Journal of Controlled Release 244 (2016) 357–365

Contents lists available at ScienceDirect

Journal of Controlled Release

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

Disulfide-functional poly(amido amine)s with tunable degradability for gene delivery

M. Rachèl Elzes [a], Niels Akeroyd [b], Johan F.J. Engbersen [a,b,*], Jos M.J. Paulusse [a,c,**]

[a] Department of Biomaterials Science and Technology, MIRA Institute for Biomedical Technology and Technical Medicine, Faculty of Science and Technology, University of Twente, P.O. Box 217, 7500 AE Enschede, The Netherlands
[b] 20Med Therapeutics, Zuidhorst 251, Drienerlolaan 5, 7522 NB Enschede, The Netherlands
[c] Department of Nuclear Medicine and Molecular Imaging, University Medical Center Groningen, P.O. Box 30.001, 9700 RB Groningen, The Netherlands

ARTICLE INFO

Article history:
Received 16 June 2016
Received in revised form 8 August 2016
Accepted 21 August 2016
Available online 24 August 2016

Keywords:
Gene delivery
Poly(amido amine)s
Reducibility
Steric hindrance
Controlled release

ABSTRACT

Controlled degradability in response to the local environment is one of the most effective strategies to achieve spatiotemporal release of genes from a polymeric carrier. Exploiting the differences in reduction potential between the extracellular and intracellular environment, disulfides are frequently incorporated into the backbone of polymeric drug delivery agents to ensure efficient intracellular release of the payload. However, although to a lesser extent, reduction of disulfides may also occur in the extracellular environment and should be prevented to avoid premature release. Accurate control over the stability of disulfide linkages enables the optimization of polymeric carriers for efficient drug delivery. Bioreducible poly(amido amine)s (PAAs) with varying degrees of steric hindrance adjacent to the disulfide bonds (0, 2 or 4 methyl groups) were prepared in order to obtain carriers with controlled stability. The degradation behavior of these PAA-polymers was evaluated under different reducing conditions and their in vitro toxicities and transfection efficiencies were assessed. Degradation of the PAA-based polyplexes consistently required higher reducing strengths as the steric hindrance near the disulfide bonds increased. Polyplexes based on 2-methyl cystamine disulfide based PAA polymer (PAA^{2m}) remained stable under extracellular glutathione concentrations (0.001–0.01 mM), while degrading within 1 h under reducing conditions similar to those in the intracellular environment (1–10 mM glutathione). This polymer exhibited excellent transfection capabilities, with efficiencies up to 90% of transfected cells. PAA^{0m} showed slightly reduced transfection properties compared to PAA^{2m}, likely due to premature degradation. The severely hindered PAA^{4m}, however, displayed increased toxicity, accompanied by reduced transfection efficiency, as a result of its exceptional stability. These results demonstrate the feasibility of introducing steric hindrance near the disulfide moiety to tune polyplex stability against bioreduction, and show that PAA^{2m} is a promising polymer to be further developed for gene therapy.

1. Introduction

A vast number of diseases is caused by genetic defects or mutations; estimates range from 6000 to 7000, and new conditions are discovered every year [1]. These diseases are inherently chronic and as such, place a major, growing burden on the health care system in terms of costly therapies and specialist care [2]. Gene therapy is widely recognized as one of the most promising treatment options, targeting the cause of the disease rather than its symptoms [3,4]. Moreover, due to its versatility, gene therapy offers a potential solution to multifactorial and purely acquired diseases as well, including cancer and infections [5–7]. Successful gene therapy relies on the delivery of therapeutic nucleotides into the cell interior to either enhance or inhibit specific protein production. Despite several decades of research, this delivery remains one of the leading challenges preventing gene therapy from entering the clinics. Carrier systems have improved the delivery efficiency through shielding the genetic payload from extracellular degradation by nucleases, and by promoting transport across cellular membranes [8]. Unfortunately, development of suitable vectors still faces several significant hurdles, mainly with regard to achieving efficient and long-lasting transfection without inducing adverse effects.

Current carriers can be largely divided into viral and synthetic vectors [9,10]. Viral carriers, while being efficient delivery agents, raise serious safety concerns regarding immunogenicity and insertional mutagenesis, and large-scale production is impractical [11–13].

