Journal of Controlled Release 132 (2008) 267–272

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Effect of chemical functionalities in poly(amido amine)s for non-viral gene transfection

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ARTICLE INFO

Article history:
Received 29 May 2008
Accepted 30 June 2008
Available online 3 July 2008

Keywords:
Gene delivery
Poly(amido amine)
Structure effects
Bioreducible polymer
Buffer capacity

ABSTRACT

The development of safe and efficient gene delivery vectors is an essential prerequisite for successful gene therapy. As viral vectors suffer from inherent disadvantages, cationic polymers as non-viral vectors have great potential in gene delivery, but their practical application so far is seriously hampered due to their relatively low transfection efficiency caused by multiple extra- and intracellular gene delivery barriers. Therefore, it is important to provide cationic polymers with functionalities that can seriously influence polymeric properties which are important to overcome gene delivery barriers. In this paper, we aim to contribute to the understanding of the effect of functionalities in cationic polymers on their gene delivery properties and transfection activity. As poly(amido amine)s can be easily provided with a large variety of chemical functionalities, we have focused on this class of cationic polymers. It is shown that various structural characteristics in these peptidomimetic polymers such as charge density, rigidity, basicity, hydrophilicity/hydrophobicity, degradability and type of amino groups influence one or more gene delivery properties such as DNA binding capability, colloidal stability, endosomal escape (buffer capacity), vector unpacking, cytotoxicity, and eventual transfection efficiency. Optimal combination of the functionalities in the poly(amido amine)s may lead to significant increase of the level of gene expression. This indicates that multifunctionalized polymers like the poly(amido amine)s can evolve to the next generation of non-viral gene delivery system for gene therapy.

1. Introduction

Gene therapy holds a considerable promise for treating human diseases such as cancer and AIDS [1,2]. The concept of gene therapy is the introduction of encoded therapeutic gene into a patient's somatic cells to produce therapeutic proteins. An essential perquisite for success gene therapy is the development of safe and efficient gene delivery vectors [3]. Gene delivery vectors are classified into viral or non-viral type. Viral vectors derived from natural viruses are still actively investigated and employed in clinical gene therapy due to their inherent high transfection capability to infected cells. However, viral vectors have potential problems in cytotoxicity, insertional mutagenesis, limited cargo capacity and immune response after repeated administration [4]. Non-viral vectors such as cationic polymers do not have these inherent problems and have the additional advantage of easy manufacturing and versatile chemical modification [5]. This makes cationic polymers attractive alternatives in gene delivery. Over past decades, many cationic polymers such as polyethylenimine (pEI), poly(L-lysine) (pLL) and polyamidoamine dendrimers have been studied as non-viral vectors for gene delivery in vitro and in vivo [6–10]. However, so far clinical application of polymeric gene delivery systems is seriously hampered by the low transfection efficiency compared to viral systems.

For the design of efficient polymeric transfection vehicles, it is essential to understand the polymer-based gene delivery pathway. Although this process is not yet fully elucidated, the mechanism involving the basic steps is presented in Scheme 1. Cationic polymers condense DNA into nano-sized polymer/DNA complexes (polyplexes) by a self-assembling process due to electrostatic interaction of the positively charged polymer with the negatively charged DNA. Polyplexes with positive surface charges are formed when the number of positive charges of the polymer exceeds that of the negative charges in DNA. When polyplexes encounter the cells, they may interact with the negatively-charged cellular membrane and are taken up into the cells via endocytosis. In the intracellular environment, the polyplexes are normally located in endosomes that become acidified and finally fuse with lysosomes. In this case, DNA is prone to degradation by lysosomal enzymes. In order to transfer their DNA cargo successfully to the nucleus, polyplexes must escape from the endosome by some transmembrane mechanism or endosomolytic process like osmolysis by the "proton sponge effect" [11]. After endosomal escape, polyplexes are located in the cytoplasm. At present the fate of polyplexes in the cytoplasm is not fully understood. However, it is clear that the

