Bioreducible Polycationic Gene Vectors

Guoying Si

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BIOREDUCIBLE POLYCATIONIC GENE VECTORS

DISSERTATION

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*To my parents,
my family, and my lover*

獻給我的父母，
我的家人和我的摯愛

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Chapter 1. General Introduction

BACKGROUND

Gene delivery is emerging as a promising modality in nanomedicine and precision medicine, and eventually will improve the health and wellbeing of humans.¹⁻⁵ The biologically active genes are exogenous nucleic acids like DNA, messenger RNA (mRNA), small interfering RNA (siRNA), microRNA (miRNA) as well as antisense oligonucleotides. These nucleic acids are relatively large in molecular size, have negative surface charge, and are labile to various enzymes, which necessitates the use of gene vectors to overcome the many hurdles that externally administered genes undergo on their way to the interior of targeting cells.⁶ Viral vectors can be used for this purpose, but despite being efficient in transfection, viral vectors are plagued with various problems related to safety and manufacturing, which renders non-viral vectors as appealing alternatives.^{3,7} Among non-viral vectors, in particular cationic polymers have attracted attention owing to beneficiary features such as synthetic versatility, modification flexibility, absence of immunogenicity, and high loading capacity.⁸⁻¹⁰ However, compared to viral vectors, their transfection efficiencies are relatively low, making it appealing to pay efforts in the design and development of novel polymers with both high transfection efficiency and minimal cytotoxicity.⁴

As peptidomimetic polymers, poly(amido amine)s (PAAs) have been intensively studied as non-viral gene vectors, owing to their facile preparation, ease of functionalization, low cytotoxicity, and good water solubility.^{11,12} Protonation of part of the tertiary amino groups in the polymer chain turns these polymers to polycations which readily form complexes (polyplexes) with negatively charged nucleic acids. Repetitive disulfide bonds were successfully incorporated in the PAA main chains to take advantage of the reductive intracellular environment which cleaves the disulfide bonds and releases genetic materials in an active manner. Disulfide-linked PAAs, denoted as bio-reducible PAAs, possess high buffer capacity, which facilitates endosomal escape of polyplexes of these PAAs, leading to functional activity of the delivered genes in the cells.¹³ These PAAs have been successfully employed as gene vectors for delivery of DNA,¹⁴ siRNA,¹⁵ and proteins^{16,17} in different formulations with outstanding efficiency and excellent cell viability. However, the application of these bio-reducible PAAs *in vivo* is mired by the disturbance of serum on the their polyplex stability. The attachment of hydrophilic poly(ethylene glycol) (PEGylation) to the polymers to form a hydrophilic shell around the polyplexes somehow mitigates the problem, however this approach also results in low cellular uptake and insufficient endosomal escape, both leading to lowered transfection performance.¹⁸ Thus, it is of great

interest to improve transfection efficiency of bioreducible PAAs in the presence of serum through other approaches rather than PEGylation. In general, it is of great interest to develop novel bioreducible cationic polymers as gene vectors with efficient transfection and minimal toxicity.

Although the advent and development of nanotechnology have yielded our society with numerous benefits it also has brought some concomitant risks.^{19,20} Therefore as part of this PHD thesis program in the framework of NanoNextNL, all the toxicity assays in this thesis are dedicated to a risk analysis and technology assessment (RATA) analysis.

AIM OF THE STUDY

The purpose of the study involved in this thesis is dedicated to: (i) improving transfection efficiency of disulfide-linked poly(amido amine)s (SS-PAAs) in the presence of serum through physical and chemical approaches; (ii) developing a novel class of linear bioreducible poly(amino ether)s as efficient polycationic vectors with minimal toxicities and less susceptibility to serum; and (iii) evaluating the *in vitro* cell viability of polyplexes made of above polymers with DNA in relation to a RATA analysis of novel cationic polymeric vectors.

OUTLINE OF THE THESIS

The thesis focuses on the optimization and development of bioreducible cationic polymers as efficient and non-toxic gene vectors, as well as performing RATA analysis on the developed polymeric vectors. The outline of the thesis is as follows:

Chapter 2 deals with an overview of current bioreducible polymeric gene delivery systems.

Chapter 3 presents thiourea-functionalized SS-PAAs that form stabilized polyplexes through hydrogen bonding between the SS-PAA thiourea groups and the phosphate ester groups of DNA. These polyplexes show improved transfection efficiency under serum-free and serum-present conditions.

Chapter 4 describes the introduction of fluoroalkyl groups onto SS-PAAs by the anhydride-amine reaction. Polyplexes of the fluorinated PAAs are stabilized by increased hydrophobic interactions and exhibited significant higher transfection activity under serum conditions, as compared to their non-fluorinated analogs.

Chapter 5 introduces hybrid polyplexes comprising of SS-PAAs, gold nanorods, and plasmid DNA as a novel type of gene vectors with increased colloidal stability due to gold-

sulfur interactions. Gold nanorods were first coated with SS-PAA and subsequently mixed with DNA to form polyplexes with slightly higher positive surface charge compared to normal polyplexes without gold nanorods. Hybrid polyplexes showed higher transfection activity under serum conditions, compared to normal polyplexes.

Chapter 6 introduces a novel class of cationic polymeric gene vectors, the poly(amino ether)s (PAEs) with various amounts of disulfide linkages in the main chain. The PAEs were synthesized by the polyaddition of 4-amino-1-butanol with various ratios of disulfide-containing and disulfide-free diglycidyl ether. PAEs with higher disulfide content gave higher transfection activity, indicating the favorable effect of the presence of the disulfides in the PAEs-based gene delivery system.

Chapter 7 continues Chapter 6 with the preparation of a series of disulfide-containing PAEs (SS-PAEs) with different side chains by polyaddition of various primary amines to a disulfide-containing diglycidyl ether, and the evaluation of the effect of structural variations on the transfection activity mediated by these SS-PAEs.

Chapter 8 describes a promising modular synthesis of SS-PAEs via polyaddition reaction of disulfide-containing bi(secondary)amine with various diglycidyl ethers. The SS-PAEs induce remarkable transfection without cytotoxicity in COS-7 cells, both in serum-free and serum-containing medium, underlining the promising potential of these SS-PAEs vectors for *in vivo* gene delivery.

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Chapter 2. Bioreducible Polymeric Gene Delivery Systems

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KEYWORDS

Bioreducible polymers; polycations; polydisulfide; drug delivery; nanomedicine; gene therapy.

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ABSTRACT

Gene delivery concerns the introduction of exogenous genes into cells through either viral- or non-viral gene vectors, and possesses tremendous potential in therapeutic applications. In comparison to their viral counterparts, non-viral vectors bring a number of important advantages, such as decreased cytotoxicity and immunogenicity, ease of preparation and good possibilities for modular functionalization. Among non-viral gene delivery vectors, polycationic materials are the most popular vectors, since they are able to associate genes into nanoscaled polyplexes through electrostatic interactions, and can escort genes to the cells, and eventually release genes into the cytoplasm to target the gene machinery. This however, requires conflicting colloidal stabilities of the polyplexes in the extracellular environment and the intracellular milieu (i.e. highly stable vs labile, respectively). To address this stability paradox, the high redox potential inside cells has been exploited by incorporation of bio-reducible disulfide moieties. The polycationic vectors based on disulfide containing polycations are stable outside cells but are rapidly degraded by cleaving of the disulfides in the intracellular milieu. In this chapter, various approaches to introduce bio-reducible disulfide groups in polycationic gene delivery carriers and the effect on polyplex properties, transfection efficiency, and cell toxicity will be discussed.

INTRODUCTION

Nanomedicine tackles human health issues through nanotechnology, and denotes a multidisciplinary field combining chemistry, physics, biology, medicine and engineering.¹⁻⁵ Within nanomedicine, gene delivery has attracted considerable attention owing to its therapeutic potential in treating numerous genetic and acquired diseases, among which cancers,⁶ cystic fibrosis,⁷ Parkinson's disease,⁸ immunodeficiency,⁹ chronic granulomatous disorder,¹⁰ as well as deciphering biological events at a molecular level.¹¹ The genes, also referred to as genetic materials, include DNA,^{12,13} messenger RNA (mRNA),^{14,15} small interfering RNA (siRNA),¹⁶⁻¹⁹ microRNA (miRNA)^{20,21} or antisense oligonucleotides.^{22,23} Efficacious gene delivery relies on efficient and safe vectors that are able to protect the gene from denaturation by a wide variety of enzymes, while facilitating cellular uptake and gene traffic inside the targeted cells.^{24,25} The vectors for delivering genes are categorized into two main types: viral vectors^{26,27} and non-viral vectors.^{25,28} Viral carriers, consisting of retroviruses, adenoviruses, and adeno-associated viruses, are imparted with gene to be delivered and typically display high efficiency in introducing genetic material into host cells.²⁹ However, they are plagued with insertional mutagenesis,³⁰ immunogenic

responses,³¹ and toxicity,³² all of which severely impede their widespread implementation in real applications. Non-viral carriers present an interesting alternative,³³ in particular cationic polymers.³⁴⁻³⁶ Cationic polymers are characterized and can be designed by their ease of preparation and functionalization with minimal toxicity and immunogenicity.²⁴ Cationic polymeric gene vectors form complexes with genes into so-called polyplexes through electrostatic interactions between the cationic sites in these polymers and the anionic phosphate groups present in genes.³⁷⁻³⁹

The advances in polymer chemistry⁴⁰ and nanotechnology^{41,42} allow for accurate manipulation of polymeric architectures and hence have fueled important progress in non-viral gene delivery.^{43,44} However, cationic polymeric gene delivery systems still need to overcome numerous barriers before becoming a feasible approach in gene delivery.^{25,45} Those barriers include efficient cellular entry to target cells,⁴⁶ endosomal escape,⁴⁷ gene unpacking and subsequent internalization,⁴⁸ and DNA transcription and translation.⁴⁹ Ideally, polyplexes should have prolonged systemic circulation with excellent colloidal stability, avoid reticuloendothelial clearance, possess the ability to extravasate tissues and enter target cells, while circumventing lysosomal degradation, and releasing genes to the sites of interest resulting in gene expression.⁵⁰ Polyplexes should have high stability outside the cells, but should rapidly destabilize to release the gene payload after cellular entry.²⁴ These conflicting requirements between extracellular stability and intracellular stability have triggered exploiting the reductive potential across the cell membrane.⁵¹⁻⁶⁰ In secreted proteins, matrix proteins, and cell surface proteins, the cysteine amino acids mainly exist in the intracellular compartments in their (cystine) disulfide form.⁶¹ Inside the cell, glutathione (GSH) and enzymes from the thioredoxin family collectively generate a reducing cytosol.⁶² Intracellular GSH concentrations are between 1-11 mM, which is approximately 100-1000 times higher than extracellular concentrations.⁵⁹ Disulfide bonds are rapidly cleaved in the reducing cytoplasm, but remain intact outside the cell. Disulfide bonds have therefore been deliberately incorporated into polymeric gene vectors to resolve the aforementioned conflicting requirements in the stability of polyplexes.⁶⁰

Figure 1. Schematic illustration of DNA delivery via bioreducible polycations as gene vector.

Disulfide-functional cationic polymeric vectors can associate nucleic acids through electrostatic interactions into nanoscaled polyplexes. Cationic polyplexes have affinity for the negatively charged cell membrane and facilitate nucleic acid entry into the cell via endocytosis. After endosomal escape the polyplexes arrive in the reducing environment of the cytosol and dissociate to release nucleic acid after intracellular cleavage of disulfide linkages, resulting in transcription of the gene (Figure 1). Intracellular cleavage of polymers containing disulfide bonds implies reduction of the disulfide bonds into thiol groups, and the vectors are therefore also named bioreducible polymeric vectors.⁵⁹ In this chapter, an overview of bioreducible non-viral gene vectors is presented with a focus on synthetic gene vectors.

POLYETHYLENEIMINE DERIVATIVES

Various approaches of incorporation disulfide bonds

Polyethyleneimine (PEI, **1**, Figure 2) is one of the most widely used transfection agents, because of a combination of excellent gene binding abilities and good endosomal buffering capacity.⁶³⁻⁶⁶ However, its full application potential is severely impeded by its intrinsic cytotoxicity.⁶⁷⁻⁷⁰ Disulfide bonds have been introduced into PEI to prepare bioreducible gene vectors with efficient transfection ability, but with mitigated cytotoxicity. Branched

PEI (800 Da) was reacted with bifunctional disulfide-based crosslinkers (i.e. dithiobis(succinimidylpropionate) and dimethyl 3,3'-dithiobispropionimide) resulting in polymeric vectors (**2**) which exhibited transfection abilities comparable to PEI (25 kDa) as evaluated in Chinese hamster ovary (CHO) cells with substantially reduced toxicity.⁷¹ The comparable transfection activity and reduced toxicity is theorized as the polyplexes disassembled due to GSH-induced disulfide cleavage, resulting in free DNA and small polymer fragments that are easily cleared.⁷¹ Polyplexes based on disulfide-crosslinked PEI and DNA prepared at an N/P ratio of 10 showed improved biocompatibility over non-reducible branched PEI (25 kDa).⁷² Branched PEI (800 Da) was crosslinked via disulfides through either thiolation followed by oxidation (**3**)⁷³ or through Michael addition on cystamine bisacrylamide (CBA) (**4**).⁷⁴ Compared to PEI (25 kDa), the disulfide-linked branched PEI (800 Da) gave higher transfection activity, lower cytotoxicity, and increased serum-stability as revealed by *in vitro* experiments.^{73,74} The transfection activity of disulfide-linked branched PEI (1.8 kDa) was further increased through conjugation with the endosomolytic protein listeriolysin O from the intracellular pathogen *Listeria monocytogenes*.⁷⁵ Zhong *et al.* reported the modification of branched PEI (1.8 kDa) with hydrophobic lipoic acids via carbodiimide chemistry (**5**), to give up to 500-fold increased transfection ability in HeLa and 293T cells as compared to unmodified parent groups, and up to 3-fold as compared to PEI (branched, 25 kDa).⁷⁶ The same research group later enhanced transfection activity (4-fold) and reduced toxicity of PEI (25 kDa) by introducing a bioreducible cystamine periphery on the PEI polymer through Michael-type addition (**6**).⁷⁷ Bae and coworkers thiolated branched PEI (800 Da) with 2-iminothiolane and successively oxidized the thiols to obtain bioreducible PEI as gene vectors with molecular weights ranging from 5-80 kDa (**7**), while preserving the high buffering capacities of the parent PEI.⁷⁸ The bioreducible gene vectors were demonstrated to display at least 8 times less cytotoxicity than standard PEI (25 kDa). With binding DNA into 100-200 nm-sized polyplexes with cationic surface charge (+20-35 mV), the bioreducible vectors exhibited approximately 1200 to 1500-fold and 7-fold higher transfection activities than parent PEI (800 kDa) and standard PEI (25 kDa) respectively as revealed from *in vitro* transfection experiments.⁷⁸ Branched PEI (1.2 kDa) was successively guanidinylated, thiolated, and oxidized, to afford bioreducible guanidinylated PEI (**8**) with enhanced cellular uptake, low cytotoxicity, and remarkable transfection activity in human breast cancer cells (MCF-7 and MDA-MB-231) and cervical cancer cells (HeLa).⁷⁹

Figure 2. Chemical structures of bio-reducible polymeric gene vectors based on polyethylenimine derivatives.

Goepferich *et al.* prepared disulfide-linked branched PEI vectors (**9**) by crosslinking linear PEI (2.6, 3.1, and 4.6 kDa) with dithiodipropionic acid or cysteine linkers through

EDC/NHS chemistry.⁸⁰ The reversibly crosslinked vectors degraded under reducing conditions into non-toxic fragments, which ensured their high biocompatibility observed in cell experiments. The bioreducible branched PEI-based vectors gave superior transfection activities with up to 70% transfection efficiency achieved in HEK cells.⁸⁰ These bioreducible vectors enabled siRNA delivery for the knockdown of EGFP (enhanced green fluorescent protein) expression in CHO-K1 cells. The cellular uptake of siRNA was boosted with increased degree of branching in the vectors.⁸¹ Also Zhao *et al.* have used the same chemistry to crosslink branched PEI (800 Da) and used the obtained bioreducible PEI successfully as delivery vector of hTERT (human telomerase reverse transcriptase) siRNA to inhibit the growth of HepG2 xenograft tumor model.⁸²

Another approach was followed by Jiang *et al.* who employed click chemistry to prepare disulfide-crosslinked branched PEI (1.8 kDa) as bioreducible nonviral gene vectors.^{83,84} The branched PEI was first functionalized with approximately four azide pendant groups, which were subsequently reacted with a disulfide-containing dialkyne linker via CuBr-catalyzed click reaction, leading to bioreducible high molecular weight PEI (**10**).⁸³ Transfections carried out on 293T and HeLa cells revealed that the bioreducible PEI exhibited superior transfection activity and substantially lower cytotoxicity as compared to conventional branched PEI (25 kDa), both under serum-free and serum-present conditions.⁸³ Multi-azido-functional branched PEI (1.8 kDa) was also prepared and reacted with mono-alkyne-terminated PEI with disulfide linkages, to give hyperbranched disulfide-containing PEI (**11**) with much lower cytotoxicity and similar transfection efficiency.⁸⁴ Mono alkyne-terminated PEI was grafted to poly(aspartic acid) with various azide pendant groups, forming a bioreducible polymer brush with minimal toxicity and high transfection activity, as demonstrated in 293T cells.⁸⁵

Fluorescence measurements to follow intracellular disulfide reduction

Bioreducible linear PEI (**12**) with molecular weights ranging from 7.0 kDa to 11.0 kDa was synthesized by oxidative polycondensation of ethylene imine oligomers with two terminal free thiols.⁸⁶ Its transfection activity increased with increasing amine density, approaching that of PEI (25 kDa). The bioreducible linear PEI exhibited low cytotoxicity, owing to the complete intracellular degradation within 3 h, as demonstrated by fluorescence microscopy using probe–probe de-quenching of BODIPY-FL fluorescent dyes.⁸⁶ Wu *et al.* labeled linear PEI (3.0 kDa) with Rhodamine B via a linker that

contained one free thiol, and applied oxidation to form disulfide linkage between two linear PEI chains. Fluorescence quenching of the Rhodamine B molecules indicated that these were closely coupled. Fluorescence was restored upon disulfide cleavage. Moreover, the plasmid was modified with a Rhodamine B fluorescence resonance energy transfer (FRET) donor of BODIPY. These fluorescent materials possess higher transfection activity and lower toxicity as compared to conventional branched PEI (25 kDa) which was exemplified on HepG2 cells. The analysis of FRET and self-dequenching of the polyplexes unveiled that (i) DNA release from the polyplexes occurred prior to disulfide cleavage; (ii) some polyplexes rapidly escaped the endosome, and (iii) cleavage of disulfides took place inside lysosomes 5 h after endocytosis.⁸⁷

Qiao and coworkers developed an autofluorescent linker with dual responsiveness towards pH and GSH that enabled traceable and controlled delivery of nucleic acids.⁸⁸⁻⁹⁰ The autofluorescent linker consisted of acetaldehyde-modified-cystine (AC) with one disulfide bond and two Schiff bases, which induce autofluorescence via the $n-\pi^*$ transition of their two C=N bonds.⁹¹⁻⁹³ The $n-\pi^*$ transition is broken upon disulfide reduction in the cytosol and imine bonds are hydrolyzed by the low pH in late endosomes, leading to different fluorescence intensities for imaging.⁸⁸ Uniform dendrimer-linked amino-functionalized silica nanoparticles with hierarchical pores (HPSNs-NH₂) were grafted with non-toxic branched PEI (800 Da) via AC linkers (Figure 3). The traceable gene vectors exhibited strong gene binding ability, negligible cytotoxicity, and transfection efficiency enhancements of 80%, 72%, and 56% in HEK293, HeLa, and CHO cells, respectively, as compared to linear PEI (22 kDa) and Lipofectamine 2000.⁸⁸ Fluorescence of the gene vectors inside the cells increased between 0.5-6 h post-transfection, due to accumulative uptake of the vectors inside cells. However, fluorescence weakened and eventually disappeared 6 h after transfection. This was ascribed to Schiff base hydrolysis and disulfide cleavage.⁸⁸ The AC linker was subsequently also employed in HPSNs/PEI hybrid vectors⁹⁰ and PEI-based nanogels⁸⁹ to obtain traceable and responsive delivery of siRNA.

Figure 3. Schematic illustration of GSH- and pH-responsive traceable gene delivery system based on HPSNs-AC-PEI (a) and its corresponding synthetic route (b).⁸⁸

Stabilization of polyplexes

PEI (branched, 25 kDa)/DNA polyplexes were endowed with robust extracellular stability against anionic competition through coating with hydrophilic poly[*N*-(2-hydroxypropyl)methacrylamide] (PHPMA) via disulfide linkages between PHPMA and PEI. DNA was fully released upon treatment with DTT (20 mM) due to disulfide reduction. The resulting polyplexes exhibited up to 100-fold higher transfection efficiencies as compared to polyplexes based on vectors coated with PHPMA through thioethers.⁹⁴ Alternatively, PEI (25 kDa)/DNA polyplexes were stabilized with disulfide-containing dithiobis(succinimidyl propionate) (DSP) as a reversible linker (**13**).^{95,96} As anticipated, the resulting disulfide-crosslinked polyplexes showed numerous merits in terms of resistance against polyanion exchange and high ionic strength, colloidal stability, and little- to no association with blood proteins and erythrocytes.⁹⁵ The crosslinked polyplexes were efficiently taken up by NIH 3T3 cells and subsequently released DNA through intracellular reduction of the vector. After intravenous administration, transfection dominated in the liver rather than in the lung (undesired), owing to higher blood levels for crosslinked polyplexes resulting from disulfide crosslinking.⁹⁷ In following studies, high molecular weight PEG (30 kDa) was conjugated onto branched PEI (25 kDa) through urea linkages, affording PEG-PEI copolymers. The copolymers efficiently complexed DNA into polyplexes that were further crosslinked by DSP. The resulting polyplexes exhibited 10-

fold improved transfection activity and substantially reduced hemolytic activity, owing to their significantly prolonged circulation *in vivo*.⁹⁶ The effect of PEGylation on transfection by bioreducible PEI-based vectors was investigated in detail by Bauhuber *et al.* on a library of 39 linear PEG-PEI copolymers with either thioether or disulfide linkages.⁹⁸ This study revealed that PEG, rather than PEI, domains dominate the physicochemical properties of polyplexes and small (<150 nm), nearly neutral polyplexes with favorable stability were formed from copolymers with PEG contents over 50%. Compared to the corresponding parent PEI, PEG-PEI copolymers linked through thioether bonds exhibited substantially reduced transfection activity that was restored upon substituting thioethers with disulfides.⁹⁸ More recently, PEI (25 kDa)/DNA polyplexes were coated with bioreducible PEG-based nanoshells containing CBA. These PEI (25 kDa)/DNA polyplexes exhibited robust colloidal stability against physiological serums and gave boosted *in vivo* transfection activity as compared to pristine polyplexes.⁹⁹

Guo and Zhou *et al.* modified the surface of gold nanorods (GNRs) with disulfide-linked short polyethylenimine (DSPEI) to achieve NIR-triggered gene delivery to glioblastoma U-87 MG cells (Figure 4).¹⁰⁰ DSPEI was functionalized with PEG and tagged with RGD targeting ligands to obtain DSPEI-PEG-RGD, which replaced CTAB on the GNR surface (termed as GNR-DSPEI-PEI-RGD). GNR-DSPEI-PEI-RGD is able to condense DNA into complexes, which can be destabilized by addition of DTT and/or applying NIR irradiation. The complexes readily transfect U-87 MG cells with enhanced transfection efficiency under NIR irradiation. The NIR-enhanced transfection is effected by photochemical-triggered endosomal escape.¹⁰⁰ After functionalization with RGD targeting ligands, this system successfully enabled delivery of small hairpin (sh)RNA delivery through active targeting, affording efficient gene silencing both *in vitro* and *in vivo*.¹⁰¹

Figure 4. Schematic illustration of NIR-triggered gene delivery carrier based on disulfide-linked PEI.¹⁰⁰

After intracellular reduction of disulfide-containing polyplexes prepared from PEI, the resulting free thiols exchange with disulfide bonds in proteins through thiol/disulfide exchange or form new disulfides with cysteine residues on proteins via oxidation, which potentially interferes with protein function and normal cellular processes.^{102,103} Dong *et al.* developed bioreducible PEI-based vectors (**14**) via crosslinking branched PEI (2 kDa) with a releasable disulfide-carbonate linker.¹⁰⁴ The cell experiment revealed that the bioreducible gene vectors possessed higher transfection ability and lower cytotoxicity as compared to control groups of PEI (25 kDa) and Lipofectamine 2000. After reductive cleavage of this linker, intramolecular cyclization takes place, leading to cleavage of the carbamate bond with degradation products being 1,3-oxathiolan-2-one rather than free thiols.^{105,106}

POLYVINYL DERIVATIVES

Poly(*N,N*-dimethylaminoethyl) methacrylate (PDMAEMA, **15**, Figure 5) is an interesting class of cationic polymeric vectors with great promise in clinical applications.^{107,108} PDMAEMA can be readily prepared through anionic¹⁰⁹ and controlled radical polymerization techniques like atom transfer radical polymerization (ATRP)¹¹⁰ and reversible addition fragmentation transfer (RAFT)¹¹¹ with tunable molecular weights, well-defined terminal groups, and different macromolecular architectures ranging from block, star, to graft. High molecular weight PDMAEMA has been demonstrated to give high transfection activity, but also displays substantial toxicity.¹⁰⁸

Figure 5. Chemical structures of bioreducible polymeric gene vectors based on polyvinyl derivatives.

Bioreducible PDMAEMA (**16**) was first reported by Oupický and coworkers in 2007.¹¹² They proposed an elegant approach based on oxidative polycondensation of oligomers with thiol endgroups.¹¹²⁻¹¹⁴ A bifunctional chain transfer agent, 1,4-bis(2-(thiobenzoylthio)prop-2-yl) benzene (BTBP), was employed in the preparation of PDMAEMA oligomers with thioester endgroups. These thioesters were converted to thiols via aminolysis, affording α , ω -dithiol-terminated PDMAEMA oligomers, which were further polymerized through oxidative polycondensation to obtain disulfide linked high molecular weight PDMAEMA (M_n =16.7 and 53 kDa). The resulting PDMAEMA exhibited up to 10-fold higher molecular weight, as well as higher polydispersities in comparison to the parent oligomers. The bioreducible PDMAEMA displayed much lower cytotoxicity, but comparable and enhanced transfection ability in a panel of cell lines as compared to non-bioreducible PDMAEMA.¹¹²

Bioreducible cationic star polymers based on PDMAEMA (sPDMAEMA) were prepared using cystamine bisacrylamides (CBA) as a core, from which PDMAEMA arms were polymerized.¹¹⁵ In an approach in which the ‘arms’ were prepared first,¹¹⁶ linear PDMAEMA precursor polymers were synthesized and subsequently crosslinked via CBA, affording bioreducible sPDMAEMA (M_n =8.3 and 28.3 kDa) with average numbers of arms of 8.2 and 10.7 respectively. The bioreducible sPDMAEMA showed enhanced DNA binding ability, and improved transfection efficiency as compared to its linear precursor. With longer and more arms, sPDMAEMA exhibited decreased cytotoxicity and enhanced transfection activity as compared to standard PEI (25 kDa).¹¹⁵ Atomic force microscopy (AFM) and time-resolved fluorescence spectroscopy revealed that sPDMAEMA/DNA polyplexes were destabilized by the steric hindrance of the arms, as well as the outward extension of the cationic arms upon decrease in pH from 7.4 to 5.0, while linear counterparts adopted a more compact polyplex morphology.¹¹⁵ Bioreducible sPDMAEMA was further employed as an siRNA vector to knock down genes in mouse calvarial preosteoblast-like cells (MC3T3-E1.4) with excellent cytocompatibility.¹¹⁷ Recently, CBA-linked PDMAEMA was applied as capping agent for siRNA-preloaded mesoporous silica nanoparticles (SS-MSN/siRNA) in siRNA delivery with minimal cytotoxicity and gene silencing efficiency comparable to Lipofectamine 2000.¹¹⁸ After injected through tail vein into mice, SS-MSN/siRNA efficiently delivered siRNA to HeLa-Luc xenograft tumor which led to tumor growth inhibition.¹¹⁸

Zhong and coworkers reported bioreducible ABA-type triblock copolymers of PDMAEMA and PEG, linked by disulfides (i.e. PDMAEMA-SS-PEG-SS-PDMAEMA, **17**).¹¹⁹ The bioreducible triblock copolymer efficiently condensed pDNA into nanoscaled (< 120 nm) polyplexes with surface charges of up to +6 mV. The polyplexes exhibited enhanced resistance against high ionic strength owing to the PEG corona around the polyplexes. pDNA was released from the polyplexes under reductive conditions as revealed by gel electrophoresis experiments. The polyplexes displayed similar cytocompatibility and 28-fold higher transfection activities on COS-7 cells than the non-reducible triblock copolymer.¹¹⁹ Low molecular weight PDMAEMA (12.7 kDa) was grafted onto poly(aspartic acid) (15.8 kDa) through disulfide spacers via azide/alkyne click chemistry, forming bioreducible brushed PDMAEMA (**18**).¹²⁰ Poly(aspartic acid) was functionalized with alkynes via disulfide linkers, and grafted by mono azido-terminated low molecular weight PDMAEMA, leading to polyaspartamide-based brushed PDMAEMAs. The bioreducible brushed PDMAEMAs exhibited much lower cytotoxicity and higher transfection capability on 293T cells than standard PEI (25 kDa) or high molecular weight PDMAEMA.¹²⁰ Pun *et al.* synthesized copolymers of DMAEMA and methoxy oligo(ethylene glycol) methacrylate (OEGMA) from a bifunctional ATRP initiator containing an internal disulfide, and subsequently used dibromomaleimide-alkyne to crosslink the obtained copolymers, affording bioreducible polycations (**19**) with molecular weights ranging from 15.4 kDa to 35.8 kDa.¹²¹ The bioreducible polycations exhibited comparable DNA complexation abilities and transfection efficiencies, but substantially lower cytotoxicity as compared to their non-reducible counterparts. Moreover, the alkyne groups in bioreducible polycations enabled azide-alkyne click functionalization with rhodamine fluorophores to investigate intracellular dynamics of the polyplexes, providing possibilities of controlled conjugation of biomolecules such as peptides and antibodies.