* Correspondence to: J. F. J. Engbersen, 20Med Therapeutics, Zuidhorst 251, Drienerlolaan 5, 7522 NB Enschede, The Netherlands.
** Correspondence to: J. M. J. Paulusse, Department of Nuclear Medicine and Molecular Imaging, University Medical Center Groningen, P.O. Box 30.001, 9700 RB Groningen, The Netherlands.
E-mail addresses: J.F.J.Engbersen@utwente.nl (J.F.J. Engbersen), J.M.J.Paulusse@utwente.nl (J.M.J. Paulusse).

http://dx.doi.org/10.1016/j.jconrel.2016.08.021
0168-3659/

Synthetic vectors, consisting primarily of cationic liposomes and cationic polymers, are more versatile in terms of design and application. Their physical and chemical properties can be easily altered [14], inclusion of a targeting moiety has been demonstrated [15–17] and large-scale production is feasible [18]. Additionally, while viral carriers have a low transgene capacity, molecules of virtually any size can be delivered using synthetic vectors [19]. Their cationic nature allows spontaneous complex formation with anionic oligonucleotides, and the resulting positively charged lipoplexes and polyplexes are easily endocytosed through multivalent interactions with the negatively charged cellular membrane. However, achieving high transfection efficiency using non-viral vectors proved to be a considerable challenge, due to stability issues, poor blood circulation and large excess of lipid or polymer required [20–22]. One of the first and most widely studied cationic polymers for gene delivery is polyethylenimine (PEI) [23]. While the branched structure of PEI (bPEI) has reported relatively efficient gene delivery, it is also a rather toxic material. Many alternatives emerged during the past decades, including poly-L-lysine, poly(*N*,*N*-dimethylaminoethyl methacrylate), dendrimers (PAMAM), cyclodextrin and poly(β-amino esters) [24–28]. A highly interesting class of cationic polymers is the poly(amido amine)s (PAAs), which have peptidomimetic moieties such as amides and their versatile preparation allows facile incorporation of various functionalities like alcohols, ethers and morpholine moieties [29–32]. We have previously shown that one of the most successful PAAs is a co-polymer of *N*,*N*′-bis(acryloyl)cystamine and 4-amino-1-butanol (pCBA-ABOL), a non-toxic degradable material with reported exceedingly high transfection efficiency [33].

Stability of the polyplexes on their route to intracellular delivery and carrier degradability once arrived in the cytosol is essential for both reducing polymer toxicity and releasing the genetic payload to ensure efficient gene delivery. An effective approach to achieve spatiotemporal control exploits the high reducing strength of the intracellular environment [34–40]. The body maintains a complex set of thiol/disulfide redox couples that determine local redox potentials, such as glutathione/glutathione disulfide (GSH/GSSG), cysteine/cystine (Cys/CySS) and thioredoxin-1 [41]. For example, the main reducing agent in the cytoplasm, GSH, is approximately 1000-fold more abundant intracellularly (~1–20 mM) than in the extracellular environment (~0.002 mM) [42,43]. When GSH comes into contact with disulfides, thiol-disulfide exchange occurs, thereby degrading disulfide containing polymers [44–47]. Ideally, this would mean prolonged systemic circulation of stable polyplexes that only degrade after reaching their site of action, which is predominantly intracellular. However, despite the popularity of this approach, further research is required on the stability of disulfides. Extracellular stability is often assumed, yet disulfide cleavage has been reported to occur in blood circulation [48–52], in vitro tumor interstitium [53–56] and on cell surfaces [57–59]. For instance, Carroll et al. observed exchange of disulfide-linked antibody-drug conjugates, with half-lives of approximately ~8 h [49]. Brülisauer et al. showed that 50% of a cationic (G_3) PAMAM dendrimer containing disulfide-linkages was degraded within ~1 h when presented to various cancer cell lines [55]. The resistance of disulfides against extracellular bioreduction has been improved through alterations of their microenvironment, either by employing electrostatic repulsion or by increasing steric hindrance [49–54,60–66]. Inclusion of negatively charged moieties near the disulfide bond repels anionic agents, such as glutathione anions, thereby reducing the rate of degradation [60–62]. Alternatively, Carroll et al. demonstrated that incorporation of a single methyl group adjacent to the disulfide moiety increased the half-life of disulfide-linked immunoconjugates from 8 h to 30 h [49]. More recently, Leroux and coworkers accomplished precise control over disulfide stability through microenvironmental effects [54], and Kellogg et al. achieved a broad range of disulfide stabilities by introducing various numbers of methyl groups around the disulfide bond [64]. They reported >22,000-fold slower disulfide cleavage under reducing conditions when employing four methyl groups compared to the unhindered analogue.