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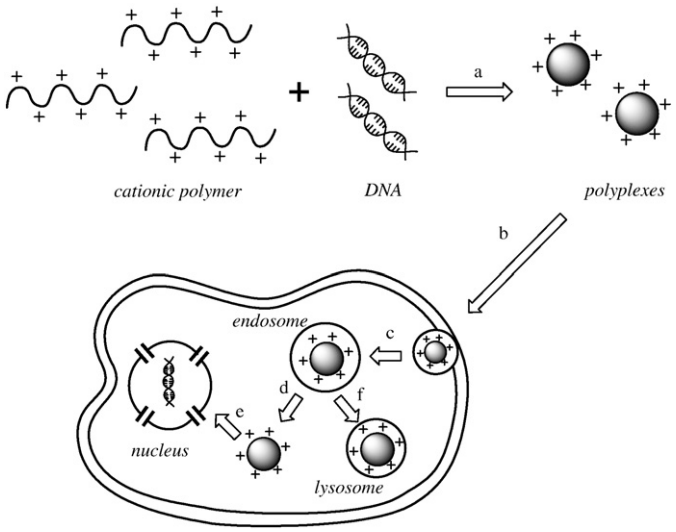
doi:10.1016/j.jconrel.2008.06.022

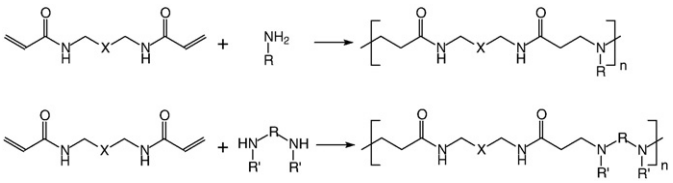


Scheme 1. Schematic illustration of cationic polymer-mediated gene delivery: a) formation of cationic polymer/DNA complexes (polyplexes); b) cellular uptake of polyplexes by endocytosis; c) endosomal pathway of polyplexes; d) endosomal escape of polyplexes; e) polyplex unpacking and nuclear translocation of DNA; f) degradation of polyplexes in lysosome.

polyplexes have to unpack DNA to deliver it to a suitable site near the nucleus or in the nucleus. After translocation of DNA into the nucleus, protein is produced by the gene expression system.

The low efficiency of polymer-mediated gene delivery may be due to the lack of mechanisms to overcome a series of extra- and intracellular gene delivery barriers in the temporal-spatial domain such as colloidal stability of vector/DNA, endosomal escape, efficient vector unpacking and nuclear translocation of DNA [12,13]. Cationic polymers need to have a multitude of functions to overcome these barriers, such as good DNA binding ability to condense DNA into positively-charged polymer/DNA complexes (polyplexes), high buffer capacity to induce endosomal escape of polyplexes and efficient intracellular vector unpacking to release DNA [13,14]. It is obvious that introduction of different functionalities in cationic polymers can importantly influence the gene delivery properties and transfection efficiency. For example, Davis et al. reported that gene delivery properties and resultant transfection efficiencies of carbohydrate-containing cationic polymers are affected by carbohydrate size, charge type and the spacing between the cationic amidine groups [15,16], and Langer et al. showed that in a library of poly(β-amino ester)s different chemical functionalities in the main chain or side chain of the polymers influence DNA condensation ability, surface charge of polyplexes and transfection efficiency [17,18]. Therefore, understanding of the correlations between polymeric functionalities and gene delivery properties is important for the rational design of efficient cationic polymeric vectors. However, although many cationic polymers with all kinds of functionalities have been reported in literature, it is difficult to correlate specific structural effects that are originating from different classes of polymers. In our approach to contribute to the understanding of the effects of various chemical functionalities in polymers on gene delivery and transfection activity, we have studied the class of poly(amido amine)s, as these polymers enable large variation in chemical functionalities within a common basic polymeric structure.