Wang *et al.* applied *in situ* deactivation enhanced ATRP (DE-ATRP)¹²²⁻¹²⁴ to prepare hyperbranched¹²⁵ or knot-shaped¹²⁶ PDMAEMA from DMAEMA and crosslinker bis(2-acryloyl)oxyethyl disulfide (BADS) with ethyl 2-bromoisobutyrate as an initiator. The branching degree of hyperbranched PDMAEMA increased upon increasing BADS content. At BADS/initiator ratios above 1, pendent vinyl groups remained intact, providing handles for functionalization with 3-morpholinopropylamine through Michael-type addition.¹²⁵ The resulting hyperbranched PDMAEMA (**20**) fragmented into smaller parts after 1 h treatment

with 20 mM GSH as revealed by size exclusion chromatography. *In vitro* experiments on HeLa cells disclosed that the transfection ability of hyperbranched PDMAEMA was higher than that of linear PDMAEMA and increased further with higher branching degrees. The hyperbranched PDMAEMA with the highest branching degree exhibited good preservation of cell viability.¹²⁵ The knot-shaped PDMAEMA with incorporated disulfides was prepared as a ‘single knot’ that self-assembled into multi-knots, whose residual vinyl moieties were converted to amines through Michael-addition to form bioreducible multi-knot PDMAEMA based gene vectors (see Figure 6).¹²⁶ The multi-knot PDMAEMA degraded into smaller fragments with one tenth of the original size through disulfide cleavage under 5 mM GSH. The multi-knot shaped polymeric vectors in particular showed high transfection activity and low cytotoxicity towards skin cells of recessive dystrophic epidermolysis bullosa (RDEB), which is superior over PEI (25 kDa) and Lipofectamine 2000. The bioreducible multi-knot vectors possess considerable potential for clinical therapy of RDEB and other wounds.¹²⁶

Figure 6. Bioreducible multi-knot PDMAEMA as gene vectors to potentially restore collagen VII expression in skin of recessive dystrophic epidermolysis bullosa (RDEB) patients.¹²⁶

Hennink and coworkers developed bioreducible decationized polyplexes with a PEG shell and a DNA-entrapping core of disulfide cross-linked poly(2-hydroxypropyl methacrylamide) (PHPMA) as an interesting gene delivery system.¹²⁷ The cationic block copolymer p(HPMA-DMAE-co-PDETMA)-b-PEG (HPMA-DMAE: carbonic acid 2-dimethylamino-ethyl ester 1-methyl-2-(2-methacryloylamino)-ethyl ester; PDETMA: *N*-[2-(2-pyridyldithio)]ethyl methacrylamide) was polymerized through controlled radical polymerization. The obtained cationic block copolymer (abbreviated as pHDP-PEG) complexed DNA into nanosized polyplexes through electrostatic inclusion at pH 8.5, followed by the formation of interchain disulfides with 3,6-dioxa-1,8-octane-dithiol and the removal of DMAE groups through hydrolysis of the carbonate ester bonds at pH 7.4 (

Figure 7). The decationized nanoparticles exhibit hydrodynamic sizes of 128 nm, surface charges of -5 mV and good preservation cell viability in HeLa cells. High transfection activity was only observed through electroporating the nanoparticles into HeLa cells, due to limited cellular uptake of these decationized nanoparticles. After functionalization with folic acid (FA) as a targeting ligand, the decationized polyplexes displayed enhanced delivery of DNA in cell lines that overexpress folate receptors (HeLa and OVCAR-3),¹²⁸ and successfully transported siRNA to human ovarian carcinoma cells.¹²⁹ These decationized polyplexes possess high biocompatibility and excellent colloidal stability in physiological milieu, indicating considerable potential for clinical application.¹²⁷⁻¹³⁰

Figure 7. Preparation of decationized polyplexes through DNA condensation, interchain disulfide crosslinking, and polyplex decationization.¹²⁷

SYNTHETIC POLYPEPTIDE DERIVATIVES

Bioreducible poly(L-Lysine)s (PLLs) (**21**, Figure 8) with molecular weights of 45 kDa and 187 kDa were prepared by oxidation of the terminal cysteinyl thiols of Cys(Lys)₁₀Cys. The resulting polymers efficiently condensed DNA into polyplexes approximately 65 nm in size and surface charges of +30 mV.¹³¹ The polyplexes were further coated with multivalent reactive copolymers of PHPMA resulting in a slight size increase of 15 nm and a substantial drop in surface charge down to -10 mV, affording steric stabilization of the polyplexes with high resistance against salt-induced aggregation. However, these laterally stabilized polyplexes exhibited much lower transfection activity in human retinoblast 911 cells compared to non-coated polyplexes. This was attributed to limited cellular uptake and

insufficient DNA unpacking.¹³¹ Bioreducible PLLs showed enhanced transfection ability of DNA and mRNA when in the presence of cationic lipids as endosomolytic agents.¹³² Histidine residues were included into bioreducible PLLs with enhanced buffer capacity, giving histidine-rich bioreducible PLLs which achieved efficient cytoplasmic delivery of plasmid DNA, mRNA, and siRNA on a panel of cells.¹³³ Bae *et al.* applied mixtures of PLL and bioreducible PLL at various ratios in the preparation of polyplexes with tunable decomplexation rates, and explored the optimal decomplexation rate for high transfection efficiency.¹³⁴ The monomer sequence in bioreducible histidine-rich PLL copolymers was found to substantially affect their DNA binding ability, buffer capacity and transfection efficiency. Differences in transfection activities of up to 2 orders of magnitude were observed for different monomer sequences.¹³⁵

Cationic block copolymers of PLL with hydrophilic PEG segments are capable to effectively condense nucleic acids into core-shell-type polyplexes with hydrophilic coronas.¹³⁶⁻¹³⁹ The micellar polyplexes exhibit remarkable transfection activity in various cell lines, as well as in liver and tumors owing to their excellent colloidal stability and prolonged blood circulation.¹⁴⁰ PEG-PLL cationic block copolymers, based on PLL segments that were thiolated through *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) spontaneously self-assemble with antisense oligonucleotides (ODN) into polyion complex (PIC) micelles with a disulfide-linked core. ODN was well protected in the micellar core against nuclease, but rapidly liberated upon GSH-induced core dissociation.¹⁴¹ The PLL segments were thiolated with either SPDP (**22**) or Traut's reagent (2-iminothiolane) (**23**) to vary both the cationic charge density and disulfide cross-linking densities of the PEG-PLL cationic blocks. The thiolated PEG-PLL associated with plasmid DNA into disulfide-linked block-cationic polyplexes with remarkable stability. Polyplexes from SPDP-modified PEG-PLL exhibited pDNA release in the reducing cytosol and induced ca. 50-fold higher transfection in 293T cells than that thiolated with Traut's reagent.¹⁴² Moreover, transfection of disulfide-linked PIC micelles was well maintained during the lyophilization-thawing procedure and did not require any lyoprotectants.^{142,143} After intravenous injection, reversible linked PIC micelles induce uniform gene expression in the liver parenchymal cells in mice.¹⁴³ The disulfide-linked PIC micelles were endowed with pH responsiveness for enhanced intracellular pDNA delivery¹⁴⁴ and further employed as efficient vectors for siRNA.^{145,146} PIC micelles were also prepared by spontaneous self-assembly of PEI-PLL thiolated with iminothiolane and siRNA. The resulting PIC micelles

showed uniform sizes of 60 nm with spherical shape and possessed high colloidal stability against high ionic strength and enzymes in the extracellular environment, but rapidly disassemble through disulfide cleavage in the cytoplasm with efficient release of siRNA. Substantially higher (100-times) gene silencing in human hepatoma (Huh7) cells was obtained as compared to non-crosslinked PIC micelles.¹⁴⁵ The disulfide-linked PIC micelles were tagged with cell-targeting cyclic RGD to achieve actively targeted systematic delivery siRNA to experimental cancers¹⁴⁷ and solid tumors.¹⁴⁸ Shuai *et al.* used siRNA loaded PIC micelles made from PLL grafted with linear PEI for the targeted delivery of siRNA *in vivo* and successfully obtained Skov-3 tumor inhibition in mice.¹⁴⁹

Cass *et al.* adopted diisocyanate condensation-induced chain extension to prepare bioreducible polycations as siRNA delivery vectors.¹⁵⁰ The disulfide-centered PLL oligomers (**24**) were prepared by ring-opening polymerization of ϵ -Cbz-L-lysine NCA (*N*-carboxyanhydride) with cystamine as initiator, and subsequently were extended with 1,6-hexanediiisocyanate and terminated with PEG-amine. After removing the protecting cbz-groups, the resulting bioreducible polymers were obtained with molecular weights ranging from 12 kDa to 23 kDa, and exhibiting good complexation of siRNA. The polyplexes had superior knockdown of GFP in CHO cells as compared to Lipofectamine 2000, with a knock down up to 50% of GFP expression.¹⁵⁰

PIC micelles were also prepared analogously using nucleic acids and PEG-based block cationomers prepared from other materials. Polyaspartamide was flanked with *N*-(2-aminoethyl)-2-aminoethyl group, leading to cationic segments (P[Asp(DET)]) with high buffer capacity.¹⁵¹ Block copolymers of PEG-P[Asp(DET)] were developed as efficient and safe gene vectors. Disulfide linkage was employed to link two polymer blocks, affording PEG-SS-P[Asp(DET)] (**25**) that associated with pDNA into PIC micelles. The PIC micelles exhibited up to 3 orders of magnitude higher transfection activity and faster initiation of gene expression than those without disulfide linkages, owing to improved endosomal escape via PEG detachment in the endosome.¹⁵² Shuai *et al.* prepared a GSH and pH-induced intracellular delivery system based on a triblock copolymer of PEG, 2-mercaptoethylamine (MEA)-grafted aspartamide (P[Asp(MEA)]), and 2-(diisopropylamino)ethylamine (DIP)-grafted polyaspartamide (P[Asp(DIP)]) (**26**). The triblock copolymer self-assembles into micelles with a disulfide-crosslinked interlayer and P[Asp(DIP)] as the core.¹⁵³ The micelles were subsequently applied as vector for long-

circulating and tumor-targeted siRNA delivery,¹⁵⁴ and co-delivery of siRNA and DOX for synergistic cancer therapy *in vivo*.¹⁵⁵

Figure 8. Chemical structures of bioreducible polymeric gene vectors based on synthetic polypeptides and poly(amino ester)s derivatives.

POLY(AMINO ESTER)S DERIVATIVES

Poly(amino ester)s (**27**, Figure 8) are a class of biodegradable cationic polymers intensively explored by Langer and coworkers¹⁵⁶ for non-viral gene delivery in the past decade.¹⁵⁷⁻¹⁶⁰ A large library of structurally diverse polymers was readily screened through high-throughput processes^{161,162} to identify optimal structures that result in efficient gene delivery to a myriad of cell lines *in vitro*. Also poly(amino ester)s were efficiently for gene delivery in the vitreous of eyes and tumors *in vivo*.^{163,164} Bio-reducible poly(amino ester)s (**28**) were first prepared through Michael addition between diacrylate monomers with 2-(pyridyldithio)-ethylamine (PDA).¹⁶⁵ The pendent pyridyldithio groups were readily reacted with thiol-containing moieties, such as mercaptoethylamine (MEA) and RGDC peptide. The obtained derivatives exhibited strong DNA binding ability that was substantially diminished in 10 mM GSH. The transfection experiments in human hepatocellular carcinoma cells illustrated the derivatives induced transfection efficiency comparable to standard PEI (25 kDa), however with much lower cellular toxicity.¹⁶⁵

Cystamine-terminated poly(amino ester)s were found to be efficient vectors in the knockdown of genes for enhanced osteogenic differentiation in human mesenchymal stem cells (up to 91%).¹⁶⁶ Kozielski *et al.* designed bio-reducible poly(amino ester)s as efficient and safe siRNA vehicles for nearly full knockdown of fluorescent marker genes in human glioblastoma cells.^{167,168} The bio-reducible cationic polymer was prepared by Michael-type polymerization of 4-amino-1-butanol with mixtures of 2,2'-disulfanediylbis(ethane-2,1-diyl) diacrylate and hexane-1,6-diyl diacrylate, followed by end-capping with 1-(3-aminopropyl)-4-methylpiperazine (Figure 9). The obtained cationic polymers self-assembled with siRNA to 100 nm sized polyplexes, and actively liberated siRNA from polyplexes through reductive cleavage of disulfides in the cytosol to induce gene silencing. Gene silencing experiments in primary human glioblastoma (GBM 319) cells expressing GFP (GFP⁺ GBM 319) demonstrated gene silencing efficiency up to 91% without significant cytotoxicity ($6 \pm 12\%$) compared to 40% knockdown induced by Lipofectamine 2000 based polyplexes at 20 nM siRNA dosing. The gene silencing was more effective in GBM 319 cells (up to 97%) than in human fetal neural progenitor cells (fNPC 34s, up to 27%), identifying cancer specificity of the polyplexes prepared from these poly(amino ester)s.¹⁶⁸

Figure 9. Cytoplasmic gene silencing in human brain cancer cells enabled by siRNA with vectors of bioreducible cationic poly(amino ester)s.¹⁶⁸

POLY(AMIDO AMINE) DERIVATIVES

Poly(amido amine)s (PAAs) are peptidomimetic polymers which have attracted considerable attention in drug and gene delivery, as well as tissue engineering, owing to their excellent water solubility, low toxicity, high biocompatibility, and synthetic versatility. PAAs are easily prepared via Michael-type polyaddition of various primary or secondary amines to bisacrylamide monomers under mild reaction conditions, to give PAAs with broad functionality in both the main polymer chain, as well as in the side groups.^{169,170}

Engbersen's group developed bioreducible PAAs with disulfides in the main chain (SS-PAAs) as non-viral gene vectors. The SS-PAAs were prepared via Michael addition of 1-(2-aminoethyl)piperazine (AEP) to bisacrylamide mixtures with various amounts of disulfide-containing bisacrylamide (CBA) (Figure 10A). *In vitro* experiments on COS-7 cells revealed that SS-PAAs exhibit higher levels of gene expression and reduced cytotoxicity as compared to PAAs without disulfides, owing to their high DNA binding ability, buffer capacity, and excellent biocompatibility.¹⁷¹ Later, various pendant groups were incorporated into SS-PAAs to tune their DNA complexation ability, buffer capacity, and transfection activity (Figure 10A).¹⁷² The amine moieties in the side groups of SS-PAAs afford stronger DNA binding ability and determine buffer capacity of the polymers. SS-PAAs with histamine (HIS), 5-amino-1-pentanol (APOL), and 4-amino-1-butanol (ABOL) moieties exhibit enhanced transfection activities without imposing any cytotoxicity to COS-7 cells, as compared to standard PEI (25 kDa), demonstrating that the side groups have strong influence on the transfection performance.^{172,173} Oligoamines with

varying amine content and alkyl spacer length were incorporated into SS-PAAAs as side groups through protective-group chemistry, and were shown to significantly improve buffer capacity, DNA complexation ability, transgene potency, and cytotoxicity of the resulting polymers (Figure 10A).¹⁷⁴ SS-PAAAs with oligoamine pendant groups were later used as vectors of Fas siRNA to suppress Fas expression in human mesenchymal stem cells (hMSCs) and therefore inhibit hypoxia-induced apoptosis in the enlarged hMSC spheroids, suggesting therapeutic potential in ischemic disease.¹⁷⁵ Oupický and coworkers incorporated macrocyclic amines into SS-PAAAs through Michael-type addition and prepared plasmid DNA delivery systems with PEI imaging modularity¹⁷⁶ and CXCR4 antagonism.¹⁷⁷

Figure 10. Chemical structures of bioreducible poly(amido amine)s (SS-PAA)s with different pendant groups (A) and alternative synthetic approach through polymerizing *N,N'*-dimethylcystamine (DMC) as amine monomers to various bisacrylamide monomers (B).

SS-PAA)s with pendant primary amine groups (pCBA-DAB) (Figure 10A) afford diverse functionalization opportunities, excellent DNA binding capacity, but also exhibit high

toxicities associated with excess cationic charges. After modification with hydrophobic moieties via benzoylation and acetylation, the modified SS-PAAAs exhibit less cytotoxicity and higher transfection activities on COS-7 cells due to the resulting polyplexes displaying hydrophobic stabilization and improved endosomolytic properties.¹⁷⁸ In continued optimization, through polymerization of amine mixtures comprising of ABOL and defined functionalized amine monomers, SS-PAAAs were successfully synthesized as vectors combining attributes of enhanced transfection performance and minimal toxicity through introduction of boronic acid groups,^{179,180} nicotinamide groups,^{181,182} as well as charge-reversal groups.¹⁸³ An alternative modular synthesis of SS-PAAAs was developed through polymerization of *N,N'*-dimethylcystamine (DMC) with various bisacrylamide monomers (Figure 10B). The resulting SS-PAAAs displayed high buffer capacity owing to the somewhat lower basicity of the tertiary nitrogens of DMC induced by the closer proximity of the disulfide moiety, and exhibit improved transfection without noticeable toxicity.¹⁸⁴

Kim *et al.* together with Engbersen *et al.* developed branched bio-reducible peptidomimetic polymers, coined poly(amido ethylenimine)s (SS-PAEI), through Michael type polyaddition between CBA and three ethylene amine monomers, i.e. ethylenediamine (EDA), diethylenetriamine (DETA), and triethylenetetramine (TETA) (Figure 11A).¹⁸⁵ All three SS-PAEIs associated with DNA into sub-200 nm and positively charged polyplexes, which induced approximately 20-fold higher transfection efficiencies in comparison to standard PEI (25 kDa) as revealed in mouse embryonic fibroblast cells (NIH3T3), primary bovine aortic endothelial cells (BAEC), and rat aortic smooth muscle cells (A7R5).¹⁸⁵ One of these three polymers, poly(amido ethylenimine) (SS-PAED), was applied as vector to deliver RTP-VEGF in a rabbit myocardial infarct model with significant VEGF expression, indicating the potential to promote neo-vascular formation and improve tissue function in ischemic myocardium.¹⁸⁶ Transfection efficiency of SS-PAEI was affected by serum proteins, though still significantly higher than standard PEI (25 kDa).¹⁸⁵ PEG (2 kDa) was grafted to SS-PAEI prepared from TETA and CBA (SS-PAEI-g-PEG) to minimize interactions with serum protein and to improve carrier effectiveness under serum conditions.¹⁸⁷ The polycation-PEG ratio was readily tuned by mixing different quantities of SS-PAEI and SS-PAEI-g-PEG (i.e. 50/50 and 90/10). When no more than 10% SS-PAEI-g-PEG was used, up to 70% DNA was protected from serum nuclease degradation over 6 h. Increasing SS-PAEI-g-PEG volume to 50% and 100% resulted in reductions in DNA protection against serum protein. Polyplexes prepared with 10% SS-PAEI-g-PEG exhibited

significantly enhanced transfection *in vitro* than SS-PAEI under serum conditions.¹⁸⁷ Continued as *in vivo* study in a murine adenocarcinoma model, it was found that polyplexes prepared with 25% SS-PAEI-g-PEG exhibited the smallest size and lowest surface charges, resulting in their predominant accumulation in the liver and to a lesser degree in the spleen.¹⁸⁸ Liu *et al.* developed hyperbranched SS-PAA with tertiary amino cores through Michael addition between CBA and AEP as efficient gene vectors.¹⁸⁹ The hyperbranched SS-PAA exhibited higher transfection efficiency compared with linear counterparts, as revealed in a study of Engbersen *et al.* together with Ferruti *et al.*¹⁹⁰ Hyperbranched SS-PAA prepared through reaction between CBA and *N,N*-dimethyldipropylenetriamine effectively condensed DNA into polyplexes that were further crosslinked through disulfide exchange induced during 15 min incubation at 50 °C. The cross-linked polyplexes showed enhanced colloidal stability under physiological conditions, and induced enhanced transfection efficiency *in vitro* and *in vivo* compared with non-crosslinked groups.¹⁹¹

Figure 11. Chemical structures of bioreducible PAAs: A, poly(amido ethylenimine) (SS-PAEI); B and C, poly(disulfide amine)s (PDAs).

A series of bioreducible PAAs coined poly(disulfide amine)s (PDAs) by Kim *et al.*¹⁹² were prepared by polyaddition of CBA with oligoamine monomers, yielding polymers with different lengths of polymethylene spacer in the main chain and the side chain (Figure

11B). Polymers with longer propylene pendant spacers (i.e., poly(CBA-SP), poly(CBA-APPD), and poly(CBA-APED)) induced higher transgene activity than their counterparts (i.e., poly(CBA-AEPD), poly(CBA-TETA)) with shorter ethylene pendant spacers. Poly(CBA-SP), poly(CBA-APPD), poly(CBA-APED) with increasing main chain spacers achieved similar transfection efficiencies, implying a smaller effect of polymer main chain length on transfection efficiency.¹⁹² Moreover, transfection results of the employed polymers varied for different cell lines, with poly(CBA-SP) inducing highest transfection in the C2C12 cell line, and poly(CBA-APED) delivering the highest transfection in the HeLa cell line. The longer, more hydrophobic alkyl side chains and more flexible backbones of these polymers improved the buffering capacity, protonation degree of tertiary amine groups, basicity and charge density of the polymers, and therefore elevated the gene transfection efficiency.¹⁹²

Another type of bioreducible PAAs was prepared via Michael addition between CBA and three *N*-Boc protected diamines (*N*-Boc-1,2-diaminoethane, *N*-Boc-1,4-diaminobutane, and *N*-Boc-1,6-diaminohexane) followed by *N*-Boc removal (Figure 11C). The side-chain spacer length substantially influenced transfection induced by polyplexes prepared from these polymers. Cell experiments revealed that poly(CBA-DAH) with a hexamethylene pendant spacer induced comparable or higher transfection efficiency *in vitro* compared to standard PEI (25 kDa).¹⁹³ After modification with prostaglandin E₂ (PGE₂), Fas siRNA was formulated and transported by poly(CBA-DAH) to rat cardiomyocytes (H9C2 cells) through PGE₂ receptor-mediated endocytosis, affording enhanced Fas gene silencing and substantial inhibition of cardiomyocyte apoptosis without inducing interferon- α in peripheral blood mononuclear cells.¹⁹⁴

Taking advantage of their excellent cell-penetrating abilities,¹⁹⁵⁻¹⁹⁸ arginine and guanidine groups have been merged into bioreducible polymers to achieve enhanced transfection activity. Arginine-grafted p(CBA-DAH) (ABP) was prepared with a molecular weight of 4.5 kDa.¹⁹⁹ Cell experiments in mammalian cells revealed that ABP had no significant cytotoxicity and substantially enhanced transfection efficiency as compared to p(CBA-DAH) and standard PEI (25 kDa). The remarkable increase in transfection of ABP was not only contributed to its high cellular penetrating ability, but mediated by other factors as well, such as good nuclear localization ability.¹⁹⁹ The arginine moieties in ABP were later found to directly penetrate the endosome membrane, which facilitated endosomal escape of

the polyplexes.²⁰⁰ ABP polymer was used in gene delivery of plasmid human EPP (phEPO) for prolonged and controlled release of erythropoietin (EPO).²⁰¹ After transfection mediated by ABP polymer, long-term EPO expression stimulated hematopoietic progenitor cells and hence inhibited cardiomyocyte apoptosis *in vitro*.²⁰¹ After systemic injection, the ABP/phEPO complexes induced higher hematocrit levels over 60 d together with enhanced reticulocytosis and boosted EOP protein expression, due to the distinct temporal and spatial distribution of complexes. During the experiment period, the innate immune response (IL-6) was not significantly activated, in contrast to controls treated with standard PEI (25 kDa).²⁰² Guanidynylated bioreducible polymer (GBP) was obtained by converting amine groups in p(CBA-DAH) into guanidine groups with 1*H*-pyrazole-1-carboxamidine. This polymer exhibited 8-fold higher transfection activity than the precursor polymer, owing to higher cellular uptake efficiency (up to 96%) and excellent nuclear localization ability contributed by the guanidine groups.²⁰³

PAAs with much higher molecular weight than those prepared through conventional Michael-type addition have been prepared through polycondensation between disulfide-containing di-*p*-nitrophenyl esters and primary diamines (Figure 12).²⁰⁴ The cationic PAAs with disulfide and 1,4-bis(3-aminopropyl)piperazine (BAP) moieties gave comparable or higher transfection activities as observed for PEI (25 kDa) and Lipofectamine 2000, owing to their distinct properties including high buffering capacity, strong gene binding ability, and intracellular gene release ability. Intravenous administration of the bioreducible polyplexes induced higher transgene expression in mouse liver, than linear PEI (22 kDa).

Figure 12. Synthesis of linear poly(amido amine)s via polycondensation between di-*p*-nitrophenyl esters and primary diamines.²⁰⁴

CONCLUSION AND PERSPECTIVES

Bioreducible disulfide bonds have been incorporated into polycationic materials by various synthetic approaches, providing favorable features to gene delivery carriers. In particular, the introduction of reducibility in the polymers allows polyplexes that are stable in the extracellular environment and dissemble in the intracellular compartment, leading to gene unpacking in an active manner. Moreover, usually colloidal stability of the polyplexes is enhanced by the introduction of disulfide bonds due to enhanced flexibility of the polymer chains by their free rotation around S-S bonds. Encouraging advances in bioreducible gene delivery carriers have been achieved both *in vitro* and *in vivo*. Nevertheless, the transfection efficiencies mediated by bioreducible polycationic vectors are still far from optimal, and potential toxicity of polymer fragments can be a risk. In addition, as common for cationic polyplexes, the transfection performance is still susceptible to serum proteins, leading to the formation of larger aggregates through non-specific interactions with proteins, and eventually diminishing transfection efficiency. Introduction of hydrophilic PEG chains to the polymers improves the circulation times of the polyplexes, however, often at the cost of transfection efficiency. Therefore, development of novel bioreducible polycationic vectors with minimized toxicity and improved transfection, even in the presence of serum, is of key importance to bring non-viral gene therapy further to the clinic.

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Chapter 3. Thiourea Functionalization Enhances Transfection of Bioresorbable Poly(amido amine)s as Gene Vectors under Serum Conditions

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KEYWORDS

Gene delivery; thiourea; hydrogen bonding; bioresorbable; serum-tolerance.

ABSTRACT

Successful gene therapy relies on gene delivery vectors with high transfection efficiency and minimal toxicity. Bioreducible poly(amido amine)s (PAAs) with disulfide linkages along the backbone and varying amounts of thiourea moieties in the side-chains were prepared via Michael-type polyaddition of 1-(4-aminobutyl)-3-(pyridin-3-yl)thiourea (PTBA) to *N,N'*-cystamine bisacrylamide (CBA). The thiourea-containing PAAs are able to bind plasmid DNA into nanosized polyplexes with positive surface charge as characterized by dynamic light scattering (DLS) and zeta-potential measurements. The plasmid DNA is liberated from the polyplexes in reducing environment, as is shown by gel electrophoresis after treatment with the reducing agent dithiothreitol (DTT). Polyplexes of thiourea-functionalized PAAs show markedly higher transfection efficiencies on COS-7 cells compared to polyplexes of the frequently applied PEI (branched, 25 kDa) and the PAA analog obtained by polyaddition of 1-amino-4-butanol (ABOL) to CBA, pABOL, at their optimal conditions. The high transfection capacity of the thiourea-functionalized PAAs remains largely unaffected in the presence of 10% serum, while those of PEI and pABOL drops strongly under these conditions. The results demonstrate that thiourea functionalization of PAAs is a promising approach to obtain gene vectors with enhanced transfection, minimal toxicity, and good serum tolerance.

INTRODUCTION

Cationic polymers emerge as appealing materials in non-viral gene delivery vectors owing to their remarkable advantages over viral vectors.¹⁻⁴ These advantages range from versatility in synthesis,⁵⁻⁷ absence of immunogenicity,² and high flexibility in functionalization. Functionalities that can be incorporated involve ligands for targeted gene delivery,⁸⁻¹¹ imaging moieties for tracking gene biodistribution,¹²⁻¹⁵ as well as therapeutic motifs for synergistic therapy.¹⁶⁻¹⁹ The positive charges along the chains of cationic polymers are able to condense negatively charged DNA through multivalent electrostatic interactions into nanosized polyplexes with polymeric cationic corona.²⁰⁻²³ To overcome the numerous barriers along the delivery route,^{24,25} increased colloidal stability of polyplexes is desirable and may be achieved by incorporating alternative interaction forces such as hydrophobic interactions^{26,27} and hydrogen bonding,²⁸⁻³⁰ as well as tertiary components as either physical³¹ or chemical cross-linker.³²

Recently, hydrogen bonding interactions have attracted considerable attention in the construction of non-viral gene delivery vectors,^{28,29,33-37} as well as in the preparation of dynamic biomedical materials.³⁸⁻⁴³ In this respect, thiourea groups are of particular interest since they are strong hydrogen bond donors and have the interesting ability to interact with phosphate groups of DNA.⁴⁴⁻⁴⁸ Liposomes endowed with thiourea groups were applied as non-viral gene vectors with comparable transfection efficiencies, though reduced cytotoxicity, as compared with their cationic liposome analogues.^{36,48} Modification of amino groups present in aminoalkyl poly(methacrylate)s⁴⁹ and poly(ethylenimine) (PEI)⁵⁰ by reaction with isothiocyanate to introduce thiourea functionalities, resulted in derivatives with efficient plasmid DNA delivery. In addition, reliable siRNA delivery, both *in vitro* and *in vivo*, was achieved by post-modification of 25 kDa PEI (branched) with thiourea groups.^{51,52} However, although the above described gene vectors illustrate the favorable effect of thiourea functionalization, they have some inherent disadvantages. Liposomes have limited transfection efficacy due to poor stability and rapid clearance,⁵³ and may induce inflammatory or anti-inflammatory responses.⁵⁴ The non-degradability of PEI and poly(methacrylate) impose high potential toxicity to cells and tissues.⁵⁵ These problematic issues of liposomes, PEI, and poly(methacrylate) severely restrict their wide deployment in clinical applications.