Here, we evaluate the effect of steric hindrance around disulfide groups by adjacent methyl substitution on the stability of PAA-based polyplexes, in order to decrease extracellular degradation and to optimize gene delivery efficiency. Analogues of pCBA-ABOL (denoted here as PAA^{0m}, where the suffix 0m stands for zero methyl groups) with two (PAA^{2m}) or four (PAA^{4m}) methyl groups adjacent to the disulfide bond were prepared and their stabilities were assessed. Toxicity and transfection efficiency of polyplexes of the three polymers was quantified to appraise their potential for gene delivery. Furthermore, to determine optimal stability, polyplex responsiveness to elevated extracellular GSH levels was investigated.

2. Materials and methods

2.1. Materials

All chemicals were obtained from commercial sources and used without further purification unless stated otherwise. Cystamine dihydrochloride (96%), 1,3-dibromo-5,5-dimethylhydantoin (DBDMH, 98%), sodium hydroxide (98%), acryloyl chloride (97%), 4-amino-1-butanol (98%), DL-dithiothreitol (DTT, 98%), L-glutathione (98%) and heparin sodium salt from porcine intestinal mucosa (Grade I-A, ≥180 USP units/mg) were purchased from Aldrich; dimethyl sulfoxide (DMSO, 99.6%), methanol (100%) and dichloromethane (99%) were purchased from VWR and 1-amino-2-methyl-2-propanethiol hydrochloride (98%) was purchased from BOC Sciences. Synthesis was monitored by thin layer chromatography (TLC). Spots on TLC-plates were visualized with vanillin, ninhydrin or potassium permanganate stains, or UV light. ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) spectra were recorded on a Bruker Ascend 400 MHz NMR-spectrometer. Electrospray Ionization Mass Spectrometry (ESI-MS) spectra were obtained with a Micromass LCT, using a Harvard apparatus Pump 11 Elite for sample injection. Acid-base titration was performed on a Metrohm 702 SM Titrino autotitrator. Particle size and surface charge were measured on a Zetasizer 4000 and a Zetasizer 2000 instrument respectively (Malvern Instruments). Size exclusion chromatography (SEC) was performed on a Waters Alliance e2695 separation module equipped with a Waters 2998 PDA detector, a Waters 2914 RI detector and a Polymer Laboratories Gel Mixed D column. *N*,*N*-dimethylformamide was used as eluent at a flow rate of 0.7 mL/min at 35 °C, employing polyethylene glycol standards. Stained plasmid DNA (pDNA) fluorescence was visualized using a FluorChem M system (excitation 475 nm, emission 537 nm, ProteinSimple). UV–Vis absorption was measured on an Infinite M200 Pro plate-reader (TECAN). An EVOS FL microscope was used to record fluorescence microscopy images. Fluorescence-Activated Cell Sorting (FACS) analysis was performed using a Becton Dickinson FACSCalibur, with a BD FACSRinse Solution as running buffer.

2.2. Synthesis of bisacrylamide monomers

2.2.1. *N*,*N*′-bis(acryloyl)cystamine (**2a**)

Cystamine dihydrochloride (2.05 g, 9.11 mmol) was dissolved in water (10 mL). A solution of sodium hydroxide (1.81 g, 45.3 mmol) in water (10 mL) and a solution of acryloyl chloride (2.2 mL, 27 mmol) in dichloromethane (6 mL) were added dropwise at 0 °C. After stirring overnight at room temperature, pH was adjusted to pH = 8 and the white precipitate was filtered off. The reaction mixture was extracted three times using dichloromethane and the organic phase was dried using magnesium sulfate. Solvents were removed under reduced pressure and the crude product was purified by recrystallization from boiling ethyl acetate to afford *N*,*N*′-bis(acryloyl)cystamine (**2a**) as a white solid (1.30 g, 55%).

^{1}H NMR (DMSO-d_6): δ = 8.33 (2H, t, J = 5.6 Hz, NH), 6.19, 6.12 (4H, 2 m, CH_2=CH), 5.61 (2H, m, CH_2=CH), 3.42 (4H, q, J = 6.8 Hz, $NHCH_2$), 2.82 (4H, t, J = 6.8 Hz, SCH_2).

^{13}C NMR (DMSO-d_6): δ = 164.8 (C(O)NH), 131.6 (CH_2=CH), 125.4 (CH_2=CH), 37.9 ($NHCH_2$), 37.1 (SCH_2).