Poly(amido amine)s (PAAs) can be readily synthesized by a Michael-type addition reaction of amine compounds to bisacrylamides (Scheme 2). The polymerization reaction is tolerable with a large variation in structural fragments in the amine and bisacrylamide monomers, thus allowing large variation of chemical functionalities in the main chain and side chain of the polymers. PAAs are peptidomimetic polymers that generally have good water solubility, relatively low cytotoxicity and good long-term biodegradability [19]. These properties make that PAAs have great potential for biomedical applications, including drug [20] and gene delivery as well as tissue engineering [21,22]. In this paper, we systematically discuss the effects of chemical functionalities in PAAs on various factors that are important for their gene delivery properties, such as colloidal stability of polyplexes, DNA binding ability, buffer capacity (endosomal escape), polyplex unpacking, cytotoxicity, and transfection efficiency.



Scheme 2. General synthesis route of linear poly(amido amine)s in which X, R and R' can be a large variety of groups with different chemical functionalities.

2. Effects of chemical functionalities on gene vector properties of poly(amido amine)s

2.1. Effects of structural variation in the main chain of poly(amido amine)s

It order to understand the effects of polymeric functionalities on physicochemical and colloidal properties of polyplexes, Garnett et al. investigated four structurally related linear PAAs (Fig. 1) with various charge density and chain stiffness in terms of their DNA binding, colloidal stability and transfection activity [23]. It was found that these PAAs show a structure-dependant DNA binding behavior. Gel electrophoresis retardation assay indicated that the polymer MBA-MMA with one tertiary amino group in the repeating unit has lower DNA binding capability than the polymers (MBA-DMEDA or MBA-2MP) with two tertiary amino groups. However, the replacement of the methylene moiety in the diacrylamide segment by a piperazine ring leads to a significant decrease in DNA binding capability (MBA-2MP vs. BAP-2MP). The reason may be that the more rigid polymer backbone caused by the presence of two piperazine rings per repeating unit in BAP-2MP prevents the polymer from reaching DNA closely. Further study on the polyplexes of these PAAs showed that the smallest particle size (<200 nm in diameter) was obtained for polyplexes of MBA-DMEDA and these polyplexes have the greatest stability to resist aggregation at physiological salt concentration. This polymer combines relatively high charge density and flexibility in the series. The physiochemical properties of the polyplexes of these PAAs showed a

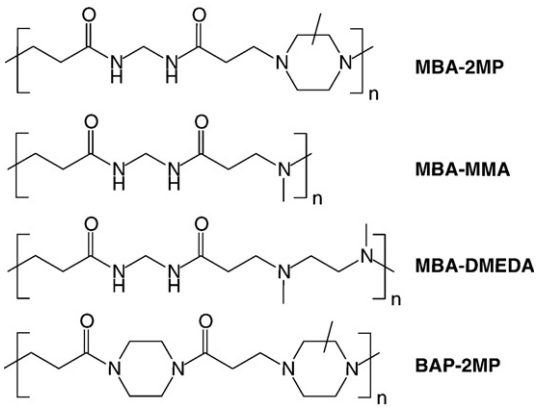


Fig. 1. Chemical structure of poly(amido amine)s with different charge density and polymer backbone stiffness.

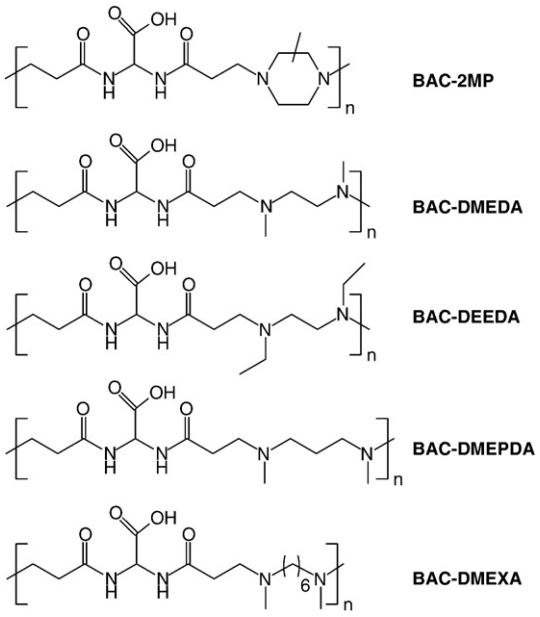


Fig. 2. Chemical structure of amphoteric poly(amido amine)s.