Peptidomimetic poly(amido amine)s (PAAs) are biodegradable, biocompatible, and non-toxic, rendering PAAs excellent materials for gene delivery.⁵⁶ Especially when disulfide linkages are incorporated in the PAA backbone the PAAs take advantage of the difference in intra- and extracellular redox potential, resulting in efficient intracellular delivery of plasmid DNA,⁵⁶⁻⁶⁰ siRNA⁶¹ and even proteins.⁶² PAAs are synthesized by Michael addition of amines to bisacrylamides under very mild conditions.⁶³ The resulting tertiary amines in the polymer backbone are prone to protonation in the neutral and endosomal pH range, endowing PAAs with overall positive charge and excellent water solubility.⁶³ However, PAAs bear moderate DNA binding ability and significantly susceptibility to serum protein interference,⁶⁴⁻⁶⁶ making it interesting to introduce functionalities which increases their DNA binding ability and resistance to serum interference.

In this chapter, we describe incorporation of thiourea moieties into disulfide-linked PAAs to increase polyplex stability and resulting transfection efficiency through hydrogen bonding between thiourea and phosphate groups in DNA (Scheme 1). Thiourea-functional

PAAs were prepared through stepwise polymerization of thiourea-containing amines with CBA. The obtained polymers were characterized in detail, their ability to form stable polyplexes was evaluated, and cytotoxicity and transfection efficiency to COS-7 cells were evaluated under both serum-free and serum conditions. The transfection efficiency of leading candidates were qualitatively screened by fluorescence images and further quantitatively evaluated by flow cytometry.

Scheme 1. Schematic representation of thiourea-functionalized disulfide-linked poly(amido amine)s for enhanced polyplex stability through hydrogen bonding between thiourea and phosphate in DNA.

EXPERIMENTAL PROCEDURES

Materials and Methods

All monomers, 4-amino-1-butanol (ABOL, Sigma-Aldrich), *N, N'*-cystaminebisacrylamide (CBA, Polysciences), 3-pyridyl isothiocyanate (PyNCS, 95.0 %, Acros), dithiothreitol (DTT, $\geq 99.5\%$, Sigma-Aldrich) and *N*-Boc-1,4-butanediamine (Boc-DAB, Sigma-Aldrich) were purchased in the highest purity and used without further purification. HEPES sodium salt and LiCl were purchased in the highest purity from Sigma-Aldrich. All water used in the described experiments was treated by a Milli-Q Gradient System (Millipore, Bedford, MA). Plasmid DNA, plasmid pCMV-GFP was purchased from Plasmid Factory (Bielefeld, Germany). Branched polyethylenimine (PEI, M_w 25 kDa) was purchased from Sigma-Aldrich.

Monomers and polymers were characterized by NMR spectroscopy on a Bruker 400 MHz spectrometer in deuterated solvent. Molecular weights were determined by gel permeation chromatography (GPC) relative to PEG standards, using a Waters Alliance 2695 separation module equipped with a PL-aquagel-OH 8 micron Mixed-M column (300 x 7.5 mm) with *N,N*-dimethylformamide (DMF) containing LiCl (50 mM) as eluent at 35 °C.

Synthesis of 1-(4-aminobutyl)-3-(pyridin-3-yl)thiourea (PTBA, monomer 1)

To a solution of *N*-Boc-1,4-butanediamine (2.63 g, 14.0 mmol, 1.0 eq.) in methanol (50 mL), 3-pyridyl isothiocyanate (2.00 g, 14.0 mmol, 1.0 eq.) was added slowly. After stirring at ambient temperature for 16 h, the obtained mixture was condensed under reduced pressure. The solids were dissolved in a mixture of methanol and dichloromethane (1/1, v/v, 80 mL) and hydrochloric acid (g) was led through the solution for 30 min. The obtained white solid was filtered off, washed with dichloromethane, and finally dried at 40 °C in a vacuum oven overnight to yield the hydrochloric acid salt as a white powder (3.30 g, 88.1%). ¹H NMR (DMSO-D₆, 400 MHz): δ 1.62 (d, 4H, NHCSNHCH₂CH₂CH₂CH₂NH₂), 2.80 (d, 2H, NHCSNHCH₂CH₂CH₂CH₂NH₂), 3.51 (d, 2H, NHCSNHCH₂CH₂CH₂CH₂NH₂), 7.93 (m, 1H, CHNCHCHCH-pyridyl ring), 8.54 (m, 2H, CHNCHCHCH-pyridyl ring), 9.06 (t, 1H, NHCSNHCH₂CH₂CH₂CH₂NH₂), 9.40 (d, 1H, CHNCHCHCH-pyridyl ring), 11.48 (s, 1H, NHCSNHCH₂CH₂CH₂CH₂NH₂). ¹³C NMR (DMSO-D₆, 400 MHz): δ 24.48, 25.24, 38.43, 43.16, 126.75, 133.62, 135.65, 136.26, 139.80, 180.57.

Synthesis of polymers of p(ABOL_m/PTBA_n)

In a typical experiment (i.e., synthesis of p(ABOL₇₂/PTBA₂₈)), *N*, *N*'-cystaminebisacrylamide (0.520 g, 2.00 mmol), 4-amino-1-butanol (0.156 g, 1.75 mmol), 1-(4-aminobutyl)-3-(pyridin-3-yl)thiourea (75 mg, 0.25 mmol), and triethylamine (0.140 mL, 1.00 mmol) were dissolved in a mixture of methanol and water (3/1, v/v, 1.5 mL). After 8 d stirring at 50 °C, the resulting viscous mixture was charged with additional 4-amino-1-butanol (16 mg, 0.18 mmol), 1-(4-aminobutyl)-3-(pyridin-3-yl)thiourea (9.0 mg, 0.030 mmol) and triethylamine (0.017 mL, 0.12 mmol) to terminate the polymerization. After stirring at 50 °C for another 2 d, the mixture was diluted in water, acidified with aqueous hydrochloric acid (4.0 M) to pH 4, and dialyzed with 1000 Da MWCO membrane against water overnight. After lyophilization, the polymer p(ABOL₇₂/PTBA₂₈) was obtained as an amorphous solid. Other polymers were prepared analogously with varying ratios of 4-

amino-1-butanol (ABOL) and 1-(4-aminobutyl)-3-(pyridin-3-yl)thiourea (PTBA), namely 0/100, 50/50, 87.5/12.5, and 100/0.

P(ABOL₀/PTBA₁₀₀): 0.267 g, 36.4 % yield. ¹H NMR (D₂O, 400 MHz): δ 1.67-1.76 (m, 4H, RNCH₂CH₂CH₂CH₂NHCSNH), 2.68-2.78 (m, 8H, 2 x NHCOCH₂ + 2 x SSCH₂CH₂), 3.17-3.58 (m, 12H, RNCH₂CH₂CH₂CH₂NHCSNH + RNCH₂CH₂CH₂CH₂NHCSNH + CH₂CH₂NCH₂CH₂ + 2 x SSCH₂CH₂), 7.46 (1H, **pyridyl ring**), 7.81 (1H, **pyridyl ring**), 8.36 (1H, **pyridyl ring**), and 8.46 (1H, **pyridyl ring**).

P(ABOL₅₀/PTBA₅₀): 0.400 g, 53.4 % yield. ¹H NMR (D₂O, 400 MHz): δ 1.60-1.79 (m, 4H, RNCH₂CH₂CH₂CH₂NHCSNH/RNCH₂CH₂CH₂CH₂OH), 2.62-2.78 (m, 8H, 2 x NHCOCH₂ + 2 x SSCH₂CH₂), 3.07-3.61 (m, 12H, RNCH₂CH₂CH₂CH₂NHCSNH + RNCH₂CH₂CH₂CH₂NHCSNH/RNCH₂CH₂CH₂CH₂OH + CH₂CH₂NCH₂CH₂ + 2 x SSCH₂CH₂ + RNCH₂CH₂CH₂CH₂OH), 7.49 (1H, **pyridyl ring**), 7.83 (1H, **pyridyl ring**), 8.39 (1H, **pyridyl ring**), and 8.49 (1H, **pyridyl ring**).

P(ABOL₇₂/PTBA₂₈): 0.363 g, 45.4 % yield. ¹H NMR (D₂O, 400 MHz): 1.60-1.79 (m, 4H, RNCH₂CH₂CH₂CH₂NHCSNH/RNCH₂CH₂CH₂CH₂OH), 2.62-2.78 (m, 8H, 2 x NHCOCH₂ + 2 x SSCH₂CH₂), 3.07-3.61 (m, 12H, RNCH₂CH₂CH₂CH₂NHCSNH + RNCH₂CH₂CH₂CH₂NHCSNH/RNCH₂CH₂CH₂CH₂OH + CH₂CH₂NCH₂CH₂ + 2 x SSCH₂CH₂ + RNCH₂CH₂CH₂CH₂OH), 7.49 (1H, **pyridyl ring**), 7.83 (1H, **pyridyl ring**), 8.39 (1H, **pyridyl ring**), and 8.49 (1H, **pyridyl ring**).

P(ABOL₉₀/PTBA₁₀): 0.363 g, 45.4 % yield. ¹H NMR (D₂O, 400 MHz): 1.60-1.79 (4H, RNCH₂CH₂CH₂CH₂NHCSNH/RNCH₂CH₂CH₂CH₂OH), 2.62-2.78 (8H, 2 x NHCOCH₂ + 2 x SSCH₂CH₂), 3.07-3.61 (12H, RNCH₂CH₂CH₂CH₂NHCSNH + RNCH₂CH₂CH₂CH₂NHCSNH/RNCH₂CH₂CH₂CH₂OH + CH₂CH₂NCH₂CH₂ + 2 x SSCH₂CH₂ + RNCH₂CH₂CH₂CH₂OH), 7.49 (1H, **pyridyl ring**), 7.83 (1H, **pyridyl ring**), 8.39 (1H, **pyridyl ring**), and 8.49 (1H, **pyridyl ring**).

P(ABOL₁₀₀/PTBA₀): 0.320 g, 40.4 % yield. ¹H NMR (D₂O, 400 MHz): δ 1.33 (s, 9H, R(CH₃)₃), 1.62 (m, 2H, RNCH₂CH₂), 1.78 (m, 2H, RNCH₂CH₂CH₂CH₂OH), 2.74 (t, 4H, 2 x NHCOCH₂), 2.84 (t, 4H, 2 x SSCH₂CH₂), 3.20 (t, 2H, RNCH₂CH₂CH₂CH₂OH), 3.41 (t,

4H, CH₂CH₂NCH₂CH₂), 3.51 (t, 4H, 2 x SSCH₂CH₂), 3.62 (t, 2H, RNCH₂CH₂CH₂CH₂OH).

Polyplex preparation

Polyplexes were prepared in HEPES buffer (20 mM, pH 7.4, supplemented with 5.0 wt% glucose). A typical procedure to prepare polyplexes at a mass ratio of 48/1 (polymer/DNA) is as follows: polymer solution (800 μ L, 900 μ g/mL) was added to the DNA solution (200 μ L, 75 μ g/mL), followed by 5 s vortexing and 30 min incubation at room temperature. Size and surface charge of the polyplexes were measured at 25 °C with a Zetasizer Nano ZS (Malvern Instruments, Malvern). Recorded values are the mean of three measurements, standard deviations are indicated as error bars.

Agarose gel retardation

Polyplexes were prepared at polymer/DNA mass ratio 48 as described previously. DTT solution in HEPES buffer was added to the polyplex solutions to result in a final DTT concentration of 2.5 mM. After 30 min incubation at ambient temperature, 6X loading buffer containing bromophenol (Ferments) was mixed with resulting dispersions. The solutions (10 μ L) were loaded on a 0.8% w/v agarose gel containing 1x SYBR[®] Safe DNA Gel Stain (Invitrogen[™]). Electrophoresis was performed at 90 V for 60 min in a TBE running buffer. After development of the gel, the picture was acquired using FluorChem (Proteinsimple, Westburg, Leusden, the Netherlands) under UV excitation.

***In Vitro* Transfection and Cell Viability Assays**

Transfection experiments were performed on COS-7 cells (SV-40 transformed African green monkey kidney cells) using the plasmid pCMV-GFP as a reporter gene. Transfections and cell viabilities were conducted in parallel using polyplexes formed at polymer/plasmid DNA mass ratios ranging from 6 to 48.

Cells were seeded in at a density of 10⁴ cells per well (2.94 $\times$ 10⁴ cells/cm²) and maintained in complete medium at 37 °C in a humidified atmosphere containing 5% CO₂ until 60-80% confluency. In a standard transfection experiment, cells were first incubated with medium (100 μ L, with/without serum) for 30 min and then loaded with 100 μ L polyplex dispersions (1 μ g DNA per well). After 1 h incubation, polyplexes were removed, and fresh complete culture medium (100 μ L) was added and cells were cultured for an additional 48 h. Transfection efficiency was determined qualitatively through fluorescence

imaging via an EVOS digital inverted microscope (EMS, Wageningen, the Netherlands). A reference was prepared from PEI (25 KDa)/DNA at a polymer/DNA mass ratio of 1.33/1. MTT assays were conducted to determine the number of viable cells, with a value of 100% cell viability for untreated cells.

Quantitative determination of transfection efficiency was performed by flow cytometry. Cells were seeded in 48-well plates with densities of ca. 1.6×10^4 cells per well (1.6×10^4 cells/cm²) and maintained in complete medium at 37 °C in a humidified atmosphere containing 5% CO₂ until 60-80% confluency. Cells were first incubated with medium (200 µL, with/without serum) for 30 min and then loaded with 200 µL polyplex dispersions (1 µg DNA per well). After 1 h incubation, the polyplexes were aspirated, fresh and warm complete culture medium (200 µL) was added and eventually cells were cultured for 48 h. The cells were fixed by adding trypsin solution (0.25%, 150 µL), and centrifuged (600 g, 5 min, ambient temperature). The pellets were resuspended in HBSS buffer (200 µL), and measured by the FACSCalibur (Becton-Dickinson, Breda, the Netherlands) at an excitation wavelength of 488 nm and an emission wavelength of 530 nm. Data was processed by the FACS Cellquest Software. A PEI (25 K)/DNA formulation prepared at a polymer/DNA mass ratio of 1.33/1 was used as a reference. MTT assay was conducted to characterize the number of viable cells with MTT value of 100% cell viability for untreated cells (i.e., cells not exposed to transfection systems).

RESULTS AND DISCUSSION

Monomer and copolymers preparation and characterization

The thiourea-present primary amine monomer PTBA was synthesized via reaction of pyridylisothiocyanate with mono Boc-protected butanediamine (Boc-DAB). Subsequent deprotection of the Boc group yielded the monomer as white solid in 88.1% yield. Fourier transform infrared (FT-IR) spectra (see Figure S2) shows a strong signal at 1354 cm⁻¹, which corresponds to the characteristic C=S stretching absorption, validating the presence of thiourea bonds in the monomer of **PTBA**. Both NMR and FT-IR results confirm the successful preparation of thiourea-functionalized monomer **PTBA**.

Scheme 2. Synthesis of thiourea-functional PAAs through Michael-type addition of CBA to ABOL and PTBA.

Polymers **P1-P5** were synthesized via Michael type polyaddition of *N, N'*-cystaminebisacrylamide (**CBA**) to mixtures of 4-amino-1-butanol (**ABOL**) and **PTBA** in equimolar amounts of total primary amines (Scheme 2). The ratios of thiourea functionalities were varied by altering the feed ratio of **ABOL** and **PTBA**. In order to perform Michael type polymerization, TEA was added to neutralize the **PTBA** to endow free primary amine groups to couple with acrylamide groups from CBA. Excess of primary amine was added to the polymerization mixture at the end of the polymerization to consume any residual acrylamide groups, which may cause toxicity towards cells.^{67,68} The NMR spectra of the isolated polymers are in good agreement with their structures. The absence of signals between 5.00 and 7.00 ppm in all NMR spectra indicates complete consumption of acrylamide groups. The presence and intensity of the signals between 7.00 and 9.00 ppm, assigned to the pyridyl groups, implies the successful incorporation of **PTBA** monomer into the polymers according to the feed ratio and indicates the tunable incorporation of thiourea functionalities into the polymers. The ratio of **PTBA/ABOL** groups in the polymers was determined based on integral ratio between the two different ethylene groups (RNCH₂CH₂CH₂CH₂NHCSNH-Py / RNCH₂CH₂CH₂CH₂OH) in the polymers. All polymers incorporated with thiourea moieties exhibit signals at 1354 cm⁻¹ (C=S stretching absorption), further confirming the successful incorporation of PTBA into the polymers. Size exclusion chromatography analysis on the polymers revealed molecular

weights ranging from 6.0 to 9.2 kDa with relatively narrow PDIs of approximately 1.5, most likely due to elimination of low molecular weight oligomers during the dialysis step (Table 1).

Table 1. Characteristics of poly(amido amine)s functionalized with various thiourea groups

Entry No.	Polymer Acronym	ABOL/PTBA mole ratio		M_w (kDa) ^b	M_w/M_n ^b
		feed	found ^a		
P1	pABOL	100/0	100/0	5.3	1.61
P2	p(ABOL ₉₀ /PTBA ₁₀)	87.5/12.5	90/10	6.0	1.59
P3	p(ABOL ₇₂ /PTBA ₂₈)	75/25	72/28	6.8	1.45
P4	p(ABOL ₅₀ /PTBA ₅₀)	50/50	50/50	6.0	1.47
P5	pPTBA	0/100	0/100	9.2	1.54

^a determined by integral ratios of NMR spectra; ^b determined via GPC.

Polyplex Preparation and Characterization

The performance of polymeric gene vectors is dictated by their ability to form polyplexes and guide DNA through various barriers to arrive at the target site.^{2,69} Polymers **P4** and **P5** showed limited solubility and formation of large aggregates. Therefore, these polymers are excluded in the following study and only the thiourea functionalized polymers **P2** and **P3**, together with **P1** as the control polymer lacking thiourea functionalities, are further investigated. As illustrated in Figure 1, all these three polymers are able to condense plasmid DNA into polyplexes with hydrodynamic diameters ranging from 126 nm to 400 nm, with positive charge of +10 to +30 mV when the polymer/DNA mass ratio is above 12. The size of polyplexes decreases and the zeta potential of the polyplexes increases with increasing polymer to DNA ratio, which can be rationalized by the fact that more positive charge carriers become available to condense DNA with increasing polymer/DNA mass ratio.^{23,56} Generally, the surface charge of polyplexes is closely related to their cytotoxicity profiles and their cellular internalization across the anionic cell membrane.⁷⁰ At the same mass ratio, the polymers with higher percentage of thiourea functionalities form smaller polyplexes with similar surface charge. The formation of smaller polyplexes can be attributed to additional hydrogen bonding interactions between the thiourea moieties

(strong hydrogen bonding donor) in the polymer and the phosphate groups present in DNA, besides the electrostatic interactions.

Figure 1. Characterization of polyplexes prepared under various polymer/DNA mass ratios through DLS. (A) Hydrodynamic sizes versus mass ratios; (B) Zeta-potentials versus mass ratios.

Successful gene delivery requires efficient DNA unpacking after polyplexes have entered the cell.^{25,71} In order to evaluate the responsiveness of the polyplexes to the reducing intracellular environment,^{72,73} these conditions were mimicked by incubation of the polyplexes with 2.5 mM DTT for 30 min. Based on the gel electrophoresis (Figure S3, in supporting information), free DNA bands are distinctively observed for DTT-treated polyplexes, while no DNA bands are distinguished in the absence of DTT. The results demonstrate that DNA is well retained by the PAAs, but rapidly liberated under reducing conditions through fragmentation of the PAAs by disulfide bond cleavage. We and others have previously shown that disulfide-containing PAA-based polyplexes show efficient nucleotide unpacking under reducing conditions which renders these polymers promising gene delivery systems.^{56,57,74}

***In vitro* transfection efficiency and cytotoxicity**

The transfection efficiency of the polyplexes was evaluated in COS-7 cells with plasmid DNA encoding for green fluorescent protein (GFP) as reporter gene. The preliminary screening of the PAAs was performed on 96-well plates at four different polymer/DNA mass ratios (i.e., 48, 24, 12, and 6) at both serum-free and 10% serum conditions. The transfection results were first qualitatively evaluated with confocal fluorescence microscopy and are illustrated in Figure 2 and Figure 3. GFP expression was observed in COS-7 cells both under serum-free and 10% serum conditions when polymer/DNA mass ratios were above 6. All polymers show enhanced GFP expression in COS-7 cells under serum-free condition, compared with common standard transfection agent of PEI (25 k) at

its optimal condition (N/P ratio 10). Moreover, the presence of thiourea functionalities (10% for **P2** and 28% for **P3**) in PAAs results in increased transfection efficiency compared with pABOL (**P1**), especially at the lower polymer/DNA mass ratios (12 and 6), suggesting the beneficiary effect of thiourea groups on transfection efficiency. This beneficiary effect is augmented under serum-present transfection conditions. In 10% serum presence, GFP expression is severely impeded in the case of cells exposed to PEI and **P1**, while pronounced GFP expression is maintained in the case of cells treated with the thiourea-PAAs-based polyplexes of polymer/DNA mass ratios 6 and 12.

Figure 2. Overlaid GFP images of COS-7 cells transfected by pCMV-GFP via PAAs or PEI after 2 d incubation under 0% serum conditions. 4 x magnification, bars = 1000 μ m (horizontal number denotes polymer/DNA mass ratios).

Figure 3. Overlaid GFP images of COS-7 cells transfected by pCMV-GFP via PAAs or PEI after 2 d incubation under 10% serum conditions. 4X magnification, bars = 1000 μ m (horizontal number denotes polymer/DNA mass ratios).

Cell viabilities of COS-7 cells exposed to the polyplexes were evaluated through MTT assays and are presented in Figure 4. Cell viabilities of over 90% were found for cells exposed to polyplexes prepared from PAA throughout polymer/DNA mass ratios of 6, 12, 24, and 48 at serum-free conditions. Higher viabilities were observed for polyplexes in the presence of 10% serum as compared to serum-free conditions. The viabilities of cells treated by PAA-polyplexes (over 90%) are substantially higher than those of cells exposed to PEI-polyplexes (60% viability for 0% serum and 75% viability for 10% serum). The lower cell viability observed upon exposing cells to PEI-based polyplexes is attributed to the intrinsic toxicity of PEI, as has been reported before.⁷⁵

Figure 4. Cell viability of COS-7 cells treated with polyplexes at different polymer/DNA mass weights via MTT assay under 0% serum (A) and 10% serum (B).

The transfection performance of polymers **P1**, **P2** and **P3** was further evaluated in a quantitative manner via fluorescence-activated cell sorting (FACS). In Figure 5, the transfection performances of the three polymers are presented as percentages of GFP-positive cells of the total cells. As displayed, **P2** and **P3** deliver 6.5-fold to 4.5-fold higher GFP expression in cells at polymer/DNA mass ratios of 12 as compared to **P1** under serum-free conditions. The presence of serum (10%) substantially decreases the transfection efficiencies of **P1** and PEI, but only a minor effect is observed for the transfection efficiencies of **P2** and **P3**.

Figure 5. Transfection efficiency of **P1**, **P2**, **P3**, and PEI in COS-7 cells via FACS under both serum-free and serum-present conditions at polymer/DNA mass weight ratios of 24 (A) and 12 (B) respectively.

CONCLUSIONS

A thiourea-functionalized amine monomer could be successfully incorporated into PAA polymers via Michael type polyaddition, yielding a series of disulfide-backboned cationic polymers with varying percentages of thiourea-moieties closely according to the feed ratio of the monomers. The thiourea-containing polymers were demonstrated to form stable nanosized polyplexes with DNA, with enhanced colloidal stability under physiological conditions, which is attributed to hydrogen bonding between polymer and DNA. Polymers with 10% and 28% thiourea groups delivered up to 6-fold and 15-fold transfection enhancement compared to the control polymer (pABOL) under serum-free and serum-present conditions, respectively, without introducing cytotoxicity. These results demonstrate that the presence of thiourea groups in PAAs is beneficial for polyplex stability and transfection efficiency and enhances the serum tolerance of PAA polyplexes without compromising their biological activity. These results indicate that (co-)functionalization of polymeric gene vectors with thiourea groups may be a new avenue to enhance the transfection capabilities of polymers with minimal introduction of toxicity.

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SUPPORTING INFORMATION

NMR

Figure S1. ^1H -NMR spectra of PAAs in D_2O (400 MHz). From bottom to top: **P1**, **P2**, **P3**, **P4** and **P5** respectively.

Figure S2. FT-IR spectra of monomer PTUBA, and synthesized PAAs with different thiourea functionalities of 100%, 50%, 28%, 10% and 0%.

Figure S3 Agarose gel electrophoresis of polyplexes (w/w=48) treated without (B) and with DTT (A) (s.c. = supercoiled, o.c. = open circular).

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Chapter 4. Effects of Incorporation of Fluoroalkyl Groups in Bio reducible Poly(amido amine)s on Gene Delivery

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KEYWORDS

Gene delivery; cationic polymers; fluorination; bio reducible; serum tolerance.

ABSTRACT

The full potential of cationic polymers as non-viral gene vectors is largely impeded due to low transfection efficiency and the interference by serum proteins. Herein, we modified a promising class of gene vectors consisting of bioreducible poly(amido amine)s (PAAs) with fluoroalkyl moieties at various functionalization ratios (100%, 50%, 25%, 10% and 0%) via the amine-anhydride reaction. Fluorinated PAAs showed excellent solubility in water at pH 7.4, independent of the degree of fluorination, and compacted plasmid DNA (pCMV-GFP) into nanosized polyplexes with diameters from several micrometers down to 150 nm, and zeta-potential from +10 mV to +30 mV upon increasing polymer/DNA mass ratios from 6 to 48. DNA was fully trapped by the cationic polymers, while liberated upon addition of DTT as corroborated with gel electrophoresis. Fluorination resulted in enhanced biocompatibility of the PAAs towards COS-7 cells as revealed by MTT assays. The potency of fluorinated PAAs in transfection of COS-7 was enhanced up to 8 fold under serum-free conditions and remained satisfactory even in the presence of serum (10%) as compared to non-fluorinated PAA analogues. In addition, the fluorinated PAAs are superior over standard transfection agent of PEI both in terms of preserving cell viability and transfection efficiency, suggesting that introduction of fluoroalkyl substitutes is an effective strategy to design effective non-viral gene vectors.

INTRODUCTION

Gene therapy remains a major challenge due to the lack of effective delivery carriers with minimal toxicities.¹⁻⁵ Although displaying high transfection efficiencies, viral carriers are plagued with poor biosafety,^{6,7} limited DNA packaging capacity,⁸ and complicated scale-up.⁹ Non-viral carriers have therefore been proposed as alternatives to avoid aforementioned shortcomings. Although their clinical potency has so far remained low,^{10,11} cationic polymers have received wide attention,¹² owing to their ability to condense DNA into positively charged polyplexes through electrostatic interactions.¹³⁻¹⁵ The polyplexes aid DNA to enter into cells,^{16,17} promote DNA to escape from the endosome,¹⁸⁻²⁰ and ultimately deliver the genetic cargo inside the nucleus to achieve gene expression.²¹⁻²³

A myriad of cationic polymers has been devised as non-viral gene carrier, usually with moderate transfection efficacies concomitant with considerable degrees of toxicity.^{24,25} Residual cationic polymer may destroy the integrity of cell membrane,²⁶ impair the mitochondria,²⁷ and eventually reduce the cell viability.^{26,28} Various approaches have been

proposed to achieve the ideal balance of cationic charge for high transfection efficiency and decreased toxicity, such as introducing cationic motifs with amino acids,²⁹ peptides,³⁰ and additional polymer poly(L-lysine),³¹ and even superparamagnetic iron oxide nanoparticles³² to enhance transfection efficiency and maintain toxicity to a minimum. From our previous work,³³ it has been shown that bio-reducible poly(amido amine)s (PAAs) equipped with primary amines as pendant groups along the polymer chain are interesting degradable cationic polymers that fragmentate upon exposure to reducing milieu. The primary amines from this polymer have been functionalized with boronic acid groups,^{34,35} nicotinamide groups,^{36,37} and charge-reversal groups³⁸ to modify the pendant primary amines to reduce the positive charge density and endow the polymers with improved functionality and high tolerance to cell viability. The modified PAAs were used as carriers for DNA,³⁵⁻³⁷ siRNA,³⁵ proteins,³⁸ and as building blocks for smart hydrogels³⁹ and multilayered films⁴⁰ with excellent biocompatibility.

As described in previous chapter, hydrogen bonding was applied to increase the stability of polyplexes. In this chapter, the hydrophobic interactions were explored to stabilize the polyplexes by using the primary amine groups to introduce fluoroalkyl groups in the PAAs. Pharmacokinetic behavior of drug molecules,⁴¹ proteins,^{42,43} and cells⁴⁴ are found to be substantially altered by fluorination.⁴⁵ Fluorination also increases stability of protein against thermal unfolding, chemical denaturation, and proteolytic degradation, while preserving biological activity.⁴⁶⁻⁵⁰ Fluorinated polymers display high affinity towards cell membranes,⁵¹ and are able to transverse lipid bilayers of cell membranes and endosome/lysosome membranes more easily, thereby favoring endosomal escape of polymer and possible cargo.⁵² These beneficial properties are related to the hydrophobicity and lipophobicity of fluorinated polymers.⁵³⁻⁵⁵ For lipid-mediated gene delivery, Vierling *et al.* have shown the favorable effect of the presence of fluoroalkyl by combining fluorinated glycerophosphoethanolamine helper lipids with common cationic lipids to enhance gene transfection both *in vitro* and *in vivo*.^{56,57} Lebeau *et al.* showed that incorporation of fluorinated motifs close to the cationic lipid polar head resulted in enhancement of transfection potency through increased colloidal stability of the formed lipopolyplexes.^{58,59} Cheng *et al.* modified dendrimers^{60,61} with different fluorinated compounds⁶² to obtain enhanced transfection efficiency with reduced cytotoxicity.⁶¹ Moreover, this method was also expanded to the common standard transfection agent PEI, resulting in higher transfection efficiency and improved biocompatibility.⁶³ Fluorinated

vectors (both lipids and polymers) typically show good serum compatibility at appreciable transfection efficiencies even up to 50% serum.⁶¹

Scheme 1 Schematic representation of fluorinated disulfide-linked poly(amido amine)s for efficient gene delivery.

In this chapter, bio-reducible PAA (I) was prepared with variable ratios of units of pABOL (A) and the units containing the perfluoro-butylamide moiety (B, i.e., 0%, 10%, 25%, 50%, and 100%) (Scheme 1). The effects of fluorination of the PAAs on the transfection efficiency and cytotoxicity were investigated through *in vitro* studies on COS-7 cells. In addition, the effects of increased serum concentrations on transfection efficiency was investigated.