2.2.2. N,N′-dithiobis(2-methylethyl)bisacrylamide (**2b**)

1-Amino-2-propanethiol hydrochloride (**1b**) was prepared according to the procedure described by Fujita et al. [67]. In a three-necked flask, 1-amino-2-propanethiol hydrochloride (10.0 g, 78.7 mmol) was suspended in methanol (120 mL) and sodium hydroxide (3.34 g, 83.4 mmol) was added. Next, 1,3-dibromo-5,5-dimethylhydantoin (8.66 g, 30.3 mmol) was added, and the mixture was stirred overnight. Sodium hydroxide (13.3 g, 532 mmol) was added to the in situ formed disulfide and a solution of acryloyl chloride (27.0 mL, 332 mmol) in dichloromethane (66 mL) was added dropwise at 0 °C. After stirring overnight at room temperature, pH was adjusted to pH = 8 and the white precipitate was filtered off. The reaction mixture was extracted three times using dichloromethane and the organic phase was dried using magnesium sulfate. The crude product was purified by filtration over a slab of silica followed by recrystallization from boiling ethyl acetate. *N,N′*-dithiobis(2-methylethyl)bisacrylamide (**2b**) was obtained as a white solid (0.980 g, 8.6%).

^{1}H NMR (DMSO-d_6): δ = 8.33, 8.28 (2H, 2 t, J = 5.6 Hz, NH), 6.26, 6.11 (4H, 2 m, CH_2=CH), 5.61 (2H, m, CH_2=CH), 3.45, 3.17 (4H, 2 m, $NHCH_2$), 3.00 (2H, m, $CHCH_3$), 1.20 (6H, 2d, J = 2.4 Hz, CH_3).

^{13}C NMR (DMSO-d_6): δ = 164.8164.79 (C(O)NH), 131.5, 131.5 (CH_2=CH), 125.5, 125.4 (CH_2=CH), 45.6, 45.0 ($NHCH_2$), 43.6, 43.55 ($CHCH_3$), 18.0 (CH_3).

ESI-MS (m/z): 289.38 $[M + H]^+$, 311.34 $[M + Na]^+$, 327.32 $[M + Na]^+$.

2.2.3. N,N′-dithiobis(2,2-dimethylethyl)bisacrylamide (**2c**)

1-Amino-2-methyl-2-propanethiol hydrochloride (**1c**) (4.75 g, 33.5 mmol) was suspended in methanol and sodium hydroxide was added, this formed a purple solution. After addition of DBDMH the solution turned yellow within 15 min. The protocol for the synthesis of **2b** was followed to yield *N,N′*-dithiobis(2,2-dimethylethyl)bisacrylamide (**2c**) as a white solid (2.16 g, 41%).

^{1}H NMR (DMSO-d_6): δ = 8.13 (2H, t, J = 6.4 Hz, NH), 6.37–6.07 (4H, m, CH_2=CH), 5.61 (2H, m, CH_2=CH), 3.27 (4H, d, J = 6.0 Hz, $NHCH_2$), 1.20 (12H, s, $(CH_3)_2$).

^{13}C NMR (DMSO-d_6): δ = 164.9 (C(O)NH), 131.6 (CH_2=CH), 125.5 (CH_2=CH), 49.7 ($NHCH_2$), 48.1 ($C(CH_3)_2$), 25.7 ($(CH_3)_2$).

ESI-MS (m/z): 317.44 $[M + H]^+$, 339.40 $[M + Na]^+$, 355.40 $[M + Na]^+$.

2.3. Synthesis of poly(amido amine)s

2.3.1. PAA^{0m} (**3a**)

N,N′-bis(acryloyl)cystamine (**2a**) (1.91 mmol) and 4-amino-1-butanol (ABOL, 1.91 mmol) were dissolved in demi water (2 mL) and methanol (4 mL), the solution was stirred at 50 °C for 7 days. ABOL (0.982 mmol) was added and allowed to react for 2 days to end-cap the polymer. The pH was lowered until a clear solution was obtained, this solution was dialyzed over a 1 kDa molecular weight cut-off membrane for 24 h, followed by lyophilization. PAA^{0m} (**3a**) was obtained as a white powder (151 mg, 23%).

^{1}H NMR (DMSO-d_6): δ = 8.83 (C(O)NH), 3.40, 3.33 (CH_2OH, CH_2CH_2S), 3.07 ($CH_2CH_2C(O)$), 2.86, 2.78 ($NCH_2CH_2CH_2$, $CH_2C(O)$), 1.59 ($CH_2CH_2CH_2OH$), 1.42 (CH_2CH_2OH).

^{13}C NMR (DMSO-d_6): δ = 169.9 (C(O)), 60.2, 60.0 (CH_2NCH_2, CH_2OH), 52.3 (SCH_2), 48.7 ($NHCH_2$), 38.0, 37.0 ($CH_2CH_2CH_2OH$), 29.7 ($C(O)CH_2$).