correlation with their efficiency of gene transfection, using A549 cells. The polyplexes of MBA-MMA and BAP-2MP show inferior transfection due to their poor DNA binding ability and colloid-stability. Moreover, polyplexes of MBA-DMEDA induce a transfection that is much higher than that of MBA-2MP and is comparable with lipofectamine that was used as a positive control. This difference is correlated with the fact that the former have much smaller particle size and better colloid-stability than the latter. These results suggest that not only ionic interactions, which primarily depend on the charge density of the polymers, but also structural flexibility influences DNA binding capability of polymers and colloid-stability of polyplexes and, consequently, the transfection activity.

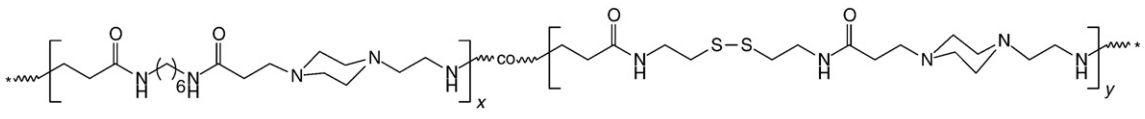
2.2. Effects of a carboxylic acid moiety in the bisacrylamide segment of poly(amido amine)s

After endocytosis, polyplexes located in the endosomes may undergo degradation during the acidification process of endosomes from early to late endosomes and the final fusion with lysosomes. Genes are also easily degraded by enzymes present in the acidic endosomes (pH 5–7) and lysosomes (pH 4.5) [24]. Escape of the polyplexes from the endosomes into the cytosol is required for eventual delivery of their cargo into the nucleus and this process is one of the major barriers in gene delivery. Some viruses are found to utilize specific fusogenic peptides to disrupt the endosomal membrane, enabling their escape from endosomes. Various synthetic fusogenic peptides as endosomolytic agents have been developed, including melitin [25], INF-7 [26] and KALA [27]. These fusogenic peptides are sensitive to the environmental pH and can undergo conformational changes from a random coil at neutral pH to an α-helix conformation at low pH. Ferruti et al. developed a series of amphoteric poly(amido amine)s possessing a carboxylic acid group in the bisacrylamide unit (Fig. 2). These amphoteric PAAs also display a pH-dependant conformational change upon protonation of the carboxylate and amino groups in these polymers, a behavior similar to fusogenic peptides, as determined by small-angle neutron scattering [28]. Therefore, they cause more hemolysis of erythrocytes at pH 5.5 (endosomal pH) than at pH 7.4 (physiological pH) [29]. In this series, BAC-2MP shows the greatest hemolysis at pH 6.5, probably due to the highest structural rigidity of this polymer. This polymer also shows "stealth" properties after intravenous administration [30]. BAC-2MP induces a comparable transfection efficiency to that of 25 kDa branched pEI in HepG2 cells and higher than the analog MBA-2MP (Fig. 1) lacking the carboxylic acid groups. These results indicate structural segments that induce pH-dependent conformational changes, as illustrated by the amphoteric PAAs, can be exploited to promote endosomolytic activity, enhanced gene transfection [31].