EXPERIMENTAL PROCEDURES

Materials and Methods

All monomers, *N*, *N'*-cystaminebisacrylamide (**CBA**, Polysciences), *N*-Boc-1,4-butanediamine (**Boc-DAB**, Sigma-Aldrich), 4-amino-1-butanol (**ABOL**, Sigma-Aldrich), heptafluorobutyric anhydride (**F7**, $\geq 99.0\%$, Sigma-Aldrich), dithiothreitol (DTT, $\geq 99.5\%$, Sigma-Aldrich), branched polyethylenimine (PEI, Mw 25 kDa, Sigma-Aldrich) and

triethylamine (TEA, $\geq 99\%$, Sigma-Aldrich) were used as received without further purification. All solvents used are of analytical grade. Water used in these experiments is treated through a Milli-Q Gradient System (Millipore, Bedford, MA). Plasmid DNAs, pCMV-GFP and pCMV- Δ GFP were purchased from Plasmid Factory (Bielefeld, Germany).

^1H NMR and ^{19}F NMR were taken on a Bruker Spectrometer (400 MHz) in deuterated solvents. Molecular weights were determined by gel permeation chromatography (GPC, Waters Alliance 2695) relative to PEG standards, using a Mixed-M column (PL-aquagel-OH 8 micron, 300 x 7.5 mm) with mobile phase of *N,N*-dimethylformamide (DMF) containing LiCl (50 mM).

Synthesis of fluorinated PAAs p(ABOL_m/DABF7_n). The procedure to prepare fluorinated PAAs is exemplified for p(ABOL₅₀/DABF7₅₀). Into a glass vial with methanol/water (v/v, 3/1, 5.00 mL), **CBA** (2.62 g, 10.0 mmol), **Boc-DAB** (0.945 g, 5.00 mmol) and **ABOL** (0.450 g, 5.00 mmol) were consecutively charged. After stirring at 50 °C for 7 d, **Boc-DAB** (0.189 g, 1.00 mmol) was added to the homogeneous viscous mixture. After 2 d stirring at 50 °C, the mixture was cooled down to ambient temperature and diluted with methanol (50.0 mL) and subsequently purged with HCl gas for 30 min to deprotect the primary amine groups from the **Boc-DAB** moieties. The mixture was concentrated under reduced pressure and resuspended in methanol (50.0 mL), followed by addition of excess of triethylamine (TEA) and excess of heptafluorobutyric anhydride (**F7**). After stirring overnight at room temperature and evaporating solvent under reduced pressure, the viscous residue was diluted with water (25.0 mL), acidified to pH 4, purified by dialysis (MWCO 1000 Da), and lyophilized to give the fluorinated polymer p(ABOL₅₀/DABF7₅₀) as an amorphous solid (1.89 g, 46.8 % yield). ^1H NMR (D_2O , 400 MHz): δ_{H} 1.56-1.66 (m, 4H, $\text{RNCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NHCOF}_7$), 2.44 (t, 4H, 2 x NHCOCH_2), 2.55 (m, 4H, $\text{RNCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NHCOF}_7$), 2.84 (m, 8H, 2 x $\text{SSCH}_2\text{CH}_2 + \text{CH}_2\text{CH}_2\text{NCH}_2\text{CH}_2$), 3.00 (t, 2H, $\text{RNCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NHCOF}_7$), 3.50 (t, 4H, 2 x SSCH_2CH_2). ^{19}F NMR (D_2O , 400 MHz): δ_{F} -81.4 (t, 3F, CF_3), -121.5 (m, 2F, CF_2CF_3), -128.0 (t, 2F, COCF_2).

Polyplex preparation

Polyplexes were prepared in HEPES buffer (20 mM, pH 7.4). The typical procedure to prepare polyplex of mass ratio 48/1 is described as below: polymer solution (800 μL , 900

µg/mL) was added into DNA solution (200 µL, 75 µg/mL), followed by 5 s vortexing and 30 min incubation at room temperature. Sizes and surface charges of polyplexes were measured at 25 °C with a Zetasizer Nano ZS (Malvern Instruments, Malvern). The values are recorded as the mean of three measurements with their standard deviation as error bar.

Agarose gel retardation

Polyplexes were prepared at polymer/DNA mass ratio 48 as described previously. DTT solution in HEPES buffer was added to polyplex solutions to give the final DTT concentration of 2.5 mM. After 30 min incubation at ambient temperature, 6X loading buffer containing bromophenol (Ferments) was mixed with resulting dispersions. The dispersions (10 µL) was loaded on a 0.8% w/v agarose gel containing 1x SYBR® Safe DNA Gel Stain (Invitrogen™). Electrophoresis was performed at 90 V for 60 min in a TBE (Tris-borate-EDTA, 1X) running buffer. After gel development, the picture was acquired using FluorChem (Proteinsimple, Westburg, Leusden, the Netherlands) under UV excitation.

***In Vitro* Transfection and Cell Viability Assays**

Transfection experiments were performed on COS-7 cells (SV-40 transformed African green monkey kidney cells) using the plasmid pCMV-GFP as a reporter gene. Transfections and cell viabilities were conducted in parallel using polyplexes formed at polymer/plasmid DNA mass ratios ranging from 6 to 48.

Gene expression experiments and cell viability evaluations were carried out in separate 96-well plates. Cells were seeded in at a density of 10^4 cells per well (2.94×10^4 cells/cm²) and maintained in complete medium at 37 °C in a humidified atmosphere containing 5% CO₂ until 60% - 80 % confluency. In a standard transfection experiment, cells were first incubated with medium (100 µL, with/without serum) for 30 min and then loaded with 100 µL polyplex dispersions (1 µg DNA per well). After 1 h incubation, polyplexes were removed, and fresh complete culture medium (100 µL) was added and cells were cultured for an additional 48 h. Transfection efficiency was determined qualitatively through fluorescence imaging via an EVOS digital inverted microscope (EMS, Wageningen, the Netherlands). A reference was prepared from PEI (25 KDa)/DNA at a polymer/DNA mass ratio of 1.33/1. MTT assays were conducted to determine the number of viable cells, with a value of 100% cell viability for untreated cells.

Quantitative determination of transfection efficiency was performed by flow cytometry. Cells were seeded in 48-well plates with densities of ca. 1.6×10^4 cells per well (1.6×10^4 cells/cm²) and maintained in complete medium at 37 °C in a humidified atmosphere containing 5% CO₂ until 60% - 80% confluency. Cells were first incubated with medium (200 µL, with/without serum) for 30 min and then loaded with 200 µL polyplex dispersions (1 µg DNA per well). After 1 h incubation, the polyplexes were aspirated, fresh and warm complete culture medium (200 µL) was added and eventually cells were cultured for 48 h. The cells were fixed by adding trypsin solution (0.25%, 150 µL), and centrifuged (600 g, 5 min, ambient temperature). The pellets were resuspended in HBSS buffer (200 µL), and measured by the FACSCalibur (Becton-Dickinson, Breda, the Netherlands) at an excitation wavelength of 488 nm and an emission wavelength of 530 nm. Data was processed by the FACS Cellquest Software. A PEI (25 K)/DNA formulation prepared at a polymer/DNA mass ratio of 1.33/1 was used as a reference.

RESULTS AND DISCUSSION

Polymer synthesis and characterization

Poly(amido amine) polymers incorporating different percentages (100%, 50%, 25%, 12%, and 0%) of Boc protected aminobutyl side chains next to hydroxybutyl side chains were prepared via Michael-type polyaddition of *N*, *N'*-cystaminebisacrylamide (**CBA**) with mixtures of *N*-Boc-1,4-butanediamine (**Boc-DAB**) and 4-amino-1-butanol (**ABOL**) at the appropriate molar ratios. After removal of the Boc-protecting groups of the obtained polymers, the resulting free amine groups were reacted with heptafluorobutyric anhydride (**F7**) to afford the fluorinated PAAs p(ABOL_m/DABF7_n) (Scheme 2).

Scheme 2. Synthetic route of preparation of fluorinated PAAs.

In line with our previous reports,^{33,36} the viscosity of the reaction mixture during the polymerization increased gradually without any occurrence of gelation, indicating the formation of linear polymers. ¹H NMR spectroscopy indicated the disappearance of signals at 5-7 ppm confirming complete consumption of acrylamide groups that are potentially toxic to cells.^{64,65} Extensive dialysis followed by lyophilization yielded the fluorinated PAAs as HCl salts. The molecular composition of the fluorinated PAAs is determined by ¹H NMR spectroscopy. The methylene group proximal to the hydroxyl group of ABOL, with a chemical shift of $\delta = 3.62$ ppm, was compared with the methylene signal close to the amide moiety, with a chemical shift of $\delta = 3.50$. The composition of the PAAs is in good agreement with the feed ratios, demonstrating the ease of altering the polymer composition. The ¹⁹F NMR spectra of the products confirmed the successful introduction of the fluoroalkyl moieties in the PAAs. GPC analysis revealed mono-modal size distributions, with molecular weights ranging from 5.2-8.1 kDa and polydispersity indices (PDI) between 1.5-1.7 (Table 1). The PDI of the polymer is counterintuitively small, which is likely due to the removal of small oligomers during the dialysis procedure.

Table 1. Characteristics of fluoroalkyl-substituted poly(amido amine)s with various functionalization extents

Polymer Acronym	Fluorination Percentage (%)		M _w (kDa) ^b	PDI ^b
	feed	real ^a		
pDABF7	100	100	8.2	1.49
p(ABOL ₅₀ /DABF7 ₅₀)	50	50	7.5	1.54
p(ABOL ₇₅ /DABF7 ₂₅)	25	25	7.3	1.67
p(ABOL ₉₀ /PABF7 ₁₀)	12.5	10	6.8	1.47
pABOL	0	0	5.3	1.61

^a determined by integral ratios of NMR spectra; ^b determined via GPC.

Polyplex preparation and characterization

The characteristics of the polyplexes, prepared from the polymers at various polymer/DNA mass ratios at pH 7.4 were evaluated and are presented in Figure 1. The polyplexes displayed diameters ranging from as large as 1.4 μ m to ca. 100 nm with surface charges ranging from +3 mV to +38 mV. As the polymer/DNA mass ratio increases, the polyplexes typically become smaller in size and display higher surface charge, underlining the

dominant influence of electrostatic interactions during DNA condensation.^{13,66} As shown in Figure 1, increased fluorination of the PAAs results in polyplexes that are larger in diameter, and have decreased surface charge. This could be partially due to the increased molecular weight the polymer per protonable nitrogen, rendering less cationic charges available for polyplex formation. However, at polymer/DNA ratio 24 and 48, polyplexes made with PAAs containing 10% fluorinated side groups have smaller size, but similar zeta-potential. This discrepancy is ascribed to that hydrophobic F7 group which facilitates DNA condensation through hydrophobic interactions in addition to electrostatic interactions.⁶⁷ When the degree of fluorination increases, this hydrophobic interaction fails to compensate the loss in charge density, resulting in larger polyplexes with reduced surface charge.

Figure 1. Characterization of polyplexes prepared under various polymer/DNA mass ratios through DLS. (A) Hydrodynamic sizes versus mass ratios; (B) Zeta-potentials versus mass ratios.

Polyplex stability was further investigated by agarose gel electrophoresis. As displayed in Figure 2, no DNA migration is discerned for the polyplexes, implying DNA has been completely entrapped in the wells through binding by the polymers. The polyplexes require high colloidal stability when at extracellular environment to retain and protect DNA, while they have to undergo rapid disintegration inside the cells to release the DNA cargo.^{5,25} All PAA polymers contain reducible disulfide linkages, which enables rapid polyplex disassembly upon entering the reducing interior of cells.^{68,69} This reducing environment was mimicked by DTT (2.5 mM). After incubating the polyplexes in the presence of DTT, polyplexes were subjected to gel electrophoresis displaying migrating DNA bands, similar to free DNA. These results demonstrate DNA is tightly packed by the fluorinated PAAs, but is also rapidly released upon exposure to reducing conditions through cleavage of the disulfide bonds in the polymer chain.

Figure 2. Agarose gel electrophoresis of polyplexes (w/w=48) treated without DTT (A) and with DTT (B) (s.c. = supercoiled, o.c. = open circular).

***In vitro* transfection efficiency and cytotoxicity**

Transfection efficiency of pDNA/polymer polyplexes with different percentage of fluoroalkyl groups was investigated on COS-7 cells with GFP as the reporter gene under serum-free and 10% serum conditions. First, the transfection efficiency was evaluated at varying polymer/DNA mass ratios of 6, 12, 24, and 48 under serum-free conditions. Overlaid GFP fluorescence images of transfected COS-7 cells are presented in Figure 3. Substantial improvement of GFP expression is observed for polyplexes containing only 10% fluorinated-PAA at all polymer/DNA mass ratios. However, for polyplexes prepared from 25% fluorinated PAAs significant GFP expression at mass ratios above 6, while in the case of polyplexes based on 50% fluorinated PAAs, mass ratios above 12 are required. For polyplexes from the PAAs with 100% fluorinated side group, at mass ratio 48 only a low GFP expression is observed which is probably related to their poor biophysical properties such as large particulate size and small surface charge. Importantly, transfection results of fluorinated PAAs (except for 100% fluorinated) outperform non-fluorinated PAAs (0%) at the same polymer/DNA mass ratio. Notably, as can be seen in Figure 4 the

transfection performance of fluorinated PAAs is only marginally reduced in the presence of 10% serum as compared to non-fluorinated PAAs and reference PEI.

Figure 3. Overlaid GFP images of COS-7 cells transfected by pCMV-GFP in PAA or PEI polyplexes after 48 h incubation under 0% serum conditions. The percentages on the Y-axis represent the percentage of fluoro-containing side groups in the PAAs, and the horizontal numbers denote the polymer/DNA mass ratios (4X magnification, bars = 1000 μ m).

Figure 4. Overlaid GFP images of COS-7 cells transfected by pCMV-GFP in PAA or PEI polyplexes after 48 h incubation under 10% serum conditions. The percentages on the Y-axis represent the percentage of fluoro-containing side groups in the PAAs, and the horizontal numbers denote the polymer/DNA mass ratios (4X magnification, bars = 1000 μ m).

The transfection performance of the fluorinated PAAs (10%, 25% and 50%) was further evaluated quantitatively by flow cytometry for polyplexes at polymer/DNA mass ratios of 6 and 12 (Figure 5A and 5B). Comparison of the Figure 5A and 4B show that transfection efficiencies increase with increasing polymer/DNA ratio. Most likely this is due to increasing favorable biophysical characteristics, such as small hydrodynamic size and positive surface charge of these polyplexes. At polymer/DNA mass ratio 12 (Figure 5A) and in absence of serum, the transfection efficiency of 10% fluorinated PAAs is nearly 3-fold higher of that of the non-fluorinated PAAs (84% vs 30% transfection efficiency respectively). The presence of 10% serum only reduces the transfection efficiency of the fluorinated polymers from 84%, 65%, and 42% to 78%, 58%, and 34% respectively, whereas significant reduction of the non-fluorinated PAA from 30% to 12% is observed. Similar transfection results and serum effects are observed for polyplexes at polymer/DNA mass ratios of 6 (Figure 5B).

Figure 5. Transfection efficiencies of poly(amido amine)s functionalized with 50%, 25%, 10%, and 0% fluorination at polymer/DNA mass ratios 12 (A) and 6 (B). (PEI, 25 k branched-PEI, as reference; fluorination degree displayed as X-axis.)

Translation of polymeric gene vectors to the clinic is substantially hurdled by the interaction with serum proteins that cause premature unpacking of DNA, therefore preventing effective transfection.^{70,71} Therefore the transfection performance of the 10%-fluorinated PAAs at polymer/DNA mass ratios 6 and 12 was further investigated under different serum concentrations (0%, 10%, 25%, and 50%). As shown in Figure 6, at polymer/DNA mass ratio 12, over 50% transfection is achieved at serum concentration of 25%, and still 20% transfection takes place under 50% serum condition. When the polymer/DNA mass ratio drops to 6, the transfection efficiency is slightly reduced, but still reaches 75%, 70%, 39%, and 10% in transfection efficiency at serum concentrations of 0%, 10%, 25%, and 50%, respectively. The excellent transfection capability of the 10%

fluorinated PAAs under serum conditions make them promising vectors for *in vivo* application.

Figure 6. Transfection efficiency of COS-7 cells by polyplexes from 10% fluorinated poly(amido amine)s under different serum conditions as determined by flow cytometry.

Effective transfection agents should bear high transfection ability, together with minimal toxicity.⁷² As shown in Figure 7, the fluorinated PAAs based polyplexes maintained excellent cell viability of the COS-7 cells at all polymer/DNA mass ratios. Cell viabilities are well over 90% for all polymer/DNA mass ratios under serum-absent condition (Figure 7A). This is much better than polyplexes from the commonly employed cationic polymer PEI which show only 56% cell viability. In the presence of 10% serum the biocompatibility of polyplexes based on fluorinated PAAs is even slightly further enhanced, and no sign of cytotoxicity is observed (Figure 7B).

Figure 7. Cell viability of polyplexes at different polymer/DNA mass weights to COS-7 cells via MTT assay under 0% serum (A) and 10% serum (B) (the value in X-axis denotes the actual percentage of fluorination degree in polymer).

CONCLUSIONS

Poly(amido amine)s with different degrees (100%, 50%, 25%, and 10%) of perfluorobutylamide side groups next to hydroxybutyl side groups were successfully prepared. Both the fluorinated and the non-fluorinated PAAs are able to condense DNA into nanosized polyplexes with sizes as low as 120 nm and surface charges around +25 mV through both

electrostatic and hydrophobic interactions. Gel electrophoresis experiments show that DNA is effectively liberated from the polyplexes upon exposure to DTT, indicating that DNA is released from the polyplexes inside the reducing environment of the cell. After incubation with polyplexes, COS-7 cells maintain outstanding cell viability, confirming excellent biocompatibility of the polyplexes prepared from fluorinated PAAs. Moreover, significantly enhanced transfection efficiency of up to 80% is observed for the fluorinated polymer vectors, almost 3-fold higher than observed for the non-fluorinated analogues. Introduction of perfluoro-alkyl moieties is demonstrated to be effective and promising method to promote transfection efficiency, while maintaining minimal cytotoxicity.

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Chapter 5. Poly(amido amine)s/DNA Polyplexes with Incorporated Gold Nanorods as Vectors for Imaging and Gene Delivery

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KEYWORDS

Gold nanorods, disulfides, gene delivery, colloidal stability, serum, crosslinking.

ABSTRACT

Disulfide-containing poly(amido amine)s (pABOL) were prepared by Michael-type polyaddition of *N, N'*-cystaminebisacrylamide and 4-aminio-1-butanol (ABOL). Gold nanorods (GNRs) were coated with pABOL by displacing toxic surfactant of hexadecyltrimethylammonium bromide (CTAB) on the surface of GNRs by pABOL through the gold-sulfur interaction. Therefore, GNRs were exposed overnight to pABOL polymer, followed by several centrifugation and re-suspension steps, resulting in modified GNRs solution with well-preserved optical properties of GNRs. Modified pABOL-GNRs condensed plasmid DNA into polyplexes with sizes below 200 nm and positive surface charges of approximately +30 mV, affording hybrid polyplexes with absorbance in the near-infrared (NIR) range. The hybrid polyplexes exhibit smaller particles size and improved colloidal stability, as compared to regular polyplexes consisting only of pABOL and plasmid DNA. *In vitro* transfection experiments revealed that the hybrid polyplexes induce comparable transgene activity without demonstrating added cytotoxicity as compared to GNR-free polyplexes under serum-free conditions. In the presence of serum (10% fetal bovine serum , FBS), the hybrid polyplexes show 3-fold and 17-fold improved transfection efficiency compared to GNR-free polyplexes and optimized standard PEI (25 kDa) formulation, respectively.

INTRODUCTION

Gene delivery, the delivery of genetic materials into eukaryotic cells, holds great promises for the treatment of broad scope of gene-related diseases,¹ like immunodeficiency,² cystic fibrosis,³ Parkinson's disease,⁴ chronic granulomatous disorder,⁵ and cancer.⁶ For successful application, gene delivery systems should meet various criteria. Maintaining intactness of genetic materials (plasmid DNA,^{7,8} mRNA,^{9,10} siRNA¹¹⁻¹³ or microRNA¹⁴) during the delivery route requires gene vectors,⁸ which are able to encapsulate the genetic materials into nanosized complexes¹⁵ which provide protection against degradation.¹⁶ Upon intravenous administration, minimal interaction with vascular endothelial cells and blood components is needed,¹⁷ and furthermore the delivery vehicles should survive the reticuloendothelial system.¹⁸ Furthermore, the gene vectors should allow cellular uptake via the negatively charged cell membrane¹⁹ and escape from the lysosomal pathway to arrive in the cytosol and deliver the therapeutic payload in the cytosol (mRNA, siRNA, and miRNA) or for DNA through nuclear membrane.²⁰ Two types of gene vectors are distinguished: viral vectors and non-viral vectors.²¹ Viral vectors show high transfection

efficiency,²² however these vectors struggle with a number of discerned difficulties such as difficult manufacturing and storage,²³ low gene loading, and potential side effects (insertional mutagenesis,²⁴ cytotoxicity,²⁵ immunogenicity,²⁶ and carcinogenicity²⁷). These severe limitations have prompted strong efforts in the development of non-viral vectors with aid of innovative nanotechnology.²⁸

Due to their relative ease of synthesis and modification, a large variety of polymers have been developed to evaluate as non-viral gene delivery vectors.²⁹ Among these are poly(ethylenimine),³⁰ poly(L-lysine),³¹ poly(β -amino esters),³² poly((2-(dimethylamino)ethyl methacrylate),³³ polyamidoamine dendrimers,³⁴ chitosan,^{35,36} and stealth polymers like Pluronic^{37,38} and PEG.³⁹ More recently, several non-viral vectors with “intelligent” features,⁴⁰ being responsive to specific stimulus, such as pH,⁴¹ temperature,⁴² redox potential,⁴³ enzymes,⁴⁴ and infections⁴⁵ have been developed. In addition, the ease to modify polymers allows the addition of new functionalities to gene delivery systems,¹⁶ for example, linking of targeting groups to realize specific delivery,⁴⁶ coupling of imaging motifs to trace vectors,⁴⁷ and incorporation of multiple payloads for synergistic performance.⁴⁸ Among various polymers, poly(amido amine)s (PAAs) are especially interesting polymers owing to their excellent water solubility, minimal toxicity, remarkable biodegradability and biocompatibility, and sophisticated synthetic modularity.⁴⁹ PAAs with disulfides along the backbone are liable to space-selective cleavage due to the glutathione concentration gradient outside and inside cells, and have been successfully employed as polymeric vectors for plasmid DNA,⁵⁰ siRNA,⁵¹ and even proteins.⁵²

Usually, serum proteins cause aggregation of cationic polyplexes, leading to reduced transfection efficiency due to insufficient cellular uptake of polyplexes.^{50,53,54} To minimize polyplexes aggregation, polyplexes have been stabilized by providing the polyplexes with a hydrophilic corona by grafting with hydrophilic groups, such as poly(ethylene glycol) (PEG),⁵⁵⁻⁵⁷ dextran,⁵⁸ hyaluronic acid,⁵⁹ and silica nanogels.^{60,61} The presence of hydrophilic groups was found to reduce cytotoxicity, enhance water solubility, diminish interaction with blood components, and can accommodate additional ligands. However, these hydrophilic groups shield the interaction of such polyplexes with cell membranes, resulting in reduced cellular uptake and thus low transfection efficiency.⁶² Chemical crosslinking was proposed to improve the colloidal stability of polyplexes through a myriad of crosslinking agents with different responsive linkages, such as redox potential,⁶³⁻

⁶⁵ and pH.⁶⁶ However, these crosslinking stabilization approaches demand tedious efforts during the crosslinking procedure⁶³ and retard the DNA unpacking from the gene vectors resulting in much lowered transfection efficiency^{63,67} Non-covalent crosslinking emerged as an appealing alternative to improve colloidal stability⁶⁸ but has not been fully explored yet.

Gold nanorods (GNRs) have become of interest in gene delivery due to their unique chemical and physical properties of being biologically inert, large surface areas, functionalization flexibility, and customizable optical property.^{69,70} However hexadecyltrimethylammonium bromide (CTAB), a crucial surfactant during the synthesis, exhibits potential cytotoxicity, which necessitates surface modification.⁷¹⁻⁷⁵ The surface modification procedure relies on either a cumbersome layer-by-layer assembly,⁷⁶⁻⁸⁰ tedious CTAB displacement with various materials with higher biocompatibility,⁸¹⁻⁸⁴ or expensive functionalization with nucleotide motifs,⁸⁵⁻⁸⁷ which substantially impedes the translation potential into the clinic. In this chapter, a facile GNRs surface functionalization is proposed with disulfide-containing PAAs by simple mixing. The PAA-modified GNRs are able to condense DNA under the formation of hybrid polyplexes (DNA/PAA-GNR). The embedded GNRs form physical crosslinks in the DNA-PAA matrix, which enhance colloidal stability, which is expected to become translated in increased stability in the presence of serum, leading to improved transfection under these conditions.

Scheme 1. Preparation of hybrid polyplexes consisted of GNRs (yellow cylinder), disulfide-containing PAAs, and plasmid DNA via self-assembly.

Besides the hydrogen bonding and hydrophobic interactions to stabilize the polyplexes as described in the previous two chapters, physical crosslinking of GNRs was used in this chapter. The GNRs were prepared by the seed-mediated growth method, and subsequently

were modified with disulfide-linked PAAs. After mixing with DNA, self-assembled hybrid polyplexes were formed (Scheme 1). The hybrid polyplexes were characterized in hydrodynamic size, zeta-potential, morphology, and optical properties. The transfection efficiency mediated by the hybrid polyplexes was evaluated on COS-7 cells together with cell viabilities.

EXPERIMENTAL PROCEDURE

Materials and Methods

Tetrachloroauric acid ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, 99.99%, Acros), sodium borohydride (NaBH_4 , 99%, Sigma-Aldrich), ascorbic acid (AA, 99%, Sigma-Aldrich), hexadecyltrimethylammonium bromide (CTAB, $\geq 96.0\%$, Fluka), dithiothreitol (DTT, Sigma-Aldrich) and silver nitrate (AgNO_3 , 99.8%, Merck) were directly used without further purification. All monomers, 4-amino-1-butanol (ABOL, 98%, Sigma-Aldrich), *N*, *N'*-cystaminebisacrylamide (CBA, Polysciences), and *tert*-butylamine (tBA, Sigma-Aldrich) were purchased in the highest purity and used without further purification. All solvents used were of analytical grade unless otherwise specified. Water used in these experiments was purified with a Milli-Q Gradient System (Millipore, Bedford, MA). Plasmid DNA, plasmid pCMV-LacZ, pCMV-GFP, and pCMV- Δ GFP were purchased from Plasmid Factory (Bielefeld, Germany). Branched polyethylenimine (PEI, M_w 25 kDa) was purchased from Sigma-Aldrich.

^1H NMR and ^{13}C NMR were taken on a Varian Inova Spectrometer (400 MHz) in deuterated solvents. Molecular weights were determined by gel permeation chromatography (GPC) relative to PEG standards, using a GPCmax with an acetate buffer pH 4.5 containing 30% (v/v) methanol as eluent.

Gold nanorod synthesis

Preparation of growth solution: To a gold salt (HAuCl_4) solution (5.0 mL, 10 mM), a CTAB solution was added (95 mL, 0.1 mM) with gently shaking to yield a dark yellow solution. A freshly prepared silver nitrate solution (700 μL , 0.01 M) was pipetted into the above mixture and subsequently a freshly prepared ascorbic acid solution (550 μL , 0.1 M) was added under mild stirring (approximate 325 rpm) to yield a colorless growth solution.

Preparation of gold seed solution: To a gold salt (HAuCl_4) solution (0.25 mL, 10 mM), a CTAB solution was added (9.5 mL, 0.1 mM) under mild stirring. Subsequently, a cold (4.0 $^\circ\text{C}$) and freshly prepared sodium borohydride (NaBH_4) solution (0.6 mL, 0.01 M) was

pipetted to the mixture all at once under vigorous stirring (1100 rpm) which was continued for 2 min. The resulting light-brownish seed solution was added within 5 min to the growth solution.

Growth phase: The seed solution (120 μ L) was pipetted into the growth solution followed by gentle stirring (325 rpm) for 10 s. Subsequently, the resultant mixture was transferred to a water bath and kept at 27 °C. The mixture turned from colorless to brown within 10-20 min, and was left undisturbed in the water bath overnight. Next day the suspension was centrifuged at 8500 rpm/25 min and the supernatants was decanted. The pellet was resuspended in water and the previous procedure was repeated. Finally the pellet was resuspended in 2.0 mL water to yield a stock GNR solution which was stored at 4° C for further use.

Synthesis of pABOL. CBA (0.520 g, 2.00 mmol) and ABOL (0.182 g, 2.00 mmol) were successively added into a brown reaction flask and dissolved in methanol/water (3/1, v/v) CaCl_2 solution (200 mM, 1.00 mL). Polymerization was proceeded in the dark at 70 °C under stirring. The polymerization mixture became homogeneous within 20 min and polymerization was allowed to react further for 24 h to yield a viscous mixture. Subsequently, 3 equivalents of tBA (0.448 g, 6.00 mmol) were added to consume any unreacted acrylamide groups and the reaction was stirred for another 24 h at 70 °C. The resulting milky suspension was diluted with water to a volume of approximately 20.0 mL, acidified with 4 M HCl solution to pH $\sim$ 4, and purified by Amicon ultrafiltration (MWCO 1000 Da) with acidic water (pH $\sim$ 4). After lyophilization, pABOL was obtained in its HCl-salt form (0.364 g, 40.2% yield). The number average molecular weight (M_n) of the synthesized polymer pABOL was calculated from the NMR spectrum using the integrated areas of two methylene peaks of the butanol side groups (1.62 ppm and 1.78 ppm) and the methyl peaks of the *tert*-butyl end group (1.33 ppm) with M_n of $\sim$ 6.1 KDa (average number of repeat units of 16). ^1H NMR (D_2O , 400 MHz): δ 1.33 (s, 9H, $\text{R}(\text{CH}_3)_3$), 1.62 (m, 2H, RNCH_2CH_2), 1.78 (m, 2H, $\text{RNCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$), 2.74 (t, 4H, 2 x NHCOCH_2), 2.84 (t, 4H, 2 x SSCH_2CH_2), 3.20 (t, 2H, $\text{RNCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$), 3.41 (t, 4H, $\text{CH}_2\text{CH}_2\text{NCH}_2\text{CH}_2$), 3.51 (t, 4H, 2 x SSCH_2CH_2), 3.62 (t, 2H, $\text{RNCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$).