2.3.2. PAA^{2m} (**3b**)

PAA^{2m} was prepared under the same conditions as PAA^{0m}. The scale of the reaction was 1.94 mmol, to yield PAA^{2m} (**3b**) as a white powder (177 mg, 24%).

^{1}H NMR (DMSO-d_6): δ = 8.51 (NH), 3.42 (CH_2OH), 3.37 ($NCH_2CH_2CH_2$), 3.28 ($CH_2CH_2C(O)$), 3.07 ($CHCH_2$), 2.97 (CH), 2.70 ($CH_2C(O)$), 1.70 ($CH_2CH_2CH_2OH$), 1.43 (CH_2CH_2OH), 1.20 (CH_3).

^{13}C NMR (DMSO-d_6): δ = 169.2 (C(O)), 60.0 (CH_2OH), 52.1 (SCH), 48.4 ($NHCH_2$), 45.4, 43.7 (CH_2NCH_2), 29.3, 29.1 ($CH_2CH_2CH_2OH$), 20.0 ($C(O)CH_2$), 18.0 (CH_3).

2.3.3. PAA^{4m} (**3c**)

PAA^{4m} was prepared under the same conditions as PAA^{0m}. The scale of the reaction was 1.74 mmol, to yield PAA^{4m} (**3c**) as a white powder (231 mg, 33%).

^{1}H NMR (DMSO-d_6): δ = 8.30 (NH), 3.42 (CH_2OH), 3.28 ($NHCH_2$), 3.20 ($CH_2CH_2C(O)$), 3.06 ($NCH_2CH_2CH_2$), 2.72 ($CH_2C(O)$), 1.70 ($CH_2CH_2CH_2OH$), 1.44 (CH_2CH_2OH), 1.20 ($(CH_3)_2$).

^{13}C NMR (DMSO-d_6): δ = 169.3 (C(O)), 59.9 (CH_2OH), 49.5 ($SCCH_2$), 48.5, 48.1 (CH_2NCH_2), 29.3, 29.0 ($CH_2CH_2CH_2OH$), 25.6 (CH_3), 20.0 ($C(O)CH_2$).

2.4. Characterization

2.4.1. Acid-base titration

The buffer capacity of the polymers was determined by dissolving polymer to achieve ~0.015 mmol protonatable amines in 0.15 M sodium chloride solution (10 mL). The pH was adjusted to pH ~ 2 using 1 M hydrochloric acid solution. The solution was titrated using 0.1 M sodium hydroxide solution until pH 12 was reached. The buffering capacity, defined as the percentage of amine groups being protonated between pH 5.1 and 7.4 (endosomal pH range), was calculated using the following equation:

$$\text{Buffer capacity}\,(\%) = \frac{\Delta OH^-_{pol} - \Delta OH^-_{NaCl}}{\text{mole N}} \times 100\%$$

where ΔOH^-_{pol} and ΔOH^-_{NaCl} are the molar equivalents of hydroxides needed to increase the pH from 5.1 to 7.4 of the polymer solution and pure 0.15 M NaCl solution, respectively, and mole N is the total moles of amines in solution.

2.4.2. Dynamic light scattering (DLS)

Polyplexes were prepared by adding 30 μL of plasmid DNA solution (1 mg/mL, pCMV-GFP, Plasmid Factory) into a cuvette. Subsequently, demi-water was added and the solution was mixed thoroughly. Polymer solutions (6 mg/mL) were added, the solution was homogenized and incubated for 10 min. at room temperature. To achieve the ratios 6.25, 12.5, 25, 50 and 100, water (938.8, 907.5, 845, 720 and 470 μL) and polymer solution (31.25, 62.5, 125, 250 and 500 μL) were added, respectively. Polyplex stability was evaluated on samples prepared by mixing 40 μL pDNA solution (1 mg/mL) with 628 μL water (pure or containing 154.3 mg DTT to achieve a final concentration of 1 M) and 334 μL polymer solution (6 mg/mL). All samples were measured during 20 h with 5 min intervals.