2.3. Effects of disulfide bonds in poly(amido amine)s

The use of the disulfide bond as bioreducible linker has received much attention in recent years in chemistry and biology. The disulfide bond can be cleaved inside the cells 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 in plasma (intracellular vs. extracellular glutathione concentration 0.5–10 mM vs. 2–20 μM) [32], the disulfide bond is relatively stable in the extracellular environment, but rapidly degradable inside the cells due to the presence of high amounts of thiols. This feature invoked the design of disulfide-containing cationic polymers of which the polyplexes are stable in the extracellular environment but readily release the genes intracellularly due to the cleavage of the disulfide segments in the polymer. Read and Seymour et al. showed that bioreducible pLL derivatives give hundred times higher transfection than their non-reducible analogs [33]. Lee et al. revealed that disulfide-containing pEIs have improved gene vector properties compared to 25 kDa branched pEI, as similar transfection levels can be attained with considerably lower cytotoxicity [34].

To explore the effect of disulfide bonds in PAAs in relation to facilitated intracellular vector unpacking and gene transfection, we have designed a series of bioreducible PAAs containing various amounts of disulfide linkages in the main chain [35]. Using 1-(2-aminoethyl)piperazine (AEP) as the amine monomer [36], and various ratios of hexamethylene bisacrylamide (HMBA) and the disulfide-containing cystamine bisacrylamide (CBA) as the bisacrylamide monomers, we have prepared the copolymeric system p(HMBA*x*/CBA*y*-AEP) where *x* and *y* represent the percentages of HMBA and CBA, respectively (Fig. 3). Gel retardation experiments in the presence of glutathione-mimicking agent dithiothreitol show that a limited



p(HMBA-AEP) ***x/y*** **=100/0**
p(HMBA*80*/CBA*20*-AEP) ***x/y*** **= 80/20**
p(HMBA*60*/CBA*40*-AEP) ***x/y*** **= 60/40**
p(HMBA*40*/CBA*60*-AEP) ***x/y*** **= 40/60**
p(CBA-AEP) ***x/y*** **= 0/100**

Fig. 3. Linear poly(amido amine)s containing various amounts of disulfide linkages.

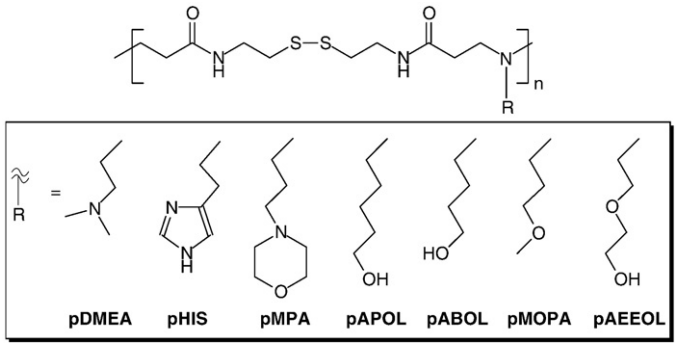


Fig. 4. Bioreducible poly(amido amine)s with various pendant groups.

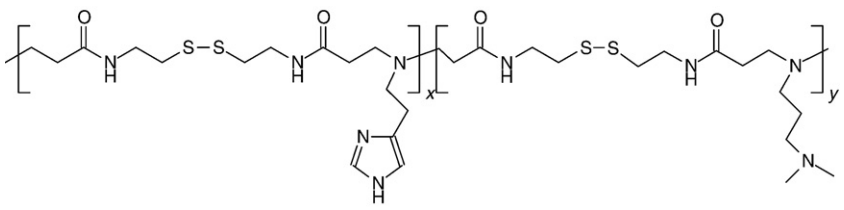
amount of disulfide linkages in the repeating bisacrylamide units ($x/y \leq 40/60$) of the copolymers is necessary to afford sufficient DNA release. Moreover, the bioreducible (co)polymers induce much higher transfection efficiencies against COS-7 cells *in vitro* and meanwhile have essentially lower cytotoxicity than their analog p(HMBA-AEP), lacking the disulfide segments. In another study, the group of Kim et al. and our group also revealed that polyplexes of branched disulfide-containing poly(amido ethylenimine)s induce much higher transfection efficiencies than 25 kDa branched pEI and lower cytotoxicity towards different cell types [37]. These studies indicate that the introduction of disulfide bonds into the backbone of polymers enables fast intracellular fragmentation of the polymers, leading to facilitated gene unpacking and improved transfection efficiency. In addition, the fast degradation of the cationic polymers leads to decreased cytotoxicity.