Polymer and gold nanoparticles modification. Stock GNR suspension (10 μ L) was dispersed in pABOL solution (1.0 mL, 2.0 mg/mL, H_2O) in an Eppendorf tube, and incubated at 4.0 °C overnight. After that, the pABOL-GNR constructs were isolated from

the free polymers in solution by centrifugation (9000 rpm, 10 min), and the resulting pellets were redispersed in water.

Hybrid polyplexes preparation. Hybrid polyplexes (DNA/pABOL-GNR) from DNA and the pABOL-GNR constructs were prepared in HEPES buffer (20 mM, pH 7.4). Therefore, the desired amount stock GNR solution was added into 800 μ L polymer solution, followed by 5 s vortexing. The resulting mixture was added into 200 μ L DNA solution, followed by 5 s vortexing and 30 min incubation at room temperature. Size and surface charge of the polyplexes were measured at 25 °C with a Zetasizer Nano ZS (Malvern Instruments, Malvern). The value is recorded as the mean of three measurements and their standard deviation is given in the error bar in the figures. The extinction spectra were recorded on Shimadzu PC3101 UV-Vis-NIR spectrophotometer. Normal polyplexes (DNA/pABOL) were also prepared following the same protocol as hybrid ones, however with skipping the step of mixing GNRs and polymer solution.

***In Vitro* Transfection**

The transfection ability of the polyplexes was evaluated on COS-7 cells with plasmid DNA green fluorescent protein (GFP) as encoding reporter gene. Cells were seeded in 48 well plates with density of 1.6×10^4 per well and cultured in complete medium (DMEM with 10% FBS, 200 μ L) at 37 °C under humidified atmosphere with 5% CO₂ to reach 60-80% confluency. Cell medium was refreshed with medium (200 μ L) with designed serum concentration, and cells were maintained in the new medium for 30 min, after which polyplex dispersion (200 μ L, 1.0 μ g DNA per well) was added. After 60 min incubation at 37°C, the polyplexes were replaced with fresh warm complete medium (200 μ L), and cells were kept in the medium for another 48 h. After this period, the medium was replaced by trypsin solution (0.25%, 200 μ L), and cells were spun down (600 g, 5 min, R.T.), resuspended in HBSS buffer (200 μ L), and measured by with FACS (FACSCalibur, Becton-Dickinson, Breda, the Netherlands) at an excitation wavelength of 488 nm and emission wavelength of 530 nm. FACS Cellquest Software was applied to process data. A PEI (25 K)/DNA formulation prepared at its optimal condition (N/P=10) was used as a reference.

Cell Viability Assays

The cell viability of COS-7 cells exposed to polyplexes was evaluated through MTT assays. In 48 well plates, cells were seeded with density of 1.6×10^4 per well and cultured

in complete medium (DMEM with 10% FBS, 200 μ L) at 37 °C under humidified atmosphere with 5% CO₂ to reach confluency ranged from 60-80%. Cells were refreshed with warm medium (200 μ L) with designed serum concentration, and maintained in the new medium for 30 min after which polyplex dispersions (200 μ L, 1.0 μ g DNA per well) was added. After 60 min incubation at 37 °C, the polyplexes were replaced with fresh warm complete medium (200 μ L), and cells were allowed to grow for another 48 h. From the cells untreated with polyplexes, half of the cells were killed by incubating in 100X triton (4%, 20 μ L) for 15 min (viability 0%), and residual half was used as control with viability of 100%. All cells were washed with DPBS (200 μ L), and successively incubated in MTT solution (0.5 mg/mL, 200 μ L) at 37 °C under 5% CO₂ condition for 4 h. After refreshing MTT with DMSO (200 μ L), the resulting formazone crystals were quantified with a plate reader (Tecan Infinite M200) at wavelength 540 nm with reference 680 nm. All measurements are performed in triplet.

RESULTS AND DISCUSSION

Gold nanorod synthesis. Gold nanorods (GNRs) were synthesized in via the seed-mediated growth method (Figure 1A).^{88,89} In this wet-chemistry method, preformed gold nanospheres serve as the seeds on which metal is grown along preferential directions directed by the surfactant hexadecyltrimethylammonium bromide (CTAB) in the presence of silver nitrate (AgNO₃). The optical spectra of the synthesized GNRs possess two absorption peaks, one at approximately 513 nm and one at 773 nm (Figure 1B). This is in correspondence with literature report of GNRs being hemispherically capped cylinders with two plasmon peaks due to asymmetry.⁹⁰ One peak, assigned as the longitudinal plasmon peak, is due to oscillation of electrons along the longitudinal axis, and other assigned as the transverse plasmon peak due to oscillations along the transverse axis.⁹¹ The transverse plasmon peak is like in spheres, located around 520 nm, whereas the longitudinal plasmon peak is at longer wavelength and dependent of the aspect ratio of the particle. The concentration of GNRs was determined by the optical spectra, defined as optical density (O.D.). The extinction coefficient value of the longitudinal plasmon peak has been taken into consideration for calculating the number of particles per mL using Lambert-Beer law.

Figure 1. Synthesis and characterization of gold nanorods (GNRs) via the seed-mediated growth method. A) Schematic illustration of GNRs synthetic route; GNRs suspension in water after twice centrifugation and resuspension; B) the absorption spectra of GNR suspension in water; and C) morphology of GNRs by atomic force microscopy (scale bar equals 100 nm).

Atomic force microscopy (AFM) was applied to characterize the purified gold nanorods. From the AFM image, it can be seen that uniform gold nanorods with good morphology were obtained with average diameter of 13 nm and average length of 68 nm (Figure 1C).

Polymer synthesis. The bioreducible poly(amido amine) pABOL with repetitive disulfide bonds along its backbone was synthesized via Michael type addition of the primary amine monomer 1-amino-4-butanol (ABOL) to *N,N'*-cystaminebisacrylamide (CBA) under catalysis of CaCl_2 (Figure 2A).

Figure 2. Synthesis of pABOL. A) Synthetic route and structure of pABOL; B) Characterization of pABOL through gel permeation chromatography (GPC).

During the polymerization a gradual viscosity rise was observed without any occurrence of gelation. After 24 h polymerization, excess of *tert*-butylamine was added into the reaction mixture to consume any residual acrylamide groups. The polymer was isolated through exhaustive dialysis (1000 Da cut-off), followed by freeze-drying. The resulting polymer has good solubility in water. The ^1H NMR spectra are in full accordance with the expected structure. The complete disappearance of the signals between 5 and 7 ppm, assigned to the acrylamide group, indicated that pABOL is only end-capped with amino groups. Gel permeation chromatography (GPC) measurements showed a single peak with PDI of 1.24 (Figure 2B).

Polymers coating gold nanorods

Disulfide groups have a relatively strong affinity for gold surfaces and the disulfide bonds along pABOL enable the polymer to displace the cytotoxic CTAB from the gold nanorods, and thus make the GNRs more compatible with biological material. The displacement of CTAB by pABOL proceeds smoothly by incubation the CTAB-GNR suspension with pABOL solution overnight. The resulting pABOL-GNR particles can be purified by centrifugation of the mixture and redispersion of the pellet in water, yielding a colloidal stable solution without further color change (Figure 3A). The extinction spectra of polymer-coated GNRs and original GNRs are presented in Figure 3B. The polymer-coated GNRs showed two absorption bands that were similar to the original GNRs solution, however with a 10 nm red-shift of the longitudinal plasmon band (Figure 3B). The 10 nm red-shift due to the displacement of CTAB with pABOL was similar to the red-shift observed upon PEGylation of GNRs with thiol-functional poly(ethylene glycol).⁹² Gold nanorods without polymer are irreversibly aggregated during the centrifugation, and it is

clear that the encapsulation of the GNRs in pABOL is responsible for the stability of the GNRs in solution. This greatly enhances the utility of the gold nanorods for several applications.

Figure 3. Coating with GNRs with pABOL. A) Photographic images of solution of GNRs after and before pABOL coating; and B) the extinction spectra of GNRs.

Hybrid polyplexes

A requirement for cationic polymers to function as gene delivery vectors is that they are able to condense genetic materials into nanosized polyplexes.⁹³ From previous research it has appeared that pABOL complexes with DNA to nanosized particles (polyplexes) at pABOL/DNA weight ratio 48/1.⁵⁰ Therefore, pABOL and DNA weight ratio in these experiments was also set to 48/1 for preparation of the hybrid polyplexes. For the normal DNA/pABOL polyplexes (without GNR), the average size and zeta-potential were 142 nm and +26 mV, respectively. For the DNA/pABOL-GNR polyplexes, the average size and zeta-potential were 126 nm and +28 mV, respectively. Thus the introduction of GNRs gives stronger condensing of DNA, resulting in smaller polyplexes. It can be assumed that the GNRs' action as chemical crosslinkers in the polyplexes networks and the binding affinity of the GNRs for pABOL is responsible for the higher packing density in these polyplexes.

The stability of both polyplexes was investigated by following their size in time by DLS measurements. The size of the DNA/pABOL-GNR polyplexes and DNA/pABOL polyplexes increases in time in pH 7.4 HEPES buffer. This can be attributed to slow degradation of polymers due to hydrolysis of amide bonds along the pABOL chain.⁹⁴ As illustrated in Figure 4A, the size of the hybrid polyplexes remains within 200 nm within 24 h storage while the DNA/pABOL polyplexes increase to sizes over 200 nm already after 6 h. The slower size increase of the DNA/pABOL-GNR polyplexes can again be attributed

to the cross-linking stability of the GNRs with the disulfide bonds of pABOL.^{69,89} The stability observed upon 3-fold dilution with HEPES buffer also demonstrated that the hybrid polyplexes are much more stable than polyplexes without GNRs (Figure 4B).

Figure 4. Hybrid polyplexes and interaction between GNRs and pABOL through DLS. A) Size evolution of hybrid polyplexes and normal polyplexes in pH 7.4 HEPES buffer; B) Size distribution diagram of hybrid- and normal- polyplexes upon dilution; C) Effect of different amount of GNRs during hybrid polyplexes preparation; and D) Size distribution diagrams of GNRs solution and hybrid polyplexes.

DLS analysis was applied to obtain information about the nature of the GNR in the hybrid polyplexes. Particles with non-spherical shapes, such as nanorods, can be more precisely characterized by multiple angle or depolarized DLS measurements. As depicted in Figure 4B and Figure 4C, the GNR solution showed two peaks in the size distribution diagram, one with an average hydrodynamic diameter at 5-6 nm, and one at 70-80 nm. These peaks are ascribed to the rod-shape of the GNRs. The small size peak is related to the rotational diffusion of the nanorods rather than to an actual dimension of the nanorods.⁹⁵ As the hybrid polyplexes show a single size distribution peak at 105 nm, it can be concluded that this implies a homogenous particle system. With different amounts of GNRs, two peaks in size distribution diagram appeared again when GNRs (O.D.=0.9) applied during hybrid polyplexes (Figure 4C). In all, based on the DLS results, the homogenous hybrid polyplexes were successfully prepared.

Extinction spectra of obtained hybrid polyplexes were recorded on UV-Vis spectrometry (Figure 5A). From the spectra, the hybrid polyplexes showed strong absorption in NIR wavelength as well as absorption in 520 nm from transverse plasmon band. Compared to

the original GNRs (Figure 1B), the red-shift in the absorption spectra of hybrid polyplexes was ascribed to change of dielectric constant of the environment around the GNRs. These results confirm that the GNRs have been incorporated into the polyplexes without compromising their morphologies.

Finally, the hybrid polyplexes were characterized through transmission electron microscopy (TEM). TEM measurements (Figure 5B) show that the hybrid polyplexes have size of around 100-200 nm and one uniform and hemispherically capped shape. These size and morphology of GNRs obtained from TEM is in agreement with the results of AFM. The larger size of the hybrid polyplexes obtained from DLS can be ascribed to different sample conditions, as DLS measures the particles in aqueous solution.

Figure 5. Characterization of hybrid polyplexes with GNRs (0.6 O.D.). A) Extinction spectra of GNRs solution and hybrid polyplexes; B) TEM image of hybrid polyplexes (Scale bar= 100 nm).

***In vitro* transfection and cytotoxicity**

The transfection activities of the hybrid polyplexes at polymer/DNA mass ratios 6, 12, 24, and 48 were evaluated in COS-7 cells with plasmid DNA encoding for green fluorescent protein (GFP) as a reporter gene. The transfection efficiency is presented as the percentage of GFP-positive cells in total cells based on fluorescence-activated cell sorting (FACS) experiments. As shown in Figure 6, the transfection efficiency increases with increasing polymer/DNA mass ratio for hybrid polyplexes as well as normal polyplexes. This effect can be related to the smaller size and higher surface charge of polyplexes at higher polymer/DNA mass ratios. The transfection mediated by hybrid polyplexes exhibits comparable values as observed for GNR-free polyplexes, but both higher than optimized PEI (branched, 25 kDa) formulations with polymer/DNA mass ratios above 6. When the transfection experiment was carried out in the presence of 10% fetal bovine serum (FBS),

hybrid polyplexes induced substantially higher (up to nearly 3-fold) transfection activities as compared to GNR-free polyplexes, while transfection mediated by PEI polyplexes at their optimal polymer/DNA ratio becomes minimal. The introduction of fetal bovine serum (FBS, 10%) induces aggregation of polyplexes, leading to the reduction in transfection of polyplexes.⁵³ It is clear that the hybrid polyplexes exhibit significantly less serum interference than GNR-free polyplexes and PEI-based formulations, which is evidently the stabilization effect of the GNRs in these polyplexes.

Figure 6. Transfection efficiency mediated by hybrid-and normal-polyplexes at serum-free (left) and serum-present (right) conditions with PEI as reference.

For potential utility in clinical application, gene vectors should possess low cytotoxicity. Evaluation by MTT assays showed that the cells remained their viability over 90% after treatment with hybrid polyplexes and GNR-free polyplexes, while cell viabilities of only ca. 60% were observed after treatment with PEI-based polyplexes under serum-free conditions. Also in the presence of serum excellent cell viabilities were obtained for hybrid and GNR-free polyplexes. The results demonstrate that hybrid polyplexes and GNR-free polyplexes are essentially non-toxic. This makes the hybrid polyplexes interesting gene vectors with unique optical properties in NIR range, which may endow light-triggered transfection with concomitant imaging modularity.

Figure 7. Cell viability of cells treated with hybrid polyplexes, normal polyplexes, or PEI-based polyplexes at serum-free (right) and serum-present (left) conditions via MTT assays.

CONCLUSIONS

Gold nanorods (GNRs) were prepared through the seed-mediated growth method with sizes of 13 x 68 nm and a NIR ranged surface plasmon band at 773 nm. The GNRs could be readily incorporated in the disulfide-containing pABOL network without compromising their colloidal stability and optical properties. Through mixing GNRs, pABOL, and plasmid DNA, hybrid polyplexes were prepared of nanosized dimensions (< 200 nm) and positive surface charge (+28 mV). The polyplexes exhibited strong absorption in NIR window (700-850 nm). Compared with normal polyplexes comprising plasmid DNA and pABOL, hybrid polyplexes of DNA/pABOL-GNR shown smaller size, slightly higher surface charge, and enhanced colloidal stability, owing to the action of GNRs as stabilizing crosslinker in these polyplexes. The hybrid polyplexes exhibit transfection efficiency in COS-7 cells comparable to normal polyplexes without inducing cytotoxicity under serum-free conditions. Under serum conditions (10% FBS), hybrid polyplexes induce ca. 3-fold and 17-fold higher transfection efficiencies as compared to GNR-free polyplexes and optimized PEI (25 kDa) respectively. The remarkably high transfection activities mediated of the hybrid polyplexes under the serum conditions are most likely related with their enhanced colloidal stability.

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Chapter 6. Novel Poly(amino ether)s with Various Disulfide Content as Gene Vectors

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KEYWORDS

Disulfides, cationic polymers, gene delivery, poly(amine ether)s, serum tolerance.

Part of this chapter has been in preparation for publication.

ABSTRACT

A class of novel poly(amino ether)s (PAEs) with varying amounts of disulfide linkages in their main chain were prepared by reaction of 4-amino-1-butanol (**ABOL**) with varying ratios of 2-hydroxyethyl disulfide diglycidyl ether (**SS-DE**) and 1,4-butanediol diglycidyl ether (**CC-DE**). The PAEs display high buffer capacities in pH range 5.1-7.4 and efficiently condense DNA into nanosized (< 100 nm) and positively charged (+30-+44 mV) polyplexes. Transfection experiments on COS-7 cells revealed that the transfection efficiency of polyplexes based on PAEs dramatically increases with increasing disulfide content. MTT assays showed the PAE polyplexes are essentially non-toxic. In addition, in the presence of serum, PAEs proved to be effective gene vectors.

INTRODUCTION

The success of gene therapy is still impeded by the lack of efficient and safe delivery vectors.^{1,2} Viral gene vectors constitute approximately 70% of the vectors used in ongoing clinical trials.¹ However, these vectors are associated with several limitations, such as immunogenicity, low DNA loading capacity, and technical barriers for manufacturing on large scale.³⁻⁷ These limitations have encouraged strong efforts in the development of a great variety of non-viral vectors,⁵⁻⁹ in particular cationic polymer-based gene vectors.¹⁰⁻¹⁸

A diverse collection of polymers was developed as gene vectors, ranging from effective, though rather toxic poly (L-lysine),¹⁹⁻²² polyethylenimine (PEI),²³ and poly(2-dimethylaminoethyl)methacrylate (pDMAEMA),^{24,25} to the versatile and considerably less toxic poly(amino ester)s,^{26,27} poly(amido amine)s,²⁸⁻³⁰ and others.^{16,31,32} Michael addition of amines to bisacrylates and bisacrylamides was adopted in the preparation of poly(amino ester)s^{26,33-35} and poly(amido amine)s^{29,36} respectively, resulting in efficient gene delivery vectors with remarkable biocompatibility. The reaction of epoxides with amines is ubiquitously found in common epoxy resins³⁷ and has recently gained renewed attention from polymer chemistry due to controlled polymerization of linear poly(amino ether)s (PAEs).³⁸⁻⁴¹ Cationic PAEs have already been successfully applied as gene vectors without compromising cell viability.⁴²⁻⁴⁵ However these PAEs lack degradability, which may inflict considerable risks to their wide deployment in clinical application.⁴⁵ Incorporating degradable motifs in the polymer chain such as ketal, ester, and other linkages has shown its feasibility in other non-degradable cationic polymers.^{18,46,47} Disulfide bonds are receiving increasing attention to render polymeric gene vectors bio-responsive, owing to their

cleavage in the reductive cytoplasmic compartment.^{28,46,48-55} Here, we present the synthesis and characterization of PAEs with varying amounts of disulfide linkages and *in vitro* studies to evaluate their abilities to form polyplexes with DNA and performance in gene delivery (Scheme 1).

Scheme 1. Schematic illustration of cationic polymeric gene vectors with various disulfide content through epoxy-amine chemistry.

EXPERIMENTAL PROCEDURES

Materials and Methods

Epichlorohydrin ($\geq 98\%$, Sigma-Aldrich), 2-hydroxyethyl disulfide (technical grade, Sigma-Aldrich), tetrabutylammonium bromide (TBAB, $\geq 98\%$, Sigma-Aldrich), sodium hydroxide (NaOH, pellet, Sigma-Aldrich), 1,4-butanediol diglycidyl ether (CC-DE, $\geq 95\%$, Sigma-Aldrich), 4-amino-1-butanol (ABOL, Sigma-Aldrich), and dithiothreitol (DTT, Sigma-Aldrich) were used directly as received without further purification. All solvents used were analytical grade and used without further purification. Water used in these experiments was treated through a Milli-Q Gradient System (Millipore, Bedford, MA). DNA plasmids, pCMV-GFP and pCMV- Δ GFP, were purchased from Plasmid Factory (Bielefeld, Germany). Branched polyethylenimine (PEI, M_w 25 kDa) was purchased from Sigma-Aldrich.

Monomers and polymers were characterized by NMR spectroscopy on a Bruker 400 MHz spectrometer in deuterated solvents. Molecular weights were determined by gel permeation chromatography (GPC) relative to PEG standards, using a Waters Alliance 2695 separation module equipped with a PL-aquagel-OH 8 micron Mixed-M column (300 x 7.5 mm) with *N,N*-dimethylformamide (DMF) containing LiCl (50 mM) as eluent at 35 °C.

Synthesis of 2-hydroxyethyl disulfide diglycidyl ether (SS-DE). The synthesis of 2-hydroxyethyl disulfide diglycidyl ether was referred to literature.⁵⁶ To a mixture of 2-hydroxyethyl disulfide (0.772 g, 5.00 mmol), sodium hydroxide (0.800 g, 20.0 mmol) and tetrabutylammonium bromide (12.37 mg, 34.8 μ mol), epichlorohydrin (1.45 mL, 18.4 mmol) was added during 5 min. The mixture was heated to 40 °C and stirred for 3 h. The resulting mixture was subjected to silica column chromatography to obtain 2-hydroxyethyl disulfide diglycidyl ether (**SS-DE**) as a clear liquid (0.700 g, 52.2 % yield). ¹H NMR (CDCl₃, 400 MHz): δ_{H} 2.62 (m, 2H, epoxide ring 2 x OCHHCHO), 2.80 (m, 2H, epoxide ring 2 x OCHHCHO), 2.90 (t, 4H, 2 x OCHHCH₂S), 3.16 (m, 2H, epoxide ring 2 x OCHHCHO), 3.40 (m, 2H, 2 x CHCHHO), 3.78 (m, 6H, 2 x CHCHHO + 2 x OCH₂CH₂S). ¹³C NMR (CDCl₃, 400 MHz): δ_{C} 38.51, 44.22, 50.77, 69.62, 71.70.

Synthesis of poly(amino ether)s. Poly(amino ether)s (PAEs) with varying disulfide content were prepared by ring open polymerization of diglycidyl ethers **SS-DE** and **CC-DE** in the appropriate ratio with equimolar amounts of 4-amino-1-butanol.

The procedure to prepare PAE **P3** with equimolar amounts of **SS-DE** (50 mol%) and **CC-DE** (50 mol%) is as follows: into a glass vial, diglycidyl ether disulfide **SS-DE** (0.266 g, 1.00 mmol), 1,4-butanediol diglycidyl ether **CC-DE** (0.202 g, 1.00 mmol), 4-amino-1-butanol (**ABOL**, 0.180 g, 2.00 mmol), and ethanol (1.00 mL) were successively loaded. The obtained mixture was stirred at ambient temperature for 72 h, yielding a viscous mixture. The resulting mixture was diluted with water to a total volume of 15.0 mL, acidified with 4 M HCl solution to pH = 4, and purified via dialysis (MWCO 1.0 kDa) against water overnight. The remaining solution was lyophilized, yielding polymer with 50 mol% disulfide (**P3**) as a white solid.

P1 (0% disulfide, 0.315 g, 48.1 % yield). ¹H NMR (D₂O, 400 MHz): δ 1.59-1.64 (m, 6H, OCH₂CH₂CH₂CH₂O + NCH₂CH₂CH₂CH₂OH), 1.78-1.82 (m, 2H, NCH₂CH₂CH₂CH₂OH),

3.28-3.42 (m, 6H, $2 \times \text{NCH}_2\text{CHOHCH}_2 + \text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$), 3.52-3.66 (m, 10H, $2 \times \text{CH}_2\text{OCH}_2 + \text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$), 4.20-4.27 (bs, 2H, $2 \times \text{OCH}_2\text{CHOHCH}_2\text{N}$).

P5 (100% disulfide, 0.347 g, 44.2 % yield). ^1H NMR (D_2O , 400 MHz): δ 1.59-1.69 (m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$), 1.74-1.83 (m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$), 2.95-2.98 (t, 4H, $2 \times \text{SCH}_2\text{CH}_2$), 3.36-3.45 (m, 6H, $2 \times \text{NCH}_2\text{CHOHCH}_2 + \text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$), 3.60-3.68 (m, 6H, $2 \times \text{OCH}_2\text{CHOHCH}_2\text{N} + \text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$), 3.81-3.90 (m, 4H, $2 \times \text{SCH}_2\text{CH}_2$), 4.29-4.31 (bs, 2H, $2 \times \text{OCH}_2\text{CHOHCH}_2\text{N}$).

The copolymers are : **P2** (25% disulfide, 0.314 g, 41.7 % yield); **P3** (50% disulfide, 0.211 g, 29.3 % yield); **P4** (75% disulfide, 0.198 g, 28.8 % yield). The ^1H NMR spectra were comparable to **P1** and **P5** with similar peak patterns but various integral values.

Polyplex preparation

The PAE/DNA polyplexes at polymer/DNA mass ratio 24 were prepared by adding polymer solution (450 $\mu\text{g/mL}$, 800 μL) into DNA solution (75 $\mu\text{g/mL}$, 200 μL), followed by 5 s vortexing and 30 min incubation at room temperature. All polymer and DNA solutions were prepared in water. Size and surface charge of the polyplexes were measured at 25 $^\circ\text{C}$ with a Zetasizer Nano ZS (Malvern Instruments, Malvern). Data is presented as mean value $\pm$ standard deviation.

Agarose gel retardation

Polyplexes were prepared at polymer/DNA mass ratios of 12 following aforementioned procedure. Next, water (without DTT or containing DTT) was added into the polyplex solutions to give the final DTT concentration of 2.5 mM. After 30 min incubation at ambient temperature, 6X loading buffer containing bromophenol (Ferments, 5.0 μL) was mixed with resulting dispersions. The obtained mixture (10 μL) was loaded on a 0.8% w/v agarose gel containing 1X SYBR® Safe DNA Gel Stain (Invitrogen™). The loaded gel was developed at 90 V for 60 min in a TBE (Tris-borate-EDTA, 1X) running buffer, and subsequently visualized using FluorChem M Imager (Proteinsimple, Westburg, Leusden, the Netherlands) under the exposure to UV light.

Buffer capacity

The buffering capacity of the polymers was determined by acid-base titration. PAE polymer was weighed to a total amount equal to 5 mmol protonable amine groups, and dissolved in

NaCl aqueous solution (150 mM, 10 mL). The pH of the polymer solution was acidified to below pH 2 and the solution was subsequently titrated with NaOH solution (0.1 M) using an automatic titrator (Metrohm 702 SM Tirino). As a reference, PEI (25 kDa) and NaCl aqueous solution were titrated following the same method. The percentage of amine groups being protonated in the pH range 5.1 to 7.4 was defined as the buffer capacity and was calculated from the following equation:⁵⁷

$$\text{Buffer capacity (\%)} = \frac{\Delta V_{\text{NaOH}} \times 0.1 \text{ M}}{N \text{ mol}} \times 100\%$$

wherein, ΔV_{NaOH} denotes the NaOH volume required to bring the pH value of the polymer solution from 5.1 to 7.4, and N mol (5 mmol) denotes the total amount of protonable amine groups in the PAE polymer.

***In Vitro* Transfection and Cell Viability Assays**

Transfection experiments were performed on COS-7 cells (SV-40 transformed African green monkey kidney cells) using reporter genes of pCMV-GFP and pCMV-ΔGFP.

Assessments of reporter gene expression (GFP expression), as well as the evaluation of cell viability by MTT assay, were carried out in separate 96-well plates. Cells were seeded in 96 well plates with densities of approximately 1.0×10^4 cells per well (2.94×10^4 cells/cm²) and maintained in complete medium at 37 °C in a humidified atmosphere containing 5% CO₂ until 60% - 80% confluency. In a standard transfection experiment, cells were first incubated with medium (100 μL, with/without serum) for 30 min and subsequently loaded with 100 μL polyplex dispersions (0.25 μg DNA per well) prepared in HEPES buffer (20 mM, pH 7.4, supplemented with 5.0 wt% glucose) (HBG). DNA concentration was 0.25 μg per well which is about 4 times lower than commonly used to keep toxicity effects to minimum. After 1 h incubation, polyplexes were removed, fresh and warm complete culture medium (100 μL) was added and cells were further cultured for 48 h. The transfection was visualized through fluorescence imaging via an EVOS digital inverted microscope (EMS, Wageningen, the Netherlands). The PEI (25 K)/DNA formulation was prepared at a polymer/DNA mass ratio 1.25/1 and used as a positive control. MTT assays were conducted to characterize the number of viable cells with MTT value of 100% cell viability for untreated cells (i.e., cells not exposed to transfection systems).

Quantitation of transfection efficiency was performed by flow cytometry on cells exposed to polyplexes prepared at polymer/DNA mass ratios 6, 12, and 24. Cells were seeded in 48-

well plates with densities of approximately 1.6×10^4 cells per well (1.6×10^4 cells/cm²) and maintained in complete medium at 37 °C in a humidified atmosphere containing 5% CO₂ until 60% - 80% confluency. Cells were incubated with medium (200 µL, with/without serum) for 30 min and subsequently loaded with 200 µL polyplex dispersions (0.5 µg DNA per well). After 1 h incubation, polyplexes were aspirated, fresh and warm complete culture medium (200 µL) was added and cells were further cultured for 48 h. The cells were detached by adding trypsin solution (0.25%, 150 µL), and were centrifuged (600 g, 5 min, ambient). The resulting pellets were resuspended in HBSS buffer (200 µL), and measured by the FACSCalibur (Becton-Dickinson, Breda, the Netherlands) at excitation of 488 nm and emission of 530 nm. The data were processed by the FACS Cellquest Software.

RESULTS AND DISCUSSION

Monomer and polymer synthesis and characterization

The disulfide-containing monomer 2-hydroxyethyl disulfide diglycidyl ether (**SS-DE**) was synthesized by reacting 2-hydroxyethyl disulfide with epichlorohydrin in the presence of tetrabutylammonium bromide as a phase-transfer catalyst. As non-reducible comonomer, the commercially available 1,4-butanediol diglycidyl ether (**CC-DE**) was used. The amine monomer employed in this study is 4-amino-1-butanol (**ABOL**), as the presence of this monomeric unit has been demonstrated earlier to display favorable properties in transfection efficiency in a series of poly(amido amine)s.²⁸

Scheme 2. Synthesis of poly(amino ether)s with varying disulfide content by epoxy-amine reaction.