2.4.3. Gel electrophoresis

Plasmid DNA (pCMV-GFP, 1 mg/mL, 2.2 μL) was diluted with MilliQ water (34.1 μL), polymer solution (36.3 μL, 3 mg/mL) was added and the solution was mixed to form polyplexes. To achieve various w/w ratios, polymer solutions of 6 to 0.375 mg/mL were used. A free pDNA control was prepared by diluting 0.5 μL pDNA with 16.1 μL MilliQ water. DTT was dissolved in MilliQ water with concentrations ranging from 0.085 to 8500 mM. Hydrazine monohydrate (65%, 18.5 mg) was diluted with MilliQ water (70.9 μL) to form an 8.5 M solution. Heparin sodium salt was dissolved in MilliQ water and diluted to obtain concentrations

of 8.5 to 0.0425 mg/mL. A 1.5% w/v agarose (BIO-RAD) gel containing 0.05% v/v SYBR® Safe DNA Gel Stain (Invitrogen™) was prepared in TAE buffer (40 mM tris acetate, 1 mM ethylenediamine tetraacetic acid). Samples were prepared by mixing 7.5 μL transfectant with 1 μL water, DTT, hydrazine or heparin solution. After incubation at room temperature for 30 min., 1.5 μL gel loading buffer (Ambion) was added and the samples were electrophoresed at 75 mA for 30 min. The pDNA was visualized by measuring fluorescence.

2.5. Cell viability

MTT assays were performed on COS-7 cells (SV-40 transformed African Green monkey kidney cells). In a typical experiment, cells were seeded in a 48-well cell culture plate (CellStar) by adding 200 μL of an $8 \cdot 10^4$ cells/mL suspension to all wells, achieving a final seeding density of $1.6 \cdot 10^4$ cells/cm^2. DMEM (Dulbecco's Modified Eagle Medium, high glucose, GlutaMAX™, Gibco, Life Technologies) was used as medium, with 10% FBS (Fetal Bovine Serum, South American Origin, Lonza). Cells were incubated overnight at 37 °C in a humidified 5.0% CO_2-containing atmosphere. Polymer solutions of 2 mg/mL in MilliQ water were filtered over a 0.2 μm filter and subsequently diluted with Dulbecco's Phosphate-Buffered Saline (DPBS, Gibco, Life Technologies) to achieve concentrations of 50, 100, 200, 400 and 800 μg/mL. As a reference, a branched polyethylene imine solution (bPEI, 25 kDa, Sigma Aldrich) of 0.1 mg/mL was prepared and diluted ten times. pCMV-GFP (1 mg/mL) was diluted with DPBS to form a 50 μg/mL solution. Plasmid DNA and polymer dilutions were mixed in a volume ratio of 1:6.25. Medium was aspirated from the wells and replaced by 145 μL fresh DMEM (either pure or containing 10% FBS). Polyplex solution, or pure DPBS for the controls, (145 μL) was added and the plates were incubated at 37 °C for 1 h. The wells were aspirated again and 0.5 mL DMEM containing 10% FBS was added. The plates were incubated for two days, after which they were analyzed. First, a 5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Molecular Probes®, Life Technologies) in HBSS (Hank's Balanced Salt Solution, Gibco, Life Technologies) was prepared and filtered over a 0.2 μm filter. This MTT solution was diluted ten times using RPMI 1640 medium (Roswell Park Memorial Institute 1640, Gibco, Life Technologies, without FBS or Phenol Red). As a negative control, 50 μL of saponin (1% in HBSS, Sigma-Aldrich) was added and cells were incubated for 15 min. Wells were aspirated and 300 μL of diluted MTT solution was added. After 4 h of incubation the solution was replaced by 300 μL DMSO and the plates were incubated for another 10 min. The absorbance of each well at 540 nm was measured and compared to the absorbance of the untreated positive control in order to quantify the viability. All experiments were carried out in triplicate.

2.6. Transfection efficiency

Transfection experiments were performed according to the same procedure as the viability experiments. During these experiments, pCMV-LacZ (Plasmid Factory) polyplexes were prepared similar to the pCMV-GFP polyplexes and employed as negative control. After the 2 day incubation period, samples were prepared by first trypsinizing the cells. Medium was aspirated and 150 μL 0.25% trypsin (Cambrex, Whitaker) was added to each well. Cells were incubated for 3 min after which the trypsin was neutralized by adding 1 mL of DMEM with 10% FBS to all wells. Suspensions were transferred into glass tubes, centrifuged (5 min at 600 rcf), decanted, 100 μL DPBS was added and the cells were resuspended. GFP fluorescence per cell was measured at 530/30 nm and quantified using FACS. All experiments were carried out in triplicate. In the resulting histograms, a 'positive' region was selected so that the negative controls had 0.5% positive cells. Results are expressed as percentage of cells in this positive region.