2.4. Effects of different pendant groups in bioreducible poly(amido amine)s

Encouraged by the promising gene delivery results obtained with disulfide-containing bioreducible poly(amido amine)s (*SS*-PAAs), we further explored the effect of different pendant functional groups in *SS*-PAAs on gene delivery properties. *SS*-PAAs having pendant groups with various basicity and hydrophobicity (Fig. 4) were compared in terms of their DNA binding capability, buffer capacity and transfection activity [38]. Again, charge density plays an important role in DNA binding behavior. The polymers with a protonated nitrogen site in the pendant group (e.g., pDMEA and pHIS) show better capability for DNA binding than polymers like pABOL, lacking protonated side groups. Remarkably, the presence of an ether oxygen in the side group appears to have a negative effect on DNA binding ability of polymers (e.g. pMPA and pAEEOL), which may be speculatively related to the poor interaction of ether groups with anions. The buffer capacity of these *SS*-PAAs, defined as the percentage of amino groups becoming protonated in the pH range from pH 7.4 to 5.1, is supposed to be a relevant parameter for endosomal escape of the polyplexes. Both the pHIS polymer, having low-basicity amino groups ($pK_a < 7$) and the pABOL and pAPOL polymers, having hydroxyalkyl side groups, show higher buffer capacities than 25 kDa branched pEI. The latter polymer is proposed to bind protons during the endosomal acidification process and subsequently promotes endosomal escape of polyplexes by osmolysis ("proton sponge effect") [11]. In contrast, the polymer pDMEA, having high-basicity amino groups ($pK_a > 7$), has lower buffer capacity than the pEI. Obviously, the pendant groups in the pDMEA polymer behave independently and are already fully protonated at physiological pH. In relation to the favorable properties of the polyplexes of these *SS*-PAAs, polyplexes of pHIS, pAPOL and pABOL induce the highest transfection efficiencies, which are even higher than those of branched pEI. In line with this observation, the low buffer capacity of pDMEA is probably responsible for the low transfection efficiency of the pDMEA based polyplexes. Moreover, it appears that the polymers with more hydrophobic side groups give higher transfection, as the transfection efficiency increases in the sequence: pAPOL>pABOL>pMOPA, although the three polymers have similar DNA binding ability and buffer capacity. Incorporation of hydrophobic moieties into cationic polymers can enhance their transfection efficiency by increasing cell membrane-polyplexes interaction and/or membrane disruption [39,40]. Recently, Kim et al. reported on a group of *SS*-PAAs with aminoalkyl pendant groups and also showed transfection efficiencies that increase with increasing length of the alkyl chains [41]. Taken together, these results show that the structural properties in terms of basicity and polarity of the pendant groups in *SS*-PAAs are relevant factors influencing gene delivery and transfection efficiency. It appears that chemical functionalities that increase the buffer capacity of the polymers and the presence of pendant chains in the polymer that influence polyplex–cell membrane interaction, like the hydroxyalkyl chains in *SS*-PAAs, can enhance the transfection efficiency.