Poly(amino ether)s (PAEs) with different ratios of disulfide-moieties were obtained via ring-opening polymerization of primary amines (**ABOL**) with a mixture of bifunctional epoxides **SS-DE** and **CC-DE** in disulfide molar ratios of 0%, 25%, 50%, 75%, and 100% (see Scheme 2). The polymerization was performed in ethanol at room temperature, resulting in the reaction mixture becoming increasingly viscous. PAEs were purified through dialysis and subsequently lyophilized to give amorphous sticky solids in 30-45% yields. The polymers were characterized by ^1H NMR spectroscopy and the signals at 2.95-2.98 ppm that can be attributed to the methylene groups adjacent to disulfide bonds confirmed the incorporation of disulfide moieties into the backbone of the PAEs. The percentage of disulfide incorporation matched the feed ratios closely, underlining the flexibility in controlling disulfide content in the polymerization. Gel permeation chromatography (GPC) was applied to determine molecular weights and polydispersity of the obtained PAEs (see Table 1). Molecular weights range from 6.0-10 kDa with polydispersities of 1.37-1.69. The relatively low polydispersities obtained in the resulting polymers could be attributed to the removal of small oligomer fractions in the dialysis step. Homopolymers **P1** (10.3 kDa) and **P5** (9.4 kDa) display higher molecular weight than the copolymers.

Table 1. Characteristics of poly(amino ether)s with various disulfide content

Poly(amino ether)	Disulfide content ^a (%)	Yield (%)	M _n ^b (kDa)	PDI ^b (-)	Degree of polymerization ^b (%)	Buffer capacity ^c (%)
P1	0	48.1	10.3	1.69	35.2	20.7
P2	25	28.8	5.2	1.37	16.9	19.1
P3	50	29.3	6.0	1.42	18.5	25.5
P4	75	41.7	6.3	1.37	18.6	25.5
P5	100	44.2	9.4	1.68	26.3	27.2

^a Determined by ^1H NMR spectroscopy; ^b Determined by GPC; ^c Determined by titration and calculated in the pH range 5.1 - 7.4.

PAEs are rich in tertiary amines in the main chain of the polymer and are anticipated to have increased buffer capacity as compared to branched PEI (PEI, 25 kDa). As shown in the titration curves of PAEs (Figure 1), all PAEs display part of plateau region (buffer region) in the pH range 5.1-7.4, which give these PAEs slightly higher buffer capacities than PEI

(18%). The buffer capacity of the PAEs is higher at increased disulfide content, which can be ascribed to the inductive effect of the disulfides which makes the amine slightly less basic.⁵⁸ The somewhat higher buffer capacity of **P1** compared to **P2** could be related to a higher tendency for aggregation of the unprotonated (parts of the) polymer of this higher molecular weight polymer (10.3 kDa) compared to **P2** with molecular weight of 5.2 kDa. The amino groups in less polar (aggregated) environment will be less readily protonated (lower pK_a). Most disulfide-containing PAEs have higher buffer capacity (25%-27%) than that **P1** (20.7%) despite the former having lower molecular weight than latter. Thus, the disulfide serves as dominant factor in determining polymer's buffer capacities over its molecular weight, which is in line with our previous observations.^{28,58} The buffer capacities of the PAEs contribute to the capability of polyplexes to escape from the endosomal compartments, which is a necessary condition for eventual gene expression.

Figure 1. Titration curves of poly(amino ether)s with various disulfide content.

Polyplexes formation and characterization

The ability to condense DNA into polyplexes with appropriate physicochemical properties determines their fate as gene delivery vectors.^{9,46} Polyplexes were prepared by combining solutions of the respective PAEs into DNA solutions at different polymer/DNA mass ratios (i.e., 3, 6, 12, and 24). Polyplexes were characterized by dynamic light scattering (DLS) to determine their hydrodynamic sizes and their surface charges. As shown in Figure 2, all PAEs condense DNA into polyplexes with hydrodynamic sizes ranging from 60-100 nm and positive surface charges of from +30 mV up to +44 mV. As polymer/DNA mass ratios increase, the polyplexes become more compact, which is in agreement with the additional

charges available to interact with DNA.^{59,60} Interesting, at higher polymer/DNA mass ratios of 12 and 24, polyplexes made from homopolymers (**P1** and **P5**) are bigger than those made from the copolymers **P2**, **P3**, and **P4**. In principle, the zeta-potentials of polyplexes increase with increasing polymer/DNA mass ratio, as is illustrated by polyplexes made of PAEs **P1**.

Figure 2. Characteristics of polyplexes prepared from poly(amino ether)s and plasmid pCMV-GFP under various polymer/DNA mass ratios. (A) Hydrodynamic sizes versus mass ratios; (B) Zeta-potentials versus mass ratios.

Agarose gel electrophoresis was performed to assess DNA complexation by the PAEs, as depicted in Figure 3. Whereas free plasmid DNA migrates in the gel under the influence of the electrical field, DNA trapped inside polyplexes remained stationary in the agarose gel. Subsequently, the response of polyplexes to a reducing environment was evaluated by incubating the polyplexes in 2.5 mM DTT. DNA was fully released from polyplexes based on disulfide-containing PAEs, but remained trapped in the wells for polyplexes based on non-reducible PAE **P1**. A disulfide content of only 25 mol% already results in sufficient polymer cleavage to release the DNA, though actual intracellular conditions may require higher incorporation ratios.

Figure 3. Agarose gel electrophoresis of untreated polyplexes (w/w=12) (left); and polyplexes treated with 2.5 mM DTT (right).

***In vitro* transfection efficiency and cytotoxicity**

The transfection efficiency of polyplexes was investigated on COS-7 cells by using the plasmid pCMV-GFP as a reporter gene, expressing fluorescent GFP, while cell viabilities were studied by MTT assays. COS-7 cells were exposed to polyplexes with polymer/DNA mass ratios of 6, 12, and 24, and transfection efficiency was assessed by fluorescence microscopy and flow cytometry. GFP expression increases with increasing polymer/DNA mass ratios, which is in line with earlier observations for other polymeric vectors, such as PEI,²³ poly(amino ester),^{26,61} and poly(amido amine)s,²⁸ owing to enhanced cellular uptake of polyplexes with smaller size and cell membrane destabilization from excess of cationic species.^{17,62} At polymer/DNA mass ratios of 24, less than 5% transfection is observed when polymers with disulfide contents of 0%, 25%, and 50% were employed, while substantial transfection occurs when the disulfide content in the polymers increases to 75% and 100% (Figure 4 and Figure S). In these cases the transfection efficiency was 25% and 85%, respectively.

Figure 4. Overlaid fluorescence images of COS-7 cells transfected with pCMV-GFP through PAEs and PEI under serum-free conditions. 4X magnification, bars=1000 μm . (the numbers aligned vertically mean polymer/DNA mass ratios).

Serum protein is known to destabilize polyplexes and consequently hamper transfection by polymeric vectors.^{63,64} The influence of serum on the transfection of COS-7 cells was investigated and fluorescence images are presented in Figure 5. When transfections are carried out in the presence of 10% serum, still appreciable transfection activity is observed,

though reduced as compared to serum-free conditions. These transfection activities demonstrate the PAEs maintain their transfection abilities. In comparison, PEI displays a substantial loss of transfection capability in the presence of serum, underling the serum-tolerance of PAEs. This serum-tolerance is attributed to the abundant hydroxyl groups resulting from oxirane ring-opening that play similar anti-fouling effect as poly(ethylene glycol).^{45,65}

Figure 5. Overlaid fluorescence images of COS-7 cells transfected with pCMV-GFP through PAEs and PEI under serum-present conditions (10% FBS). 4X magnification, bars=1000 μ m. (the numbers aligned vertically mean polymer/DNA mass ratios).

The cell viability of cells treated with PAEs-based polyplexes was studied through MTT assays. As shown in Figure 6, COS-7 cells remain excellent viabilities for all polymer/DNA mass ratios (24, 12, and 6) employed in the absence or presence of serum. Although here cell viability is also high for the polymer **P1**, lacking the disulfide moieties, it is well accepted that disulfide increased biocompatibility of cationic polymeric gene vectors.⁶⁶ These results show that PAEs with disulfide have excellent biocompatibility, which makes these polymers promising materials in gene delivery and tissue engineering.

Figure 6. Cell viabilities of COS-7 cells treated with polyplexes at different polymer/DNA mass ratios under serum-free conditions (left); and 10% serum (right). PEI-based polyplex at mass ratio of 1.25 is incorporated as a reference.

CONCLUSIONS

Linear poly(amino ether)s with varying disulfide content were readily obtained by epoxy-amine polyaddition reaction between the primary amine 4-amino-1-butanol and mixtures of 1,4-butanediol diglycidyl ether and disulfide-containing diglycidyl ether. The resulting PAEs have high buffer capacities and bear excellent DNA condensation abilities, forming nanosized and positively-charged polyplexes. The polyplexes made from PAEs with high content of disulfide (over 50%) are able to induce high transfection activity on COS-7 cells. Moreover, the presence of serum only has marginal effect on the transfection of PAEs-based polyplexes. Polyplexes formed from disulfide-containing PAEs show much higher transfection efficiency, lower cytotoxicity, and better serum tolerance than those of PEI, implying their promising potential as efficient and safe non-viral gene vectors.

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SUPPORTING INFORMATION

Figure S. Transfection efficiency of COS-7 cells by polyplexes at polymer/DNA mass ratios 6, 12, and 24 from poly(amino ether)s with various disulfide content and PEI under different serum conditions (Left: 0% FBS; Right: 10% FBS) as determined by flow cytometry (polyplexes prepared at polymer/DNA mass ratio 1.25 are used as control).

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Chapter 7. Efficient Gene Vectors Based on Bioreducible Poly(amino ether)s with Various Side Groups

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KEYWORDS

Cationic polymers; bioreducible polymers; serum tolerance; gene delivery; buffer capacity.

Part of this chapter has been in preparation for publication.

ABSTRACT

Facile and efficient epoxy-amine reaction was adopted in the preparation of novel disulfide-linked cationic poly(amino ether)s (PAEs) with structural variations, and their potential as non-viral gene delivery vectors was evaluated. PAEs prepared from polyaddition of 2-hydroxyethyl disulfide diglycidyl ether and various amines have molecular weights ranging from 4.5 to 9.0 kDa and are characterized by high buffer capacities of up to 76%. The PAEs display excellent DNA complexation, as evidenced by the formation of polyplexes with sizes below 100 nm, while being able to release DNA through disulfide cleavage upon exposure to reducing conditions. Minimal cytotoxicity was observed on COS-7 cells for all polymer/DNA mass ratios. PAEs prepared from 4-amino-1-butanol (ABOL), 5-amino-1-pentanol (APOL), and 3-morpholinopropylamine (MPA) display high transfection efficiency as observed by the expression of green fluorescent protein (GFP). The three polymers display up to 20-fold higher transfection efficiency at condition 10% serum as compared to PEI.

INTRODUCTION

Gene therapy, termed as medication of genes as drugs for curing human diseases, has gained increasing attention over the past decades owing to its high therapeutic promise in many presently incurable diseases.¹⁻⁴ However, due to the potential degradation of genes by endonucleases, direct medication of genes yields little to no effect in patients due to ineffective delivery, increasing the demand for effective gene vectors.^{5,6} Viral gene vectors are potent in transfection efficacy but come with problems in biosafety.^{7,8} Non-viral vectors can be designed to be biocompatible, but transfection efficacies are still low.⁹⁻¹¹ Developing effective, biocompatible non-viral vector is therefore of great interest.³

Cationic polymers have been extensively investigated as promising non-viral gene vectors, since they are able to condense DNA, readily accessible and highly modular.¹²⁻¹⁴ Poly(l-lysine) (PLL),¹⁵⁻¹⁹ poly(ethylene imine) (PEI),²⁰⁻²² poly[2-(dimethylamino) ethyl methacrylate] (PDMAEMA),²³⁻²⁵ poly(amido amine) (PAMAM) dendrimers,²⁶⁻²⁸ have been used as cationic polymeric gene vectors. However, high cytotoxicity associated with these polymeric vectors severely impedes realizing their full potential in clinical application. To overcome this challenge, recent efforts have been focused on the development of biocompatible and biodegradable polymers including carbohydrate-based polymers (β -cyclodextrin,^{29,30} chitosan,³¹ dextran,^{32,33} poly(glycoamido amine),³⁴⁻³⁶ etc.), phosphorus-

containing polymers,^{37,38} poly(amino ester)s,³⁹⁻⁴³ poly(4-hydroxy-L-proline ester)^{44,45} and poly[α -(4-aminobutyl)-L-glycolic acid] (PAGA).⁴⁶ Among these systems, disulfide-containing poly(amino ester)s^{47,48} and poly(amido amine)s⁴⁹⁻⁵⁴ showed encouraging *in vitro* and *in vivo* transfection efficacies. Disulfide bonds have been found to promote condensation of DNA to form stable DNA/cationic polyplexes, help the polyplexes escape from endosomal barriers, and reduce the cytotoxicities of the gene vectors.⁵⁵

The amine-epoxide addition reaction has been widely used in polymer chemistry.⁵⁶⁻⁵⁹ The reaction generates tertiary amines, which are readily protonated under physiological conditions to facilitate complexation of DNA.^{60,61} Rege and coworkers have prepared a library of cationic polymers through reaction of the epoxides of diglycidyl ethers with amines to explore these polymers for gene delivery.^{62,63} They found for polyplexes of pDNA with a number of these cationic polymers bear higher transfection efficacies and lower cytotoxicities than the common reference PEI.^{62,63} Yu's group has prepared linear⁶⁴ and branched⁶⁵ cationic polymers by copolymerizing diglycidyl ethers with various amines (secondary and primary) through amine-epoxide reaction. The linear cationic polymers showed lower cytotoxicities and higher transfection efficiencies as compared to PEI.^{64,65}

Scheme 1. Schematic illustration of disulfide-containing poly(amino ether)s as gene vectors.

As demonstrated in Chapter 6, the disulfide content variation in PAEs was found to influence the performance of PAEs in gene delivery. PAEs with 100% disulfide content have the highest transfection efficiency, a strong DNA condensation ability, high buffer capacity, efficient transfection, low cytotoxicity, and marginal interference from serum protein. In this chapter, we describe the study of the effects of different side groups on transfection activity and cytotoxicity of PAEs containing 100% disulfide bonds in their main chain prepared by epoxide-amine reaction (see Scheme 1).

EXPERIMENTAL PROCEDURES

Materials and Methods

Epichlorohydrin ($\geq 98\%$, Sigma-Aldrich), 2-hydroxyethyl disulfide (technical grade, Sigma-Aldrich), tetrabutylammonium bromide (TBAB, $\geq 98\%$, Sigma-Aldrich), sodium hydroxide (NaOH, pellet, Sigma-Aldrich), 1,4-butanediol diglycidyl ether ($\geq 95\%$, Sigma-Aldrich), and dithiothreitol (DTT, Sigma-Aldrich) were used directly as received without further purification. All monomer amines, 2-amino-1-ethanol (AEOL, Sigma-Aldrich), 3-amino propanol (APrOL, Sigma-Aldrich), 4-amino-1-butanol (ABOL, Sigma-Aldrich), 5-amino-1-pentanol (APOL, Sigma-Aldrich), 6-amino-1-hexanol (AHXL, Sigma-Aldrich), 3-pyrrolidinopropylamine (PPA, Sigma-Aldrich), 3-methoxypropylamine (MOPA, Sigma-Aldrich), 3-morpholinopropylamine (MPA, Sigma-Aldrich), and *N*-Boc-1,4-butanediamine (DAB-Boc, Sigma-Aldrich) were directly used without any further purification. All solvents used were of analytical grade. Water used in these experiments was purified with a Milli-Q Gradient System (Millipore, Bedford, MA). Plasmid DNA, plasmid pCMV-GFP was purchased from Plasmid Factory (Bielefeld, Germany). Branched polyethylenimine (PEI, M_w 25 kDa) was purchased from Sigma-Aldrich.

^1H NMR and ^{13}C NMR spectra were recorded on a Bruker NMR spectrometer (400 MHz) in deuterated solvents. Molecular weights were determined by gel permeation chromatography (GPC, Waters Alliance 2695) relative to PEG standards, using a Mixed-M column (PL-aquagel-OH 8 micron, 300 x 7.5 mm) with *N,N*-dimethylformamide (DMF) containing LiCl (50 mM) as eluent.

Synthesis of 2-hydroxyethyl disulfide diglycidyl ether. The synthetic procedure was referred to previous chapter in this thesis. To a mixture of 2-hydroxyethyl disulfide (0.772 g, 5.00 mmol), sodium hydroxide (0.800 g, 20.0 mmol) and tetrabutylammonium bromide (12.37 mg, 34.8 μmol), epichlorohydrin (1.45 mL, 18.4 mmol) was added during 5 min. The

mixture was heated to 40 °C and stirred for 3 h. The resulting mixture was subjected to column chromatography to obtain 2-hydroxyethyl disulfide diglycidyl ether (0.700 g, 52.2 % yield) as liquid. ^1H NMR (CDCl_3 , 400 MHz): δ 2.62 (m, 2H, epoxide ring 2 x OCHHCHO), 2.80 (m, 2H, epoxide ring 2 x OCHHCHO), 2.90 (t, 4H, 2 x OCHHCH_2S), 3.16 (m, 2H, epoxide ring 2 x OCHHCHO), 3.40 (m, 2H, 2 x CHCHHO), 3.78 (m, 6H, 2 x CHCHHO + 2 x $\text{OCH}_2\text{CH}_2\text{S}$). ^{13}C NMR (CDCl_3 , 400 MHz): δ 38.51, 44.22, 50.77, 69.62, 71.70.

Synthesis of poly(amino ether)s. A representative procedure is the synthesis of **pABOL**: Into a glass vial, 2-hydroxyethyl disulfide diglycidyl ether (0.533 g, 2.00 mmol), 4-amino-1-butanol (ABOL, 0.180 g, 2.00 mmol), and ethanol (1.00 mL) was successively charged. After 3 days stirring at ambient temperature, the resultant viscous mixture was diluted with water to the final volume of 15.0 mL, acidified with 4 M aqueous HCl solution to pH 4, and purified via dialysis (MWCO 1.0 kDa) against water overnight. The final polymer was obtained after lyophilization yielding a white solid (0.420 g, 56.3 % yield). ^1H NMR (D_2O , 400 MHz): 1.59-1.69 (m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$), 1.74-1.83 (m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$), 2.95-2.98 (t, 4H, 2 $\times$ SCH_2CH_2), 3.36-3.45 (m, 6H, 2 $\times$ $\text{NCH}_2\text{CHOHCH}_2$ + $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$), 3.60-3.68 (m, 6H, 2 $\times$ $\text{OCH}_2\text{CHOHCH}_2\text{N}$ + $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$), 3.81-3.90 (m, 4H, 2 $\times$ SCH_2CH_2), 4.29-4.31 (bs, 2H, 2 $\times$ $\text{OCH}_2\text{CHOHCH}_2\text{N}$). Other PAEs were prepared analogously by using different amines.

pAEOL (0.513 g, 70.5% yield): ^1H NMR (D_2O , 400 MHz): 2.96-2.99 (t, 4H, 2 $\times$ SCH_2CH_2), 3.43-3.47 (m, 6H, 2 $\times$ $\text{NCH}_2\text{CHOHCH}_2$ + $\text{NCH}_2\text{CH}_2\text{OH}$), 3.61-3.70 (m, 4H, 2 $\times$ $\text{OCH}_2\text{CHOHCH}_2\text{N}$), 3.81-3.91 (m, 4H, 2 $\times$ SCH_2CH_2), 3.95 (m, 2H, $\text{NCH}_2\text{CH}_2\text{OH}$), 4.24-4.27 (bs, 2H, 2 $\times$ $\text{OCH}_2\text{CHOHCH}_2\text{N}$).

pAPrOL (0.402 g, 53.2% yield): ^1H NMR (D_2O , 400 MHz): 1.92-2.09 (m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{OH}$), 2.96-2.99 (t, 4H, 2 $\times$ SCH_2CH_2), 3.39-3.48 (m, 6H, 2 $\times$ $\text{NCH}_2\text{CHOHCH}_2$ + $\text{NCH}_2\text{CH}_2\text{CH}_2\text{OH}$), 3.60-3.69 (m, 4H, 2 $\times$ $\text{OCH}_2\text{CHOHCH}_2\text{N}$), 3.71-3.79 (m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{OH}$), 3.80-3.91 (m, 4H, 2 $\times$ SCH_2CH_2), 4.29-4.31 (bs, 2H, 2 $\times$ $\text{OCH}_2\text{CHOHCH}_2\text{N}$).

pAPOL (0.530 g, 65.2% yield): ^1H NMR (D_2O , 400 MHz): 1.40-1.47 (m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$), 1.58-1.65 (m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$), 1.79-1.81 (m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$), 2.96-2.99 (t, 4H, 2 $\times$ SCH_2CH_2), 3.34-3.47 (m, 6H, 2 $\times$

$\text{NCH}_2\text{CHOHCH}_2 + \text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$), 3.61-3.68 (m, 6H, $2 \times \text{OCH}_2\text{CHOHCH}_2\text{N}$ + $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$), 3.81-3.91 (m, 4H, $2 \times \text{SCH}_2\text{CH}_2$), 4.23-4.30 (bs, 2H, $2 \times \text{OCH}_2\text{CHOHCH}_2\text{N}$).

pAHXL (0.326 g, 38.8% yield): ^1H NMR (D_2O , 400 MHz): 1.41-1.44 (m, 4H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$), 1.55-1.58 (m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$), 1.77 (m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$), 2.96-2.99 (t, 4H, $2 \times \text{SCH}_2\text{CH}_2$), 3.33-3.42 (m, 6H, $2 \times \text{NCH}_2\text{CHOHCH}_2 + \text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$), 3.59-3.63 (m, 6H, $2 \times \text{OCH}_2\text{CHOHCH}_2\text{N} + \text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$), 3.81-3.89 (m, 4H, $2 \times \text{SCH}_2\text{CH}_2$), 4.22-4.25 (bs, 2H, $2 \times \text{OCH}_2\text{CHOHCH}_2\text{N}$).

pMPA (0.775 g, 93.2% yield): ^1H NMR (D_2O , 400 MHz): 2.23 (s, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{N}$), 2.96-2.99 (t, 4H, $2 \times \text{SCH}_2\text{CH}_2$), 3.22-3.44 (m, 12H, $2 \times \text{CH}_2\text{N}(\text{CH}_2)_2$), 3.59-3.66 (m, 4H, $2 \times \text{OCH}_2\text{CHOHCH}_2\text{N}$), 3.82-3.90 (m, 4H, $2 \times \text{SCH}_2\text{CH}_2$), 3.98 (m, 4H, $\text{CH}_2\text{OCH}_2\text{-morpholine ring}$), 4.23-4.28 (bs, 2H, $2 \times \text{OCH}_2\text{CHOHCH}_2\text{N}$).

pPPA (0.122 g, 14.2% yield): ^1H NMR (D_2O , 400 MHz): 1.59-1.69 (m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$), 1.74-1.83 (m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$), 2.95-2.98 (t, 4H, $2 \times \text{SCH}_2\text{CH}_2$), 3.36-3.45 (m, 6H, $2 \times \text{NCH}_2\text{CHOHCH}_2 + \text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$), 3.60-3.68 (m, 6H, $2 \times \text{OCH}_2\text{CHOHCH}_2\text{N} + \text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$), 3.81-3.90 (m, 4H, $2 \times \text{SCH}_2\text{CH}_2$), 4.29-4.31 (bs, 2H, $2 \times \text{OCH}_2\text{CHOHCH}_2\text{N}$).

pMOPA (0.382 g, 48.7% yield): ^1H NMR (D_2O , 400 MHz): 1.98-2.16 (m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{OCH}_3$), 2.95-2.98 (t, 4H, $2 \times \text{SCH}_2\text{CH}_2$), 3.30-3.43 (m, 9H, $2 \times \text{NCH}_2\text{CHOHCH}_2 + \text{NCH}_2\text{CH}_2\text{CH}_2\text{OCH}_3$), 3.58-3.65 (m, 6H, $2 \times \text{OCH}_2\text{CHOHCH}_2\text{N} + \text{NCH}_2\text{CH}_2\text{CH}_2\text{OCH}_3$), 3.81-3.89 (m, 4H, $2 \times \text{SCH}_2\text{CH}_2$), 4.25-4.29 (bs, 2H, $2 \times \text{OCH}_2\text{CHOHCH}_2\text{N}$).

pDAB was prepared according to the procedure described above, followed by deprotection of the Boc-group under HCl gas purging. **pDAB-Boc** (150 mg, 0.325 mmol Boc groups) was dissolved in methanol (50.0 mL) and HCl gas was passed through the solution during 30 min. After concentration under reduced pressure, the remaining solution was diluted with water (15.0 mL), basified to pH 4 with NaOH solution (1.0 M), purified by dialysis (MWCO 1 kDa), and finally lyophilized to obtain the **pDAB** polymer as an amorphous solid (0.070 mg, 70.5% yield): ^1H NMR (D_2O , 400 MHz): 1.74-1.80 (m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_2$), 1.86-1.88 (m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_2$), 2.96-2.99 (t, 4H, $2 \times \text{SCH}_2\text{CH}_2$), 3.05-3.09 (t,

2H, NCH₂CH₂CH₂CH₂NH₂), 3.38-3.45 (m, 6H, 2 × NCH₂CHOHCH₂ + NCH₂CH₂CH₂CH₂NH₂), 3.60-3.65 (m, 6H, 2 × OCH₂CHOHCH₂N), 3.82-3.91 (m, 4H, 2 × SCH₂CH₂), 4.26-4.28 (bs, 2H, 2 × OCH₂CHOHCH₂N).

Polyplex preparation

Polyplexes were prepared in water, and characterized with a Zetasizer Nano ZS (Malvern Instruments, Malvern) at 25 °C to determine their hydrodynamic sizes and surface charges. The results are presented as averages of triplicate measurements with standard deviations as error bars. PAE/DNA polyplexes at a mass ratio of 24 were prepared by adding polymer solutions (450 µg/mL, 800 µL) into DNA solutions (75 µg/mL, 200 µL), followed by 5 s vortexing and 30 min incubation at room temperature.

Agarose gel retardation

Polyplexes were made at different polymer/DNA mass ratio 24 according to the previous procedure. Next, water buffer (with and without DTT) was added into polyplex solutions to give the final DTT concentration of 2.5 mM. After 30 min ambient incubation, 6X loading buffer containing bromophenol (Ferments) was mixed with resultant dispersions. Aliquots (10 µL) of the resultant dispersions were loaded on a 0.8% w/v agarose gel containing 1X SYBR® Safe DNA Gel Stain (Invitrogen™). Electrophoresis was performed at 90 V for 60 min in a TBE (Tris-borate-EDTA, 1X) running buffer. After development, the gel was photographed using a FluorChem M Imager (Proteinsimple, Westburg, Leusden, the Netherlands) under UV excitation.

Buffer capacity titration

The buffering capacity of the polymers was determined by acid-base titration. PAE polymer (5 mmol of protonable amine groups) was dissolved in NaCl aqueous solution (150 mM, 10 mL). The pH of the polymer solution was lowered to ≤ 2.0 and the solution was titrated with NaOH solution (0.1 M) using an automatic titrator (Metrohm 702 SM Tirino). As a reference, PEI (25 kDa) and NaCl aqueous solution were titrated following the same method. The percentage of amine groups protonated from pH 5.1 to 7.4 was defined as the buffer capacity that can be calculated from following equation:⁶⁶

$$\text{Buffer capacity (\%)} = \frac{\Delta V_{\text{NaOH}} \times 0.1 \text{ M}}{N \text{ mol}} \times 100\%$$

wherein, ΔV_{NaOH} denotes NaOH volume required to bring the pH value of the polymer solution from 5.1 to 7.4, and N mol (5 mmol) denotes the total number of protonable amine groups in the PAE polymer.

***In Vitro* Transfection and Cell Viability Assays**

Transfection experiments were performed on COS-7 cells (SV-40 transformed African green monkey kidney cells) using the plasmid pCMV-GFP as a reporter gene. Transfections and cell viabilities were conducted in parallel using polyplexes formed at polymer/plasmid DNA mass ratios of 6 and 12. Gene expression experiments and cell viability evaluations were carried out in separate 96-well plates. Cells were seeded in at a density of 10^4 cells per well (2.94×10^4 cells/cm²) and maintained in complete medium at 37 °C in a humidified atmosphere containing 5% CO₂ until 60% - 80% confluency. In a standard transfection experiment, cells were first incubated with medium (100 μ L, with/without serum) for 30 min and then loaded with 100 μ L polyplex dispersions (0.25 μ g DNA per well). After 1 h incubation, polyplexes were removed, and fresh complete culture medium (100 μ L) was added and cells were cultured for an additional 48 h. Transfection efficiency was determined qualitatively through fluorescence imaging via an EVOS digital inverted microscope (EMS, Wageningen, the Netherlands). A reference was prepared from PEI (25 K)/DNA at a polymer/DNA mass ratio of 1.25/1. MTT assays were conducted to determine the number of viable cells, with a value of 100% cell viability for untreated cells.

Quantitative determination of transfection efficiency was performed by flow cytometry. Cells were seeded in 48-well plates with densities of 1.6×10^4 cells per well (1.6×10^4 cells/cm²) and maintained in complete medium at 37 °C in a humidified atmosphere containing 5% CO₂ until 60% - 80% confluency. Cells were first incubated with medium (200 μ L, with/without serum) for 30 min and then loaded with 200 μ L polyplex dispersions (0.5 μ g DNA per well). After 1 h incubation, the polyplexes were aspirated, fresh and warm complete culture medium (200 μ L) was added and cells were cultured for an additional 48 h. The cells were fixated by adding trypsin solution (0.25%, 150 μ L), and centrifuged (600 g, 5 min, ambient). Resulting pellets were re-suspended in HBSS buffer (200 μ L), and measured by the FACSCalibur (Becton-Dickinson, Breda, the Netherlands) at an excitation wavelength of 488 nm and an emission wavelength of 530 nm. Data was processed with the FACS Cellquest Software. A PEI (25 K)/DNA formulation prepared at a polymer/DNA mass ratio of 1.25/1 was used as a reference. MTT assays were conducted to characterize the

number of viable cells with 100% cell viability for untreated cells (i.e., cells not exposed to transfection systems).