2.7. In vitro transfection under artificial GSH concentrations

Transfection experiments were carried out under similar conditions as described for the cell viability experiments. However, during the 1 h transfection period, GSH-containing medium was used. This was prepared beforehand, by dissolving GSH in DPBS (0.2 M) and subsequently diluting five times with dilution factor 10 using serum free DMEM to achieve a set of concentrations ranging from 0.002–20 mM. The COS-7 cells were transfected with 145 μL GSH-containing medium and 145 μL 25 w/w polyplexes. After the 2 day incubation period, cell viability was quantified using the MTT protocol and transfection efficiency was imaged by fluorescence microscopy.

2.8. Statistical analysis

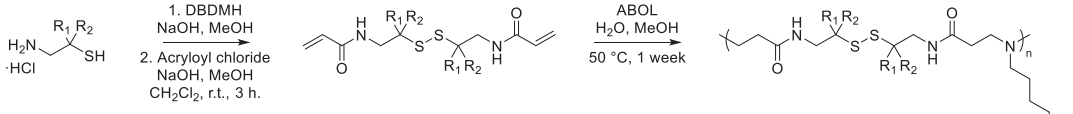
Statistical analysis ($n = 3$) was performed with SPSS 21.0, using one-way analysis of variance (ANOVA) and Tukey post hoc analysis. Differences were classified as significant ($p < 0.05$), very significant ($p < 0.01$) and extremely significant ($p < 0.001$).

3. Results and discussion

3.1. PAA polymer synthesis

Poly(amido amine)s with sterically hindered disulfides were synthesized as depicted in Scheme 1. The first step was the synthesis of the disulfide-bisacrylamide monomers **2a**–**c**. *N*,*N*′-bis(acryloyl)cystamine (**2a**) was prepared by reacting cystamine dihydrochloride with acryloyl chloride, as reported previously [68]. For the synthesis of *N*,*N*′-dithiobis(2-methylethyl)bisacrylamide (**2b**), its precursor 1-amino-2-propanethiol hydrochloride (**1b**) was prepared following the method of Fujita et al. [67]. Subsequently, thiol **1b** was oxidized to the disulfide 2,2′-dithiobis(1-propylamine) using DBDMH [69], followed by reaction with acryloyl chloride to yield the 2-methyl bisacrylamide (**2b**). Similarly, commercially available 1-amino-2-methyl-2-propanethiol hydrochloride (**1c**) was successively oxidized to 2,2′-dithiobis(2-methyl-1-propylamine) and reacted with acryloyl chloride, resulting in *N*,*N*′-dithiobis(2,2-dimethylethyl)bisacrylamide (**2c**). The structure and purity of these bisacrylamide monomers was confirmed by ^{1}H and ^{13}C NMR spectroscopy and ESI-MS.

A Michael-type polyaddition with the bisacrylamide monomers (**2a**–**c**) and ABOL was carried out for 7 days at 50 °C, followed by the addition of excess ABOL to ensure full conversion of acrylamide groups. Facile purification by dialysis and lyophilization yielded disulfide-containing poly(amido amine)s (**3a**–**c**). All polymers were analyzed by ^{1}H NMR spectroscopy, confirming successful preparation of the polymers. Further characterization by SEC indicated degrees of polymerization



1b R_1=CH_3, R_2=H
1c R_1=CH_3, R_2=CH_3

2a R_1=H, R_2=H
2b R_1=CH_3, R_2=H
2c R_1=CH_3, R_2=CH_3

3a R_1=H, R_2=H
3b R_1=CH_3, R_2=H
3c R_1=CH_3, R_2=CH_3

Scheme 1. Synthesis of disulfide-functional poly(amido amine)s. PAA^{0m} was synthesized from commercially available cystamine dihydrochloride.

Table 1
Polymer size and polydispersity index (PDI) as determined by SEC.

Polymer	#	M_n (kDa)	M_w (kDa)	PDI	DP	Buffer capacity (%)
PAA^{0m}	**3a**	7.0	10	1.45	20	61
PAA^{2m}	**3b**	4.9	6.6	1.35	13	57
PAA^{4m}	**3c**	3.8	5.4	1.41	9.4	56

(DP) of 10–20, corresponding to molecular weights of approximately 5 kDa, as shown in Table 1. The polymers displayed moderate polydispersity indices of around 1.4. Additionally, the buffering capacity of the PAAs was quantified, since this property is proposed to have a considerable influence on the proton sponge effect and therefore on endosomal release [23]. All three polymers exhibited buffer capacities of around 60% (shown in Table 1 and Fig. S1), which was considerably higher than measured for 25 kDa bPEI (21%).