2.5. Effects of variable charge density and buffer capacity of the pendant groups in SS-PAAs

As DNA binding capability and buffer capacity have appeared to be two important parameters for the gene delivery properties of polymeric vectors, we have further investigated these parameters in more detail. Therefore, a series of *SS*-PAA copolymers were synthesized that possess variable charge density and buffer capacity, starting from cystamine bisacrylamide as the bisacrylamide monomer and variable ratios of histamine (HIS) and dimethyl aminopropylamine (DMPA) as the amine monomers, yielding p(CBA-HIS*x*/DMPA*y*) [42]. The DMPA side groups have a relatively high basicity ($pK_a \sim 8.0$) and



p(CBA-DMPA)	***x/y* = 0/100**
p(CBA-HIS*30*/DMPA*70*)	***x/y* = 30/70**
p(CBA-HIS*50*/DMPA*50*)	***x/y* = 50/50**
p(CBA-HIS*70*/DMPA*30*)	***x/y* = 70/30**
p(CBA-HIS)	***x/y* =100/0**

Fig. 5. Bioreducible poly(amido amine) random copolymers with high- and low-basicity amino groups.

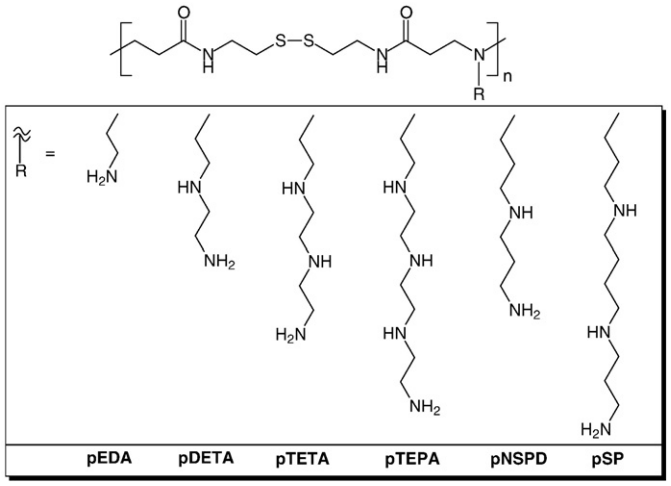


Fig. 6. Bioreducible poly(amido amine)s with various oligoamines in side groups.

are mostly protonated at physiological pH, whereas the imidazole groups in HIS have moderate basicity (pK_a~6.5) and are mostly unprotonated at physiological pH. Therefore different effects can be expected for these groups in DNA binding capacity, related to charge density, and buffer capacity, related to proton uptake capacity. (Fig. 5). It was found that the incorporation of a low percentage of DMPA moieties into the p(CBA-HIS*x*/DMPA*y*) copolymer is already sufficient to obtain a copolymer with a DNA binding capability that is comparable to the p(CBA-DMPA) homopolymer and is significantly higher than the p(CBA-HIS) homopolymer. The buffer capacity of the copolymers can be controlled by the composition of the side groups and increases with increasing HIS/DMPA mole ratio in the copolymers. The copolymer p(CBA-HIS*70*/DMPA*30*), having a HIS/DMPA mole ratio of 70/30, combines optimal DNA condensation capability and buffer capacity, and induces higher transfection efficiency than their analog p(CBA-HIS) and p(CBA-DMPA) homopolymers, both in the absence and in the presence of serum. This study indicates that systematic optimization of both DNA condensation and buffer capacity of the polymeric vector is necessary to find the optimal combination of properties for the highest gene transfection.

