PEI)</td>
<td>(Lee et al., 2007)</td>
</tr>
<tr>
<td>DTSP-Crosslinked linear PEI (2-4kDa)</td>
<td>pCMV-EGFP</td>
<td>CHO, HepG2, NIH-3T3, HeLa, HCT116, COS-7, HEK293</td>
<td>5-7 times higher than Lipofectamine 2000, JetPEI, FuGENE6, 40-70 eGFP+ %</td>
<td>(Breunig et al., 2007)</td>
</tr>
<tr>
<td>PEI-SS(x) from thiolated 800-kDa</td>
<td>pCMV-Luc, pCMV-EGFP</td>
<td>HeLa, 293T</td>
<td>10-fold higher than 25-kDa PEI (HeLa), 3-fold higher eGFP+ % (293T)</td>
<td>(Peng et al., 2008)</td>
</tr>
<tr>
<td>listeriolysinO-conjugated reducible 25kDa-PEI (LLO-SS-PEI)</td>
<td>pCMV-NGVL3 (both GFP and Luc)</td>
<td>HEK293</td>
<td>Comparable to 25-kDa PEI</td>
<td>(Choi &amp; Lee, 2008)</td>
</tr>
<tr>
<td>SS-PEI in the presence of RGD</td>
<td>pCMV-Luc and pEGFP</td>
<td>293T, HeLa</td>
<td>Comparable (293T), 8-fold higher (HeLa) than 25 kDa PEI</td>
<td>(Sun et al., 2008)</td>
</tr>
<tr>
<td>BPEI-SS-PEG-cNGR (cNGR: cyclic NRG (CNGRCK) peptide)</td>
<td>Luciferase gene pDNA</td>
<td>HEK293, HT1080</td>
<td>100-fold higher than that of 1.2kDa-BPEI</td>
<td>(Son et al., 2010)</td>
</tr>
<tr>
<td>branched poly(ethylenimine sulfide) (b-PEIS)</td>
<td>pCN-Luc or pEGFP</td>
<td>HEK293, HeLa, NIH3T3, C2C12, HUVECs</td>
<td>1000-fold higher than 6-kDa l-PEI, but comparable to 25-kDa BPEI</td>
<td>(Koo et al., 2010)</td>
</tr>
<tr>
<td>CBA-crosslinked reductable polylyspermine</td>
<td>pEGFP</td>
<td>A549</td>
<td>4-fold higher than 25-kDa PEI</td>
<td>(Jere et al., 2009)</td>
</tr>
<tr>
<td>Reducible PEI (PEI-SS-CLs) via “click” chemistry</td>
<td>pLuc, pEGFP</td>
<td>293T, HeLa</td>
<td>5-10 times higher than 25-kDa PEI</td>
<td>(J. Liu et al., 2010)</td>
</tr>
<tr>
<td>Linear PAA grafted with polyamidoamines</td>
<td>pLuc, pEGFP</td>
<td>293T, HeLa</td>
<td>Comparable (293T) or a little higher (HeLa) than 25-kDa BPEI</td>
<td>(Xue et al., 2010)</td>
</tr>
<tr>
<td>CBA-crosslinked reducible polyamines (pLPEI/pTETA/pSPE)</td>
<td>qWIZ-Luc (6.7 kb), qWIZ-GFP (5.7 kb)</td>
<td>murine brain capillary endothelial bEnd.3 cells</td>
<td>2.3-4.9 fold higher than ExGen500</td>
<td>(Zhang &amp; Vinogradov, 2010)</td>
</tr>
<tr>
<td>linear disulfide-based “click” polymer (RCP)</td>
<td>MDR1-pDNA, pEGFP</td>
<td>MCF-7, MCF-7/ADR</td>
<td>Comparable to 25kDa-BPEI</td>
<td>(Gao et al., 2010)</td>
</tr>
</tbody>
</table>

Table 1. A summary of bioreducible cationic polymers and their transfection efficiencies in different types of cell lines.
It appears that disulfide degradation mainly proceeds in the cytoplasm and in the nucleus. However, a few recent studies showed that, depending on the cell line type and the polymer constructs, the disulfide could also be degradable in those microenvironments such as the cellular surface, the endosomes and the lysosomes (Blacklock et al., 2009; Morre & Morre, 2003). Thus, further studies are certainly needed to understand the factors influencing the degradation at specific locations.

6. Conclusion

Cationic polymers with multiple functionalities are promising as non-viral vectors for gene transfection. Since more and more extracellular and intracellular gene delivery barriers are identified that seriously hamper efficient gene transfection, a number of cationic polymers have been designed that are capable of overcoming one or more gene delivery barriers, thus leading to detectable gene transfection efficiency. From those conventional non-degradable cationic polymers to current bioreducible cationic polymers, peoples have more and more reached virus-like, safe and potent polymeric gene delivery vectors. Further understanding on structure-activity relationships of cationic polymers and their intracellular fate should be indispensable, in order to achieve polymer systems that can exhibit multiple gene delivery properties for highly efficient gene transfection.

7. Acknowledgment

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8. References


Comprehensive and systematic scientific researches in cell biology and molecular biology have promoted the evolution of traditional medicine. Scientists are now able to interpret a few perplexed medical difficulties with the emphasis on molecular levels. This book focuses on frontier progress in modern biomedical science dealing with regenerative medicine, gene medicine, nanobiomedicine, and medical devices. Each chapter is intently chosen and written by professional experts in the field of biomedical science. Biomedicine is a valuable guide for the readers to become aware of current advancement in rapidly moving field of biomedicine.

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