RESULTS AND DISCUSSION

Polymer synthesis and characterization

Disulfide containing poly(amino ether)s (PAEs) were synthesized by ring-opening polymerization of 2-hydroxyethyl disulfide diglycidyl ether (SS-DE) with various amines with different pendant groups as depicted in Scheme 2. During polymerization, the mixtures become viscous, although without occurrence of gelation, indicating the formation of polymers. As shown in Table 1, most polymers are obtained in yields over 40%. The polymer structures were validated through NMR spectra, showing no residual epoxy rings in the polymers, which could be toxic in biological systems. The broad single peak located around 4.26 ppm was assigned to the proton adjacent to the secondary alcohol that was derived from the ring-opening of epoxy group, which confirms the occurrence of the epoxy-amine reaction. Molecular weight and polydispersity index of the PAEs were determined by size exclusion chromatography (Table 1) and were found to be mainly from 4.5 to 9.0 kDa, with relatively narrow size distributions, which originates from the dialysis procedure before isolation of the polymers.

Scheme 2. Synthetic route of poly(amino ether)s by the epoxy-amine reaction of 2-hydroxyethyl disulfide diglycidyl ether (SS-DE) with various amines.

The primary amines with an additional tertiary amine in the chain (**MPA** and **PPA**) were employed to introduce additional cationic charge sites to increase the DNA binding capability in these polymers. From Table 1, the amine reactivity is reduced when tertiary

amine present, leading to low molecular weight (ca. 5 kDa) compared to control groups prepared from amine without tertiary amine (7.1-9.6 kDa). The polymer **pPPA** has a molecular weight as low as 1.4 kDa, most likely due to the pyrrolidine group. These results indicate that the side groups have significant effect on the polymerization kinetics as revealed in poly(amino ester)s and poly(amino amine)s.

Table 1. Characteristics of bio reducible poly(amino ether)s prepared from different amines.

Poly(amino ether)s	Yield ^a (%)	M _n (kDa)	^b PDI ^b	Degree of polymerization (X _n)	of Buffer ^b Capacity ^c (%)
pAEOL	71	9.6	1.72	29.4	75.8
pAPrOL	53	5.2	1.48	15.1	33.8
pABOL	44	9.4	1.68	26.3	27.2
pAPOL	65	7.1	1.54	19.1	19.8
pAHXL	39	7.6	1.48	19.9	59.3
pMOPA	49	5.3	1.98	14.8	59.3
pPPA	14	1.4	1.71	3.5	47.3
pMPA	93	5.4	2.20	13.0	22.3
pDAB-Boc	66	5.6	1.93	12.7	n.d.
pDAB	50 ^d	- ^e	- ^e	12.7	27.1

^a Determined by obtained polymer and feed monomer masses; ^b Determined by GPC (DMF as eluent); ^c Determined by titration and calculated from the pH range between 5.1 and 7.4; ^d Yield after polymerization and deprotection of Boc; ^e Not measured due to limited solubility.

The polymer-DNA complexes normally follow the cellular pathways of endocytosis, and successively enter endosomal/lysosomal compartments where encapsulated DNA may degrade. It is assumed that complexes with high buffer capacity are able to escape the endosome through the so-called “proton sponge” effect.^{20,67} Buffer capacities of the PAEs were determined in the pH range 5.1-7.4 by titration of the polymers from acid solution with strong 0.1 M NaOH and listed in Table 1. The titration curves of PAEs bear relatively gentle slopes in this pH range (Figure 1), demonstrating their high buffer capacities of up to 76%, as compared to branched 25 kDa PEI (ca. 18%). Remarkably, **pAHXL** has nearly 3-fold higher buffer capacity (59.3%) than **pAPOL** (19.8%) which differs only in a single

additional methylene group ($-\text{CH}_2-$) in the pendant group, and the two have similar degrees of polymerization.

Figure 1. Titration curves of poly(amino ether)s. Titration curves of NaCl and b-PEI are added for comparison.

DNA condensation and release

The ability of bioreducible PAEs to condense DNA was evaluated by measuring size and surface charge density of polymer-DNA complexes (polyplexes) at various polymer/DNA mass ratios. The polymer/DNA mass ratios studied are 3, 6, 12, and 24. Physicochemical properties that favor polyplex transfection capability are generally small hydrodynamic size (below 200 nm) and sufficient cationic surface charge density (approximately +30 mV). The hydrodynamic diameters of the formed polyplexes are presented in Figure 2. All polyplexes display a hydrodynamic diameter below 100 nm and positive surface charge ($> +20$ mV), confirming excellent DNA condensation of the PAEs. The extent of DNA condensation increases upon enhancing polymer/DNA mass ratio, owing to increase in available charge density, which is in line with the fact that the dominant driving forces of polyplex formation are electrostatic interactions. The smallest polyplex sizes were observed for **pPPA**, **pMPA**, and **pDAB**, which all contain protonable side groups, and thus carry higher charge density as compared to other PAEs. It is reported that also hydrophobicity of pendant side groups plays an important role in the condensation ability of a polymer.⁶⁸ However, this is not apparent in **pAEOL**, **pAPrOL**, **pABOL**, **pAPOL**, and **pAHXL**. These five polymers have increasing hydrophobicity, but form polyplexes of similar size and surface charge at equal polymer/DNA mass ratio. This might be due to the relatively high extent of protonation of the amines in water, which causes that electrostatic interactions dominate the complexation.

Figure 2. Characterization of polyplexes prepared by bioreducible PAEs at various polymer/DNA mass ratios through DLS measurement: A) hydrodynamic size, and B) surface charge of polyplexes.

Formation of stable polyplexes is an important aspect to achieve efficient gene delivery. However, inefficient DNA unpacking from polyplexes may also present a major barrier. Therefore, DNA should be released from vectors before successful transfection can occur. To determine both polyplex stability and capability for DNA unpacking from the polyplexes under reducing conditions, gel electrophoresis experiments were performed. It is envisioned that under the intracellular reducing conditions disulfide cleavage in polymers containing disulfide linkers in the main chain dissociates polyplexes and thus releases DNA. These conditions were mimicked by exposing polyplexes to the mild reducing agent DTT (2.5 mM). Therefore, gel electrophoresis was carried out on the polyplexes in the absence and presence of DTT (See Figure 3). Negatively charged DNA was retarded by all PAEs at the highest polymer/DNA mass ratio 24, showing the effective encapsulation of DNA. However, after treatment with DTT, DNA migrates at a similar rate as naked DNA, indicating that polymer cleavage and polyplex disassembly releases DNA. DNA migration is observed from

polyplexes with the disulfide-present PAEs, underlining that polymer degradation is important for DNA unpacking inside the reductive cytosol. Also, dynamic light scattering showed that disulfide-containing polyplexes instantly increase their nanoscale size to micrometers (data not shown).

Figure 3. Agarose gel electrophoresis of polyplexes (w/w=24) treated without DTT (left) and with DTT (right).

***In vitro* transfection and cytotoxicity**

The cytotoxicity of the polyplexes was investigated on COS-7 cells by MTT assays. The optimal polymer/DNA mass ratio was set to 12 to keep polymer toxicity effect minimum as reported in Chapter 6. Cell viabilities of COS-7 cells exposed to polyplexes under serum-free and serum-present conditions (10% FBS) are presented in Figure 4. Cells maintained excellent viabilities under serum-free and 10% serum conditions upon exposure to the PAEs-based polyplexes. The **pDAB**-based polyplexes form an exception, leading to markedly lower cell viabilities (56% and 79% at 0% and 10% FBS respectively). The observed cytotoxicity of **pDAB**-based polyplexes can be attributed to the additional positive charges of the pendant primary amines, in line with previous observations for poly(amido amine)s and PEI.⁶⁹ The high cell viability in cells treated with bio reducible PAEs (except **pDAB**) demonstrates these PAEs are essentially non-toxic.

Figure 4. Cell viabilities of COS-7 treated with polyplexes at polymer/DNA mass ratio 12 via MTT assay under various serum conditions with PEI as references.

After evaluation of the biocompatibility of the reducible PAEs, their transfection performance was studied *in vitro* with the plasmid pCMV-GFP as a reporter gene leading to expression of GFP. The fluorescence images of COS-7 cells were employed to screen the PAE polymers using PEI as a reference (Figure 5). Based on the areas of green spots where the cells or cell clusters express GFP, the transfection efficiency is estimated in the order: **pABOL** > **pMPA** > **pAPOL**, while little or no transfection occurs in **pAHXL**, **pAPrOL**, **pAEOL**, **pMOPA**, **pPPA**, **pDAB**, and PEI. Moreover, considerable transfection activity is observed for polyplexes based on **pABOL**, **pMPA**, and **pAPOL** in the presence of serum (10%). The results coincide with previous studies associated with polymer structural variation on transfection performance of cationic polymers.⁴⁹ **pABOL**, **pAPOL**, and **pMPA** were further studied under various serum concentrations.

Figure 5. The inverted fluorescence images of COS-7 cells transfected with polyplexes from poly(amino ether)s and PEI at polymer/DNA mass ratio 12 under serum-free conditions (upper part) and serum conditions (lower part) with PEI 1.25/1 as a reference. (Scale bars = 1000 μ m).

FACS was carried out to quantitatively determine transfection efficiencies of the three polyplexes from **pABOL**, **pAPOL**, and **pMPA** under three different serum conditions (Figure 6). Transfection efficiencies are presented as percentage of GFP-expressing cells of the total cell population. Under serum-free conditions, transfection efficiencies of polyplexes based on **pABOL**, **pAPOL**, and **pMPA** at polymer/DNA mass ratio 12 are respectively 85%, 63%, and 20% respectively, as compared to polyplexes of PEI displaying only 5% transfection at this mass ratio. Generally, proteins in the serum lead to the aggregation of polyplexes, and thus significantly reduce transfection efficiency. However, in the presence of 10% serum, polyplexes based on PAEs gave transfection efficiencies which were only slightly reduced, with 80% for **pABOL**, 54% for **pAPOL** and 9% for **pMPA**, while PEI-based polyplexes did not display any significant transfection under its optimal conditions and only marginal transfection (4.0%) at the higher polymer/DNA mass ratio of 12. The reason for the potent transfection of PAEs under these 10% serum-conditions is probably the presence of abundant hydroxyl groups along the polymer chains which serve as a shielding layer to prevent interactions between serum proteins and the polyplexes.^{64,70}

Figure 6. Transfection efficiency of polyplexes prepared of bioreducible PAEs through FACS at polymer/DNA mass ratio 12 with PEI (25 kDa) as reference: A) transfection efficiency mediated by **pABOL**, **pAPOL**, and **pMPA**; B) transfection efficiency of **pABOL** at various FBS concentrations.

To further explore the effect of serum, the serum protein concentration was further elevated to mimic physiological serum conditions. Even under these conditions, transfection mediated by **pABOL**-based polyplexes remained relatively high at 60% and 30% at serum concentrations of 25% and 50% respectively (Figure 6B) This further demonstrates the notable potential of this polymer for *in vivo* application.

CONCLUSIONS

A unique and novel class of bioreducible poly(amino ether)s has been prepared by epoxy-amine reaction between disulfide-containing diglycidyl ether with a variety of primary amines, enabling large structural variation of these polymers. The resulting PAEs have much higher buffer capacity than the reference polymer PEI. PAEs condense DNA into nanosized particles of 70-90 nm with positive surface charges above +20 mV. Polyplexes of these disulfide-containing PAEs will release DNA upon exposure to reducing conditions. All PAEs show minimal- or non-toxicity to COS-7 cells, except the primary amine containing **pDAB**. From the PAEs that were evaluated for their transfection capabilities, **pABOL**, **pAPOL**, and **pMPA** display the most promising transfection efficiencies. The presence of serum was demonstrated to have only a marginal effect on transfection of polyplexes from these PAEs, while significantly reduced transfection efficiency is observed for PEI-based polyplexes. These results demonstrate that bioreducible PAEs are appealing candidates as efficient and safe gene vectors with vast translational potential for *in vivo* application.

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Chapter 8. Modular Synthesis of Bio reducible Gene Vectors through Polyaddition of *N,N'*-Dimethylcystamine to Various Diglycidyl Ethers

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KEYWORDS

Cationic polymer; epoxy-amine reaction; bio reducible; gene delivery; disulfides.

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ABSTRACT

Bioreducible linear poly(amino ether)s (PAEs) are evaluated as promising gene vectors. PAEs were synthesized by reaction of disulfide-functional monomer, *N,N'*-dimethylcystamine (DMC), with different diglycidyl ethers with molecular variations. The resulting PAEs display substantial buffer capacity (up to 64%) in the endosomal acidification region pH 7.4-5.1. PAEs condense plasmid DNA with GFP as reporter gene into 80–200 nm sized polyplexes with positive surface charges ranging from +20 to +40 mV. The polyplexes readily release DNA after exposure to reducing conditions (2.5 mM DTT) as demonstrated by agarose gel electrophoresis, due to cleavage of the disulfide groups present in the main chain of the polymers. Upon exposing COS-7 cells to polyplexes prepared at polymer/DNA ratios below 48, cell viabilities between 80%-100% were observed under serum-free conditions. These polyplexes show comparable or higher transfection efficiencies (ranging from 10%-38%), as compared to PEI (12%) under serum-free conditions. Moreover, the PAE-based polyplexes yield transfection efficiencies as high as 32% and 28% under 10% and 25% serum conditions respectively.

INTRODUCTION

Non-viral gene vectors are extensively sought after to deliver DNA as therapeutics to cure human diseases.¹⁻⁶ Non-viral gene vectors should be merited with high transfection efficiency, minimal or non-toxicity, as well as ease of manufacturing.⁷⁻⁹ Cationic polymers are interesting candidates to be developed to acquire the required properties for efficient gene delivery, using modern developments in polymer chemistry¹⁰⁻¹³ as well as nanotechnology.¹⁴⁻¹⁶ Cationic polymers can form polyplexes with DNA tightly packed inside by means of electrostatic interactions.¹⁷⁻²⁰ After entering the cells through endocytosis,²¹ the polyplexes must escape from the endosome-lysosome pathway and unpack the DNA in nucleus in order to initiate gene expression.²²⁻²⁴

An important step before the initiation of gene expression is the unpacking of DNA from the polyplex. Many cues were proposed to facilitate DNA unpacking,^{25,26} such as the use of acid-labile cationic polymers,^{13,27,28} ester-based cationic polymers^{29,30} and biologically triggered cationic polymers.³¹⁻³⁴ Among these approaches, the latter approach is arguably the most interesting, since it takes advantage of intracellular enzymes or related biomolecules.^{26,35} Glutathione, enzyme-related antioxidant dictating the reductive potential of cells, is more concentrated inside the cells than outside cells, thereby increasing the reductive potential

inside cells.³⁶⁻³⁸ By introduction of disulfide-linkages in cationic polymers,^{39,40} this higher reductive potential has been utilized as a trigger to cleave the disulfide bonds and unpack DNA from their polyplexes after successful endosomal escape of the polyplexes into the cytosol. Introduction of disulfide moieties were successfully employed in polyethylenimine (PEI),⁴¹⁻⁴⁵ poly(L-lysine) (PLL),^{46,47} peptides,^{48,49} and poly(amidoamine) dendrimers⁵⁰ resulting in increased gene transfection and improved biocompatibility. Our group, as well as other groups, reported on disulfide-linked linear^{51,52} and branched⁵³ poly(amido amine)s displaying superb transfection efficiencies and excellent biocompatibility.⁵⁴ Green and coworkers reported cationic poly(amino ester)s with disulfides along the backbone for efficient gene delivery.⁵⁵ However, clinical application of these polymers has so far been restrained due to their impaired transfection in the presence of serum.^{16,56} The introduction of hydrophilic poly(ethylene glycol) onto these vectors was found to increase the serum tolerance of these polymers only to a limited extent, failing to meet the requirements for clinical application.^{16,56,57} Recently, we developed a new class of disulfide-based poly(amino ether)s (PAEs) by epoxy-amine addition reaction of 2-hydroxyethyl disulfide diglycidyl ether with a variety of primary amines, yielding a series of PAEs with different side groups along the polymer chain (Chapter 7 in this thesis). The resulting polymers showed to be effective non-viral gene delivery vectors, with high biocompatibility and improved serum-tolerance.

Scheme 1. Schematic illustration of modular synthetic approaches to prepare disulfide-linked polycations through polyaddition reaction of *N,N'*-dimethylcystamine (DMC) to various diglycidyl ethers.

In this chapter, we report a complementary modular synthetic approach to generate disulfide-linked PAEs through polyaddition of *N,N'*-dimethylcystamine (DMC) as the fixed bifunctional diamine monomer and a series of diglycidyl ethers as the variable epoxy monomer. This has yielded bioreducible PAEs with different structural moieties in the main chain of the polymers. Transfection efficiencies and cell viabilities of these polymers were investigated on COS-7 cells through flow cytometry and MTT assay.

EXPERIMENTAL PROCEDURES

Materials and Methods

Epichlorohydrin ($\geq 98\%$, Sigma-Aldrich), 2-hydroxyethyl disulfide (technical grade, Sigma-Aldrich), tetrabutylammonium bromide (TBAB, $\geq 98\%$, Sigma-Aldrich), sodium hydroxide (NaOH, pellets, Sigma-Aldrich), 1,3-benzenedimethanol (98%, Sigma-Aldrich), *N,N'*-dimethylethylenediamine ($>97.0\%$, TCI Europe N.V.), 1,4-butanediol diglycidyl ether ($\geq 95\%$, Sigma-Aldrich), dithiothreitol (DTT, Sigma-Aldrich), triethylamine (TEA, $\geq 99\%$, Sigma-Aldrich), borane dimethyl sulfide complex (BMS, Sigma-Aldrich), *N,N'*-Bis(2-hydroxyethyl) ethylenediamine ($>95.0\%$, TCI Europe N.V.), di-*tert*-butyl dicarbonate ($>95.0\%$, TCI Europe N.V.), and thiazolidine (98%, ACROS Organics) were used directly as received without further purification. Diglycidyl ethers, 1,4-cyclohexanedimethanol

diglycidyl ether (**3**, Sigma-Aldrich) and neopentyl glycol diglycidyl ether (**4**, Sigma-Aldrich) were used as received. All solvents used were of analytical grade. Water used in these experiments was treated through a Milli-Q Gradient System (Millipore, Bedford, MA). Plasmid DNA, plasmids pCMV-GFP and pCMV-ΔGFP were purchased from Plasmid Factory (Bielefeld, Germany). Branched polyethylenimine (PEI, M_w 25 kDa) was purchased from Sigma-Aldrich.

^1H NMR and ^{13}C NMR were recorded on a Bruker NMR spectrometer (400 MHz) in deuterated solvents. Molecular weights were determined by gel permeation chromatography (GPC, Waters Alliance 2695) relative to PEG standards, using a Mixed-M column (PL-aquagel-OH 8 micron, 300 x 7.5 mm) with *N,N*-dimethylformamide (DMF) containing LiCl (50 mM) as mobile phase.

Synthesis of *N,N'*-dimethylcystamine (DMC). The monomer was synthesized in its HCl salt form based on our previous report.⁵⁸ Into a solution of thiazolidine (5.0 g, 56 mmol) in anhydrous tetrahydrofuran (THF, 20 mL) under nitrogen atmosphere, borane dimethyl sulfide complex (BMS, 10.61 g, 140 mmol) in anhydrous THF (20 mL) was slowly added under stirring and the mixture was subsequently refluxed overnight. After cooling down to ambient temperature, methanol (20 mL) was added by syringe to neutralize any residual BMS and the mixture was refluxed for an additional 3 h. After cooling down to ambient temperature, the solution was purged with HCl gas, and the mixture was concentrated under reduced pressure. The resulting liquid was consecutively dissolved in methanol (5.0 mL) and precipitated in diethyl ether (2 × 50 mL) to obtain a white oily precipitate. The precipitate was dissolved in water (7.5 mL), and titrated with saturated I_2/KI solution until obtaining a persistent yellow color. After stirring for an additional 2 h, the yellow solution turned colorless upon raising pH to 10. The solution was extracted with chloroform (4 × 30 mL). Afterwards the organic fractions were combined, dried over MgSO_4 , and concentrated under reduced pressure to yield a clear liquid. Subsequently, the liquid was dissolved in isopropanol (20 mL) and purged with HCl gas, yielding a turbid yellow mixture and a white precipitate. The precipitate was filtered off, washed with cold diethyl ether, and finally recrystallized from isopropanol/methanol to obtain the HCl salt as a white solid. (2.12 g, 30.0% yield). ^1H NMR (D_2O , 400 MHz): δ_{H} 2.76 (s, 6H, CH_2NHCH_3), 3.05 (t, 4H, SCH_2CH_2), 3.44 (t, 4H, SCH_2CH_2). ^{13}C NMR (D_2O , 400 MHz): δ_{C} 31.92, 32.83, 47.02.

Synthesis of *N,N'*-bis(2-hydroxyethyl)-*N,N'*-bis(*tert*-butoxycarbonyl) ethylenediamine.

To a mixture of *N,N'*-bis(2-hydroxyethyl) ethylenediamine (6.00 g, 40.0 mmol) and triethylamine (14.0 mL, 10.2 g, 100 mmol) in dichloromethane (100 mL), di-*tert*-butyl dicarbonate (17.6 g, 40 mmol) in dichloromethane (200 mL) was charged dropwise under stirring at 0 °C. The mixture was stirred at ambient temperature overnight. Solvent was removed under reduced pressure. The resulting solid was washed with ethyl acetate and diethyl ether and subsequently dried under vacuum, to yield a white solid (11.2 g, 80.4% yield). ¹H NMR (CDCl₃, 400 MHz): δ_H 1.45 (s, 18H), 3.25-3.60 (m, 8H), 3.71-3.82 (m, 4H).

Synthesis of diglycidyl ethers. Diglycidyl ether monomers were synthesized via reaction of epichlorohydrin with the corresponding diols. Typical example of the Boc-protected form of **1 (Boc-1)** was: into a mixture of *N,N'*-bis(2-hydroxyethyl)-*N,N'*-bis(*tert*-butoxycarbonyl) ethylenediamine (1.75 g, 5.00 mmol), sodium hydroxide (0.800 g, 20.0 mmol) and tetrabutylammonium bromide (12.37 mg, 34.8 μmol), epichlorohydrin (1.45 mL, 18.4 mmol) was added gradually. The mixture was heated to 40 °C and stirred for 3 h. The resulting mixture was subjected to column chromatography to obtain diglycidyl ether of **Boc-1** (1.95 g, 84.7 % yield). ¹H NMR (CDCl₃, 400 MHz): δ_H 1.46 (s, 18H), 2.57 (s, 2H), 2.76 (t, 2H), 3.10 (m, 2H), 3.36 (m, 10H), 3.60 (m, 4H), 3.72 (m, 2H). ¹³C NMR (CDCl₃, 400 MHz): δ 28.43, 44.12, 46.13, 47.34, 50.75, 69.89, 71.76, 79.76, 155.41.

Diglycidyl ether of **5** (1.12 g, 89.5 % yield). ¹H NMR (CDCl₃, 400 MHz): δ_H 2.62 (m, 2H, epoxide ring OCHHCHO), 2.81 (m, 2H, epoxide ring OCHHCHO), 3.20 (m, 2H, epoxide ring OCHHCHO), 3.45 (m, 2H, CHCHHO), 3.78 (m, 2H, CHCHHO), 4.59 (m, 4H, OCH₂-phenyl ring), 7.27-7.36 (m, 4H, phenyl ring). ¹³C NMR (CDCl₃, 400 MHz): δ_C 44.36, 50.93, 71.01, 73.27, 127.13, 128.64, 138.22.

Synthesis of poly(amino ether)s. Poly(amino ether)s (PAEs) were synthesized by ring open polymerization of bifunctional epoxide monomers with equimolar amounts of secondary amines. A typical procedure is given for the synthesis of **DMC1**:

Into a glass vial, diglycidyl ether **Boc-1** (0.461 g, 1.00 mmol), **DMC** (0.254 g, 1.00 mmol), triethylamine (0.400 mL, 2.87 mmol) and ethanol (0.500 mL) were charged. After 3 d stirring under ambient temperature, the resulting viscous mixture was diluted with methanol (50.0 mL), and purged with HCl gas for 30 min in order to remove the Boc protecting groups. The mixture was concentrated and the residue was dissolved in water (15.0 mL), acidified

with 4 M HCl solution to pH 4, and purified via dialysis (MWCO 1 kDa) against water overnight. Polymer **DMC1** was obtained after lyophilization as an amorphous solid. The other PAE polymers **DMC2-6** were synthesized analogously, however these polymers do not require the Boc removal step.

Polymer **DMC1** (off-white solid, 0.162 g, 31.6%): ^1H NMR (D_2O , 400 MHz): δ_{H} 2.98 (s, 6H, NCH_3), 3.10-3.16 (m, 4H, $\text{CH}_2\text{NHCH}_2\text{CH}_2\text{O}$), 3.26 (m, 4H, $\text{NCH}_2\text{CH}_2\text{S}$), 3.32-3.38 (b, 8H, $\text{CH}_2\text{NHCH}_2\text{CH}_2\text{O} + \text{OCH}_2\text{CHOHCH}_2\text{NCH}_2\text{CH}_2\text{S}$), 3.35-3.64 (m, 8H, CH_2OCH_2), 3.79 (t, 4H, $\text{OCH}_2\text{CHOHCH}_2\text{NCH}_2\text{CH}_2\text{S}$), 4.30 (bs, 2H, $\text{OCH}_2\text{CHOHCH}_2\text{NCH}_2\text{CH}_2\text{S}$).

Polymer **DMC2** (white solid, 0.132 g, 33.2%): ^1H NMR (D_2O , 400 MHz): δ_{H} 2.95 (s, 6H, NCH_3), 2.96 (m, 4H, $\text{OCH}_2\text{CH}_2\text{S}$), 3.09-3.14 (m, 4H, $\text{NCH}_2\text{CH}_2\text{S}$), 3.30 (m, 4H, $\text{CH}_2\text{OCH}_2\text{CHOHCH}_2\text{NCH}_2\text{CH}_2\text{S}$), 3.55-3.65 (m, 8H, $\text{CH}_2\text{OCH}_2\text{CHOHCH}_2\text{NCH}_2\text{CH}_2\text{S}$), 3.80-3.89 (m, 4H, $\text{OCH}_2\text{CH}_2\text{S}$), 4.25 (bs, 2H, $\text{CH}_2\text{OCH}_2\text{CHOHCH}_2\text{NCH}_2\text{CH}_2\text{S}$).

Polymer of **DMC3** (white solid, 0.086 g, 16.9% yield): ^1H NMR (D_2O , 400 MHz): δ_{H} 0.90-1.01 and 1.75-1.79 (m, 8H, $4 \times \text{CH}_2$ of cyclohexane ring), 1.29 and 1.49 (m, 2H, CH of cyclohexane ring), 2.98 (s, 6H, NCH_3), 3.11-3.18 (m, 4H, $\text{NCH}_2\text{CH}_2\text{S}$), 3.20-3.31 (m, 4H, $\text{CH}_2\text{OCH}_2\text{CHOHCH}_2\text{NCH}_2\text{CH}_2\text{S}$), 3.37-3.47 (m, 4H, $\text{CH}_2\text{OCH}_2\text{CHOHCH}_2\text{NCH}_2\text{CH}_2\text{S}$), 3.51-3.67 (m, 8H, $\text{CH}_2\text{OCH}_2\text{CHOHCH}_2\text{NCH}_2\text{CH}_2\text{S}$), 4.25 (bs, 2H, $\text{CH}_2\text{OCH}_2\text{CHOHCH}_2\text{NCH}_2\text{CH}_2\text{S}$).

Polymer **DMC4** (amorphous solid, 0.064 g, 13.9% yield): ^1H NMR (D_2O , 400 MHz): δ_{H} 0.91 (s, 6H, $\text{C}(\text{CH}_2\text{CH}_3)_2$), 2.92 (s, 6H, NCH_3), 3.08-3.12 (m, 4H, $\text{NCH}_2\text{CH}_2\text{S}$), 3.26-3.32 (m, 8H, $\text{C}(\text{CH}_2\text{CH}_3)_2 + \text{CH}_2\text{OCH}_2\text{CHOHCH}_2\text{NCH}_2\text{CH}_2\text{S}$), 3.66-3.75 (m, 8H, $\text{CH}_2\text{OCH}_2\text{CHOHCH}_2\text{NCH}_2\text{CH}_2\text{S}$), 4.25 (bs, 2H, $\text{CH}_2\text{OCH}_2\text{CHOHCH}_2\text{NCH}_2\text{CH}_2\text{S}$).

Polymer of **DMC5** (white solid, 0.220g, 43.7%): ^1H NMR (D_2O , 400 MHz): δ_{H} 2.96 (s, 6H, NCH_3), 3.09-3.12 (m, 4H, $\text{NCH}_2\text{CH}_2\text{S}$), 3.24-3.30 (m, 4H, phenyl ring- $\text{CH}_2\text{OCH}_2\text{CHOHCH}_2\text{NCH}_2\text{CH}_2\text{S}$), 3.57-3.64 (m, 8H, phenyl ring- $\text{CH}_2\text{OCH}_2\text{CHOHCH}_2\text{NCH}_2\text{CH}_2\text{S}$), 4.26 (bs, 2H, phenyl ring- $\text{CH}_2\text{OCH}_2\text{CHOHCH}_2\text{NCH}_2\text{CH}_2\text{S}$), 4.62-4.65 (m, 4H, phenyl ring- $\text{CH}_2\text{OCH}_2\text{CHOHCH}_2\text{NCH}_2\text{CH}_2\text{S}$), 7.40-7.45 (m, 4H, phenyl ring).

Polymer of **DMC6** (amorphous solid, 0.183 g, 40.1% yield): ^1H NMR (D_2O , 400 MHz): δ_{H} 1.63 (bs, 4H, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{O}$), 2.96 (s, 6H, NCH_3), 3.04-3.13 (m, 4H, $\text{NCH}_2\text{CH}_2\text{S}$),

3.23-3.30 (m, 4H, $\text{CH}_2\text{OCH}_2\text{CHOHCH}_2\text{NCH}_2\text{CH}_2\text{S}$), 3.52-3.62 (m, 12H, $\text{CH}_2\text{OCH}_2\text{CHOHCH}_2\text{NCH}_2\text{CH}_2\text{S}$), 4.25 (bs, 2H, $\text{CH}_2\text{OCH}_2\text{CHOHCH}_2\text{N}$).