2.6. Effects of amino type and amino spacer length in the pendant groups of SS-PAAs

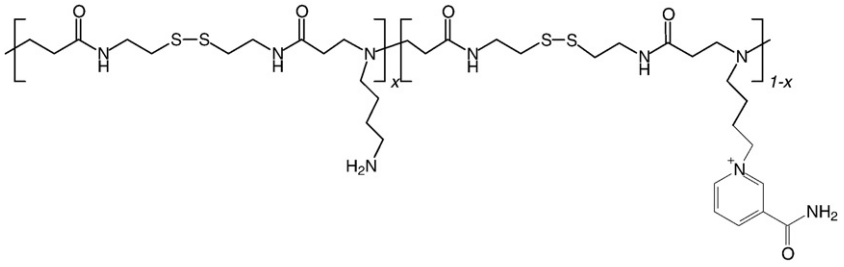
The effect of functionalities on DNA binding capability and buffer capacity was further studied in the group of bioreducible poly(amido amine)s containing different oligoamine side chains (*SS*-PAOAs) (Fig. 6) [43]. These linear polymers all show strong DNA binding capability due to the presence of protonated primary amine in the pendant chain. The *SS*-PAOAs containing secondary amino functions in the side chain (*i.e*. pDETA, pTETA and pTEPA) show high buffer capacities and are able to transfect COS-7 cells *in vitro*, with transfection efficiencies similar or even higher than those of 25 kDa branched pEI, along with very low cytotoxicity. However, increase of the amino spacer length from ethylene to propylene between the amino units in the side chains results in significantly lower transfection and increased cytotoxicity (pDETA vs. pNSPD and pTETA vs. pSP). This study indicates that type of amino group and the amino spacer length can have distinct effects on the buffer capacity, transfection efficiency and cytotoxicity profile of polymers.

2.7. Effects of intercalating groups in SS-PAAs

Polyplexes are prone to dissociation by competitive displacement of negatively-charged biomacromolecules in the extracellular space. Whereas increase of charge density in the polymeric vector can enhance DNA binding and polyplexes stability, the trade off of this approach can be a concomitant increase in cytotoxicity due to increased protein and membrane binding of the higher positively-charged polymers. Therefore, we have designed an alternative approach to enhance DNA-polymer interaction, *i.e*. developing *SS*-PAAs copolymers appended with supramolecular binding moieties that can bind to DNA by intercalation [44]. Therefore, *SS*-PAAs copolymers with different amounts of quaternary nicotinamide moieties in the side chains were synthesized (Fig. 7). It was expected that the quaternary nicotinamide moieties could bind DNA not only by electrostatic interaction but also by proximate intercalation. It was found that the presence of quaternary nicotinamide moieties in the polymer indeed results in increased DNA binding capability and improved stability of the polyplexes in the presence of heparin as a binding competitor to DNA. Moreover, these polymers show improved transfection efficiency and good cell viabilities. The *SS*-PAAs with quaternary nicotinamide pendant groups have assembled multiple chemical functionalities to optimize polymeric gene delivery properties, *i.e*. they possess good DNA binding ability, improved colloidal stability, high buffer capacity and efficient vector unpacking, thereby leading to 4-fold higher transfection efficiency than linear pEI (Exgen 500) and meanwhile having low cytotoxicity in COS-7 cells.

3. Conclusions

Although cationic polymers are regarded as promising vectors for non-viral gene delivery for many years, their clinical application is limited by the relatively low transfection efficacy. Different from viral vectors, simple cationic polyplexes do not possess all the mechanisms to efficiently overcome the extra- and intracellular barriers encountered in the temporal-spatial pathway to gene transfection. However, various chemical functionalities in cationic polymers can profoundly influence the factors that are important to overcome gene delivery barriers. In this respect, poly(amido amine)s are especially versatile polymers as different functionalities can be easily introduced while maintaining the bulk structural integrity of these polymers. Therefore, this system can be used as a unique research platform to elucidate the complicated correlations between the polymeric functionalities and



p(Nic*X*-NH$_2$), *X*= 10%, 30%, 50%

Fig. 7. Bioreducible poly(amido amine)s with quaternary nicotinamide moieties in side groups.

gene delivery properties. Functionalities with various structural characteristics as charge density, rigidity, basicity, buffer capacity, hydrophilicity/hydrophobicity, type and number of amino groups, backbone degradability and specific interaction groups are recognized to influence gene delivery properties such as DNA binding capability, colloidal stability, endosomal escape (buffer capacity), vector unpacking and cytotoxicity. Therefore, optimal combination of various functionalities is necessary to obtain multifunctional polymeric vectors that are capable to overcome the multiple gene delivery barriers and yield high transfection efficiencies.

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