Polyplex preparation

Polyplexes were prepared by adding polymer solution into DNA solution at designated polymer/DNA mass ratios. All polymer and DNA solutions were prepared in HEPES buffer (20 mM, pH 7.4). The procedure of polyplexes prepared at mass ratio 48 is as follows: In an Eppendorf tube (1.5 mL), DNA solution (75 $\mu\text{g/mL}$, 200 μL) and polymer solution (900 $\mu\text{g/mL}$, 800 μL) was successively added. The mixture was subjected to vortexing for 5 s and incubated at room temperature for 30 min to obtain the polyplex suspensions. Size and surface charge of the polyplexes were measured on a Zetasizer Nano ZS (Malvern Instruments, Malvern) at 25 °C. The data are presented as mean of three measurements with corresponding standard deviations as error bars.

Agarose gel electrophoresis

Polyplexes were prepared at polymer/DNA mass ratio 48 as described in the previous protocol. Polyplex solutions (90 μL) were diluted with either DTT HEPES solution (25 mM, 10 μL) or HEPES buffer (10 μL), and incubated under ambient conditions for 30 min. The resulting dispersions (20 μL) were mixed with 6X loading buffer containing bromophenol (Ferments, 5.0 μL), and aliquoted (10 μL) to load on agarose gel (0.8% w/v) containing 1X SYBR[®] Safe DNA Gel Stain (Invitrogen[™]). The gel was developed at 90 V for 60 min in a TBE (Tris-borate-EDTA, 1X) running buffer and consecutively imaged via FluorChem (Proteinsimple, Westburg, Leusden, the Netherlands) under UV excitation.

Buffer capacity titration

Buffering capacities of the polymers were determined via acid-base titration. The PAE polymer in amount equal to 5 mmol protonable amine groups was dissolved in NaCl aqueous solution (150 mM, 10 mL). The pH of the polymer solution was acidified to ≤ 2.0 and the solution was titrated with NaOH solution (0.1 M) using an automatic titrator (Metrohm 702 SM Tirino). As a reference, b-PEI (25 kDa) and NaCl aqueous solution were titrated following the same method. The percentage of amine groups protonated from pH 5.1 to 7.4 was defined as the buffer capacity that can be calculated from following equation:⁵⁹

$$\text{Buffer capacity (\%)} = \frac{\Delta V_{\text{NaOH}} \times 0.1 \text{ M}}{N \text{ mol}} \times 100\%$$

wherein, ΔV_{NaOH} denotes NaOH volume required to bring the pH value of the polymer solution from 5.1 to 7.4, and N mole (5 mmol) denotes the total moles of protonable amine groups in PAE polymer.

Cytotoxicity Assays

Cytotoxicity of polyplexes towards COS-7 cells was evaluated through MTT assays. In 96 well plates, ca. 10^4 cells were seeded per well and allowed to grow overnight in complete medium (100 μL , DMEM medium supplemented with 10% FBS) at 37 °C under 5% CO_2 conditions to reach 60-80% confluency. The medium was replaced with fresh medium (100 μL , w/o 10% FBS). After 30 min incubation, cells were loaded with either polyplex solutions at various polymer/DNA mass ratios (100 μL , 0.25 μg DNA per well) or HEPES buffer (20 mM, pH 7.4, supplemented with 5.0 wt% glucose) and incubated for an additional 1 h. All cells were refreshed with complete warm culture medium (100 μL), and cultured for another 48 h. Half the cells untreated with polyplexes were killed by incubating in 100X triton (4%, 10 μL) for 15 min, while the residual half were taken as a control to set viability to 100%. All cells were washed with DPBS (100 μL), and successively incubated in MTT solution (0.5 mg/mL, 100 μL) at 37 °C under 5% CO_2 condition for 4 h. After refreshing MTT with DMSO (100 μL), the resulting formazone crystals were quantified through plate reader (Tecan Infinite M200) at wavelength 540 nm with reference 680 nm. Measurements are performed in triplicate.

In Vitro Transfection Efficiency

The plasmids of pCMV-GFP and pCMV- ΔGFP were respectively used as positive and negative controls. In 48 well plates, cells were seeded at a density of 1.6×10^4 per well (1.6×10^4 cells/cm²) and cultured in complete medium (DMEM with 10% FBS, 200 μL) at 37 °C under humidified atmosphere with 5% CO_2 to reach confluencies of 60-80%. Cells were refreshed with warm medium (200 μL) with designated serum concentration, and maintained in new medium for 30 min until finally treated with polyplex solutions (200 μL , 0.5 μg DNA per well). After 60 min incubation at 37 °C, the polyplexes were replaced with fresh warm complete medium (200 μL), and cells were allow to grow for another 48 h. After replacing old medium with trypsin solution (0.25%, 200 μL), cells were spun down (600 g, 5 min,

R.T.), resuspended in HBSS buffer (200 μ L), and measured by the FACSCalibur (Becton-Dickinson, Breda, the Netherlands) at an excitation wavelength of 488 nm and emission at 530 nm. FACS Cellquest Software was applied to process data. A PEI (25 K)/DNA formulation prepared at its optimal condition (N/P=10) was used as a reference.

RESULTS AND DISCUSSION

Monomer and Polymer Preparation and Characterization

In our previous study on poly(amino ether)s (PAEs) (Chapter 6), the presence of disulfide bonds into the linear PAEs was found to improve their transfection capabilities, while maintaining excellent cell viabilities. The introduction of disulfides in this case was realized by polyaddition of a disulfide-containing diglycidyl ether monomer in combination with primary amines, yielding water-soluble bio-reducible PAEs. Cystamine disulfide forms branched or crosslinked structures with various diglycidyl ethers due to the twofold reactivity of each of the two primary amines present in this molecule.^{60,61} The resulting structures suffer from poor water solubility, impeding their application as gene vectors. In order to form linear polymers derived from cystamine disulfide, *N,N'*-dimethylcystamine disulfide (**DMC**), a cystamine analogue in which both amine groups are monomethylated, was employed as the bifunctional amine monomer. Reaction of this amine with a series of diglycidyl ethers with structural variation yields water-soluble bio-reducible linear PAEs that are expected to be well applicable to be explored for their efficiency as gene vectors (Scheme 2).

Scheme 2. Modular synthesis of bio-reducible poly(amino ether)s through amine-epoxide reaction of *N,N'*-dimethylcystamine (**DMC**) with different diglycidyl ethers.

The pool of diglycidyl ethers (**DEs**) consists of 6 different structures, as illustrated in Scheme 2. These are classified into 3 monomer groups: (i) high charge density (**1**), (ii) additional bioeducible linker (**2**), and (iii) varying hydrophobicity (**3-6**). All three factors are rationalized to play a role in transfection performance of cationic polymers. From these **DEs**, linear cationic PAEs were prepared by stirring the disulfide-containing amine **DMC** with the corresponding diglycidyl ethers in the presence of excess triethylamine. The mixtures typically became homogenous after a couple of hours and subsequently became viscous, indicating the formation polymers. No occurrence of gelation during the polymerization was observed, indicative for the formation of linear polymers. After removal of impurities and small oligomers through dialysis, the polymers were obtained in appreciable yields (ca. 33%-45%) except in the case of polymer **DMC3** (17%) and **DMC4** (14%). The relatively low yields of these polymers can be ascribed to a possible lower purity of the corresponding diglycidyl ethers since these were used as obtained from the supplier without further purification. All polymer structures were validated by ^1H NMR spectroscopy. No residual epoxide signals were observed in all cases. Molecular weights of the polymers were determined through GPC and ranged from 2.5 kDa to 4.2 kDa with polydispersities between 1.5-2.0 (see Table 1).

Table 1. Characteristics of linear bioeducible poly(amino ether)s prepared through reaction of *N,N'*-dimethylcystamine (**DMC**) with different diglycidyl ethers (**1-6**).

Poly(amino ether)s	Yield ^b (%)	M _w ^c (kDa)	PDI ^c	Degree of polymerization ^c (X _n)	Buffer capacity ^d (%)
DMC1	31.6	3.3	1.60	4.57	36.0
DMC2	33.2	4.0	2.03	4.39	50.4
DMC3	16.9	2.5	1.52	3.82	64.0
DMC4	13.9	2.9	1.73	4.06	34.4
DMC5	43.7	3.8	1.75	5.03	53.6
DMC6	40.1	4.2	1.80	6.14	36.8

^a Determined by ^1H NMR spectroscopy; ^b Isolated polymer yield; ^c Determined by GPC; ^d Determined by titration, pH range between 5.1 and 7.4.

The obtained PAEs contain tertiary amines in their main chain that are prone to protonation in acidic milieu. Protonation not only provides positive charges to condense DNA into polyplexes, but also endues buffer capacity to facilitate endosomal escape of polyplexes

through the proton sponge effect.⁶² To evaluate the capacity of these polymers to accept protons in the endosomal acidification range (pH 7.4-5.1), titration experiments were performed. The titration curves for all PAEs are displayed in Figure 1, including the titration of reference compound PEI (25 kDa) and blank NaCl solution (0.15 M). Plateaus along the titration curves in the pH 5.1-7.4 region are observed for all PAEs, implying pronounced buffer capacities. These buffer capacities range from 34% up to 64%, and are superior over PEI (18%), a commonly employed transfection agent. Since the nitrogens of the DMC units provide the buffer capacity,⁵⁸ it also confirms the successful incorporation of the disulfide units into PAEs. High buffer capacity of polymers is an excellent property to enhance endosomal escape necessary for efficient gene delivery into the cytosol.

Figure 1. Titration curves of poly(amino ether)s by NaOH (0.1 M) from pH 2-12. PEI (branched, 25 kDa) and NaCl (150 mM) are included as references.

Polyplex preparation and characterization

For efficient transfection, naked DNA requires protection by vectors, which can be realized by forming polyplexes through electrostatic interaction between DNA and cationic vectors.⁶³ Polyplexes were prepared by combining polymer solutions of **DMC1-6** with DNA solutions at mass ratio of 6, 12, 24, and 48. The resulting size and surface charge of the obtained polyplexes at these ratios are displayed in Figure 2. Polyplexes formed at polymer/DNA ratio above 6 possess hydrodynamic sizes from 80 to 200 nm and surface charge with zeta-potentials from +20 to +40 mV. At increasing polymer/DNA mass ratio, the polyplexes become smaller in dimension and carry increased positive charge, corresponding to more cationic PAEs recruited in the formation of these polyplexes.^{17,20,64} Polyplexes formed at a polymer/DNA mass ratio 6 display a relatively large size (over 400 nm) and an average surface charges of +15 mV, which is indicative for a rather loose structure. Polyplexes

prepared from **DMC1** and **DMC2** have a relatively small size compared to other polymers at the same polymer/DNA ratios. This phenomenon is related to the higher cationic charged density of polymer **DMC1** giving increased electrostatic interaction with DNA and the additional disulfide content of polymer **DMC2** making this polymer more flexible along the main chain since the disulfide bond has a low rotational barrier. The other four polymers **DMC3**, **DMC4**, **DMC5** and **DMC6** form polyplexes with similar values in size and zeta-potential at identical polymer/DNA mass ratios and no clear relation can be drawn between the polymer structure and the characteristics of the polyplexes here.

Figure 2. Characteristics of polyplexes prepared from bio reducible poly(amino ether)s and plasmid pCMV-GFP under various polymer/DNA mass ratios. (A) Hydrodynamic sizes versus mass ratios; (B) Zeta-potentials versus mass ratios.

The stability of the polyplexes was validated through agarose gel electrophoresis experiments. As illustrated in Figure 3A, DNA bands did not migrate along the electric field in the case of polyplexes based on **DMC1-6**, indicating that DNA was fully retained by the cationic PAEs through electrostatic interactions. However, it can be expected that due to the relatively high intracellular concentration of glutathione (GSH, 2.5-10 mM) inside the cell the disulfides in the polymer chain can be cleaved resulting in disassembly of the polyplexes and release the DNA content. This biological reaction was mimicked by incubation of the polyplexes with dithiothreitol (DTT, 2.5 mM) as a reducing agent for 30 min under ambient conditions. The resulting mixtures were subsequently subjected to gel electrophoresis (see Figure 3B). These polyplexes display clear bands migrating along the electric force direction, similarly to free DNA, illustrating the release of DNA from the degraded polyplexes. Also in DLS experiments the degradation of the polyplexes under the influence of DTT could be observed. Within less than 5 min after addition of DTT to polyplexes with polymer/DNA mass ratio 48 the size increases to micrometers and the count rate drops to below < 10 kCps, which is a clear sign of degradation of the nanoparticles (data not shown).

Figure 3. Agarose gel electrophoresis of polyplexes (w/w=48) treated without DTT (A) and with DTT (B) (s.c. = supercoiled, o.c. = open circular).

Cytotoxicity of polyplexes of DMC1-6

As for application *in vivo*, gene vectors require minimal toxicity and excellent biocompatibility towards cells and tissues.^{6,65} It is therefore important to evaluate cytotoxicity of the polyplexes before exploring their transfection performance. MTT assays were used to study cytotoxicity profiles of the polyplexes towards COS-7 cells under serum-free (0%) and serum-present (10%) conditions. As shown in Figure 4, the viability of COS-7 cells under serum-free conditions is above 80% when treated with polyplexes prepared at polymer/DNA ratios of 6, 12, and 24 for all polymers, except **DMC1** which has the highest cationic density in the series due to the presence of extra secondary amino groups in the chain. In contrast, PEI (25 K) is considerably more toxic at the same polymer/DNA ratios, even under its optimal conditions as described by the manufacturer. **DMC1** displays acceptable toxicity of polyplexes towards COS-7 cells at polymer/DNA ratios 6 and 12. However, toxicity becomes more pronounced at polymer/DNA ratios 24 (66%) and 48 (52%). It is worth noting that polyplexes prepared from **DMC2** exhibit even a somewhat higher viability than those prepared from the other polymers in the series. This can be attributed to the beneficial effect of the presence of extra disulfides in the polymers as it is known that the presence of these groups is beneficial for reducing cell toxicity of cationic polymers.^{36,66-68} Under 10% serum conditions, the cells show an increased viability; significant toxicity is only observed at polymer/DNA mass ratio 48. Importantly, the viability of COS-7 cells treated with polyplexes made of **DMC2** at polymer/DNA mass ratio

48 remains 81%, indicating the promising potential of **DMC2** for gene delivery under serum conditions.

Figure 4. Cell viabilities of COS-7 cells exposed to polyplexes at different polymer/DNA mass ratios, as determined via MTT assays under 0% serum conditions (A); and 10% serum conditions (B). (PEI is incorporated for comparison).

Transfection efficiency

After evaluation of the polyplex toxicity, the transfection performance of the polyplexes at polymer/DNA ratios 6 and 12 was investigated on COS-7 cells using green fluorescent protein (GFP) as the reporter gene. Three different serum conditions 0%, 10%, and 25% were evaluated. In all experiments, PEI was included as a reference both at the same polymer/DNA ratios, as well as at its optimal ratio (N/P = 10). Transfection efficiency was quantified by flow cytometry, as percentage of GFP-expressing cells of the total population. The results are shown in Figure 5. Under serum-free conditions, the transfection efficiencies of polyplexes prepared from PAEs at a polymer/DNA ratio 6 are similar or enhanced (12–25%) as compared to PEI (12.1%). Transfection efficiency increases at polymer/DNA ratio 12, reaching a highest efficiency of 36% for polyplexes based on **DMC2**. Relatively low transfection efficiencies are observed for **DMC1**, **DMC4** and **DMC6**. This is most likely related to their lower buffer capacities as compared to polymers **DMC2**, **DMC3**, and **DMC5**. At polymer/DNA ratio 6, the transfection efficiencies of **DMC2**, **DMC3**, and **DMC5** are 25%, 20%, and 21%, respectively, which increases to 36%, 26%, and 29% respectively at a polymer/DNA ratio of 12.

As illustrated in Figure 5, PAEs remain efficient in transfecting COS-7 cells with efficiency ranging from 10–32% at 10% serum, and 10–28% at 25% serum, which is considerably better than that of PEI (2.5% at 10% serum, 1.0% at 25%). The better transfection of PAEs in the presence of serum is related to the hydroxyl groups present on the polymers causing a protein repelling effect, similar to polyethylene glycol.⁶⁹ The transfection efficiency of **DMC2**-

based polyplexes at polymer/DNA mass ratio 12 in the presence of 25% serum is 32% and this is even further enhanced to 50% for polyplexes with polymer/DNA mass ratio 24 with a cell viability of up to 85% (data not shown). This underlines the high potential of this disulfide-based PAEs for non-viral gene delivery *in vivo*.

Figure 5. Transfection efficiencies of polyplexes prepared from bioreducible poly(amino ethers) **DMC1-6** and plasmid DNA at polymer/DNA mass ratios of 6 (left) and 12 (right). (PEI as reference)

CONCLUSIONS

A modular approach towards incorporation of bioreducibility into linear poly(amino ether)s was developed through reaction of *N,N'*-dimethylcystamine with various diglycidyl ethers. The obtained PAEs have good to excellent buffer capacities ranging from 40% to 67%. Upon mixing PAEs with DNA at appropriate mass ratios, polyplexes were formed with hydrodynamic diameters ranging from 80-200 nm and zeta-potentials of up to +40 mV. The polyplexes displayed no significant toxicity towards COS-7 cells at polymer/DNA ratios up to 24 with transfection of COS-7 cells with notable efficiencies of up to 36%. In addition, the presence of serum does not markedly interfere with transfection efficiency, even at serum concentrations of 10% and 25%. All transfection efficiencies and biocompatibility profiles of PAE-based polyplexes outpace the common polymeric vector PEI (branched, 25 kDa). These results present a novel modular approach towards a new generation of easily accessible cationic polymers for gene and drug delivery.

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Summary

As an important element of precision medicine, gene delivery is defined as intracellular delivery of genes into cells to interfere with or promote gene expression with therapeutic or analytic purposes by means of gene carriers. Viral carriers are predominantly used in current clinical trials, owing to their high transgene competencies. However they suffer from high toxicity and immunogenicity risks, have limited gene loading capacity, and pose significant technical difficulties in manufacturing. Non-viral gene vectors, especially polymeric vectors, form a promising alternative to circumvent aforementioned problems with additional beneficiary features, such as possibilities for structural variation and modular functionalization. However, the current polymeric gene vectors are far from ideal due to poor transfection efficiencies and toxicity issues. Optimization of available polymeric vectors and the design of novel polymeric vectors for efficient and safe *in vitro* and *in vivo* transgene activities is therefore of great interest, in order to achieve clinical translation.

The background of the work covered in this thesis is presented in **Chapter 1**. In **Chapter 2** an overview of bioreducible polycations with respect to their application in gene delivery is presented. In this chapter the basic knowledge on polycationic gene vectors and the unique features of disulfides in gene delivery is successively addressed. Then, bioreducible polymeric vectors are discussed based on different types of polycations. Finally, the perspectives on bioreducible polymeric vectors are outlined together with remarks on their potential translational capabilities. In **Chapter 3** synthesis and characterization of linear disulfide-linked poly(amido amine)s (PAAs) with varying amounts of thiourea-containing flanking groups are evaluated for colloidal stability, transfection performance and cell viability. This study demonstrates that polyplexes prepared from PAAs with 10% thiourea-functionalities induced higher transfection efficiency on COS-7 cells without bringing in extra cytotoxicities as compared to PAAs without any thiourea groups at the same polymer/DNA mass ratios. Moreover these polyplexes were still able to efficiently transfect COS-7 cells in the presence of 10% serum owing to increased polyplex stability caused by hydrogen bonding between the thiourea groups of the polymer and phosphate groups in DNA. In **Chapter 4**, the modification of bioreducible PAAs with fluoroalkyl pendant groups is described, as well as their application as transfection agent for green fluorescent protein (GFP)-encoding gene in COS-7 cells under various serum conditions (up to 50% serum). It was found that polyplexes of fluoroalkyl-functionalized PAAs exhibited much higher

transfection efficiency than non-functionalized PAAs without displaying considerable cell toxicity. Significant transfection activities were observed at much lower polymer/DNA mass ratio (as low as 6), which were not dramatically diminished under serum conditions. Transfection activities induced by PAA-based polyplexes under 10% serum conditions were further improved by polyplexes stabilized via gold nanorods (GNRs) as described in **Chapter 5**. Bioreducible PAAs were first coated with GNRs and then associated with DNA into hybrid polyplexes with smaller hydrodynamic diameter and higher surface charge as compared to conventional PAA polyplexes (i.e., GNRs-free polyplexes). Hybrid polyplexes exhibited enhanced colloidal stability through gold-sulfur interaction and are less susceptible to interference by serum proteins, leading to enhanced transfection efficiencies in COS-7 cells. The unique optical properties of GNRs endow hybrid polyplexes with potential in light-triggered traceable gene delivery.

In **Chapter 6**, the first bioreducible poly(amino ether)s (PAEs) are described as well as their application in gene delivery. PAEs with varying disulfide content were prepared through polyaddition reaction between 1-amino-4-butanol (ABOL) and epoxide mixtures comprising of 2-hydroxyethyl disulfide diglycidyl ether and 1,4-butanediol diglycidyl ether. The obtained PAEs with higher disulfide contents exhibited higher buffer capacity and induced increased transfection on COS-7 cells. The obtained PAEs were essentially non-toxic, and exhibited much higher transfection efficiency than standard PEI (25 kDa). In **Chapter 7**, bioreducible PAEs with various side groups were prepared through the epoxy-amine reaction. All PAEs showed higher buffer capacities as compared to PEI (25 kDa) and good DNA binding abilities with forming nanosized and positively charged polyplexes. The polyplexes prepared from pABOL, pMPA (2-morpholinoethylamine), and pAPOL (5-amino-1-pentanol) exhibited much higher transfection efficiencies than PEI (25 kDa) without compromising cell viabilities. Moreover, polyplexes of the PAEs showed potent transfection capabilities on COS-7 cells under serum conditions (up to 50% serum), owing to the presence of abundant hydroxyl groups along the polymer chains. In **Chapter 8**, an alternative and sophisticated synthesis of bioreducible PAEs was designed by reaction of *N,N'*-dimethylcystamine (DMC) with various diglycidyl ethers. The resultant PAEs displayed higher buffer capacities than PEI (25 kDa), and are able to efficiently condense DNA into polyplexes of nanosized dimension (around 200 nm). DNA was released through the disassembly of the polyplexes due to disulfide cleavage under reducing conditions, and eventually transcribed in the COS-7 cells with much higher efficiency than PEI (25 kDa).

In conclusion, transfection efficiency of bio reducible PAAs-based polyplexes was optimized by introducing thiourea groups, fluoroalkyl groups, or GNRs. In addition, a novel class of bio reducible PAEs has been developed that show promising properties as polymeric gene vectors for translational application. The PAEs possess several beneficiary merits, such as good water solubility, possibilities for structural fine-tuning, good DNA binding ability, high buffer capacity, active DNA release by disulfide cleavage, low cytotoxicity and potent transfection capability under serum-free as well as serum-containing conditions.

Samenvatting

Genafgifte is een belangrijk onderdeel van experimentele geneeskunde en wordt gedefinieerd als de afgifte van genen in cellen om de expressie van genen te stimuleren of te beïnvloeden met therapeutische of analytische doeleinden door gebruik te maken van gen-dragermaterialen. Vooral virale gen-dragermaterialen worden toegepast in klinisch onderzoek, vanwege de hoge transfectie efficiëntie. Het gebruik van virale gen-dragermaterialen brengt echter hoge toxiciteits- en immunogeniciteitsrisico's met zich mee. Bovendien hebben deze dragermaterialen een beperkte gen-beladingscapaciteit en is de productie van deze materialen technisch veeleisend. Niet-virale gen-dragermaterialen, en vooral polymeren, zijn een veelbelovend alternatief om de eerder genoemde problemen op te lossen. Polymeer gen-dragermaterialen bieden daarnaast meer mogelijkheden voor het aanbrengen van structurele variatie en modulaire functionalisaties. De huidige polymeer gen-dragermaterialen zijn echter verre van ideaal vanwege de lage transfectie-efficiëntie en voor sommige materialen de potentiële toxiciteit. Optimalisatie van de beschikbare polymeer gen-dragermaterialen en het ontwerpen van nieuwe materialen voor efficiënte en veilige transfectie *in-vitro* en *in-vivo* zijn daarom interessant voor een toekomstige transitie naar de kliniek.

De achtergrond van dit proefschrift wordt behandeld in **Hoofdstuk 1**. In **Hoofdstuk 2** is een overzicht van bioreduceerbare polykationen gegeven in het licht van toepassing voor genafgifte. In dit hoofdstuk worden achtereenvolgens de basisbeginselen van polykationische materialen en de mogelijkheden van disulfidebindingen in de materialen voor genafgifte behandeld. Vervolgens worden bioreduceerbare polymeer vectoren besproken aan de hand van verschillende typen kationen. Als laatste worden de perspectieven van polymeervectoren uiteengezet met betrekking tot de mogelijkheden voor klinische toepassing van deze materialen. **Hoofdstuk 3** beschrijft de synthese en karakterisering van lineaire- disulfide-bevattende poly(amido amine)s (PAAs) met variërende hoeveelheden thiourea-zijgroepen, en de evaluatie van de kolloidale stabiliteit, transfectie efficiëntie en de cel toxiciteit. Dit onderzoek toont aan dat polyplexen, gevormd met PAAs met 10% thiourea zijgroepen, een hogere transfectie efficiëntie hebben dan PAAs zonder thiourea functionaliteit in COS-7 cellen zonder een verhoogde cel toxiciteit bij dezelfde polymeer/DNA verhoudingen. Het was bovendien mogelijk COS-7 cellen te transfecteren bij een serumconcentratie van 10%. Dit kan worden verklaard door een verhoogde polyplex stabiliteit, veroorzaakt door

waterstofbruggen tussen de thiourea groepen van het polymeer en de fosfaatgroepen van het DNA. In **Hoofdstuk 4** worden de modificatie en toepassing van bioreduceerbare PAAs met fluoralkyl groepen beschreven voor het transfecteren van COS-7 cellen met een voor groen fluorescent proteïne (GFP) coderend gen bij variërende serumconcentraties (tot 50%). Bij lage polymeer/massa ratio's (tot 6) werd een hoge transfectieactiviteit waargenomen, welke niet afnam bij een serumconcentratie van 10% . De door PAAs geïnduceerde transfectieactiviteit werd verder verbeterd door polyplexen gestabiliseerd door gouden nanostaafjes (GNSs), zoals beschreven in **Hoofdstuk 5**. Op de GNSs werd eerst een PAA coating aangebracht en werden vervolgens gecomplexeerd met DNA als hybride polyplexen, welke een lager hydrodynamisch volume en een hogere oppervlaktelading bezaten dan conventionele PAA polyplexen (zonder GNSs). Door de unieke optische eigenschappen van GNSs, bieden deze hybride polyplexen perspectief voor licht-geactiveerde- en door licht detecteerbare genafgifte.

In **Hoofdstuk 6** worden voor het eerst bioreduceerbare poly(amino ether)s (PAEs) en hun toepassing in genafgifte beschreven. PAEs, met een variërende hoeveelheid disulfidebindingen, werden gemaakt door een polyadditie tussen 1-amino-4-butanol (ABOL) en 1,4-butaandiol diglycidyl ether. De verkregen PEAs met een hoge disulfide-inhoud, hadden een hogere buffercapaciteit en gaven hogere transfectiewaarden in COS-7 cellen. De verkregen PAEs waren nagenoeg niet-toxisch en lieten veel hogere transfectiewaarden zien ten opzichte van standaard poly(ethylenimine) (PEI, 25 kDa). De synthese van bioreduceerbare PAEs, door een epoxy-amine reactie, met verschillende zijgroepen wordt beschreven in **Hoofdstuk 7**. Alle PAEs bezaten een hogere buffercapaciteit dan PEI (25 kDa) en vertoonden goede bindingseigenschappen met DNA om polyplexen te vormen. Polyplexen gevormd met pABOL, pMPA (2-morpholinoethylamine) en pAPOL (5-amino-1-pentanol), bezaten een veel hogere transfectie-efficiëntie dan PEI (25 kDa) en een lage cel toxiciteit. Bovendien gaven PAE polyplexen veelbelovende transfectiewaarden bij COS-7 cellen bij serumconcentraties tot 50%, wat werd veroorzaakt door de hydroxylgroepen in het polymeer. In **Hoofdstuk 8** wordt een alternatieve en verfijnde synthesesmethode voor bioreduceerbare PAEs beschreven door de reactie met N,N'-dimethylcystamine (DMC) met verschillende diglycidylethers. De resulterende PAEs hadden een hogere buffercapaciteit dan PEI (25 kDa) en konden DNA efficiënt condenseren in polyplexen van ongeveer 200 nm. DNA werd afgegeven door het uiteenvallen van de polyplexen, vanwege het verbreken van de disulfidebindingen onder reducerende

omstandigheden, en getranscribeerd in COS-7 cellen met een hogere efficiëntie dan PEI (25 kDa).

Concluderend, de transfectie-efficiëntie van op PAA gebaseerde polyplexen is geoptimaliseerd door de introductie van thiourea, fluoralkyl of GNSs. Daarnaast is een nieuw type bioreduceerbare PAEs ontwikkeld met veelbelovende resultaten voor toepassing als vectoren voor genafgifte in translationele applicaties. De PAEs bevatten enkele goede eigenschappen, zoals goede wateroplosbaarheid, mogelijkheden voor structurele aanpassingen, goede binding met DNA, hoge buffercapaciteit, actieve DNA-afgifte bij disulfidereductie, lage cytotoxiciteit en goede transfectie-eigenschappen bij serum-vrije en serum-bevattende condities.

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司国颖

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Enschede

Curriculum Vitae

Guoying Si (司国颖) was born on 28th June, 1986 in Yancheng, Jiangsu province, P.R.China. In July 2005, he entered Soochow University (Suzhou, P.R. China) to study material science and engineering. In June 2009, he earned his Bachelor degree in engineering after accomplishing his bachelor assignment of “design and synthesis of efficient and safe polymeric gene vectors” under the supervision of Prof. Zhiyuan Zhong. Two month later, he came to Department of Biomedical Chemistry in University of Twente (the Netherlands) to start his master education in chemical engineering. In August 2011, he obtained his Master degree in science under supervision of Prof. dr. Johan F.J. Engbersen with the master thesis entitled “fabrication of multilayered thin film of poly(amido amine)s and calf-thymus DNA and their degradation profile in physiological conditions”. From September 2011 to August 2015, he remained at the same department to commerce his PhD research on optimization and development of bioreducible polycations as gene vectors with research results complied in this thesis.