3.2. Polyplex formation and stability evaluation

Polymers **3a–c** were mixed with pDNA to obtain polyplexes, at several weight ratios ranging from 6.25 to 100 w/w (polymer/pDNA). DLS measurements confirmed the formation of polyplexes, with average sizes of 50–80 nm and zeta potentials of +20 to +25 mV (Table S1 and Fig. 1). Gel electrophoresis showed increased pDNA binding with an increasing w/w ratio for all polymers (Fig. S2).

In the cell interior, PAAs are degraded by means of disulfide exchange with reducing thiols such as GSH. To assess the effect of steric hindrance on the stability of polymers **3a–c**, polyplex size and complexation ability were investigated using two techniques: DLS size evaluation and gel electrophoresis. Polyplexes were prepared at 50 w/w and exposed to the reducing agent DTT. Similar to GSH, DTT cleaves disulfide moieties in the polymer backbone, leading to polymer degradation and concomitant polyplex disassembly. In earlier work, we demonstrated PAA^{0m} dissociates rapidly in the presence of 2.5 mM DTT [70]. Polyplexes of polymers **3a–c** were subjected to 2.5 mM or 1 M DTT during the DLS measurements, and polyplex size was monitored over time. As a control, the polyplexes were measured overnight in water. The size of the polyplexes over time is presented in Fig. 2.

No notable size increase was observed for polyplexes in DTT-free water overnight. Upon addition of DTT (2.5 mM), the recorded size of

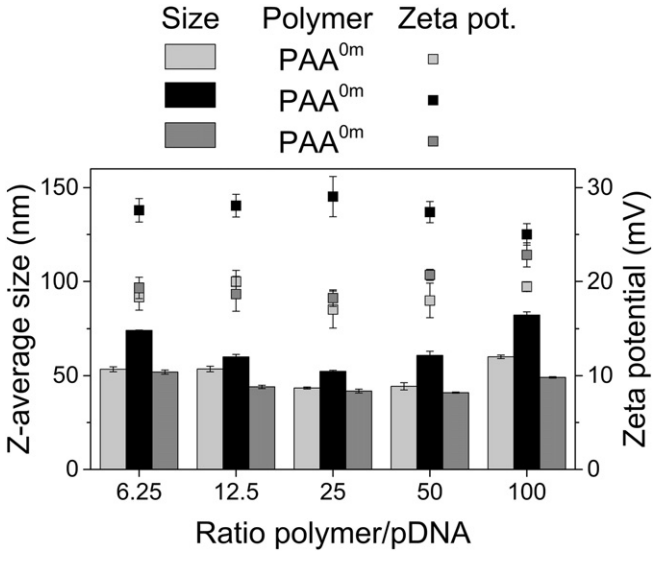


Fig. 1. The average particle size (bars) and zeta potential (squares) of polyplexes based on polymers **3a–c**, as measured by DLS (n = 3).

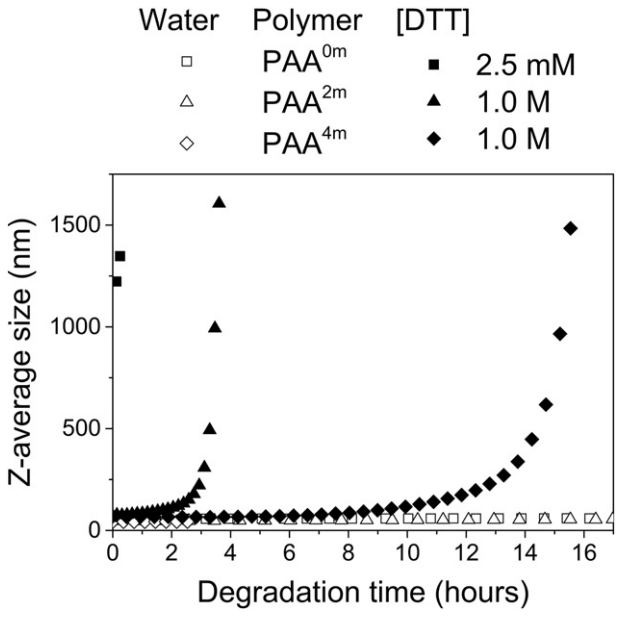


Fig. 2. Swelling behavior of 50 w/w polyplexes in water and when treated with 2.5 mM DTT (PAA^{0m}) or 1 M DTT (PAA^{2m} and PAA^{4m}); size was monitored over time using DLS.

polyplexes based on PAA^{0m} was 66 nm, however, polyplex size increased to micrometers within 5 min. The hindered-disulfide polyplexes of PAA^{2m} and PAA^{4m} showed only minor size increases after several days at this DTT concentration. Therefore, a 400-fold increase in DTT concentration (1 M) was employed to study the stability of these polyplexes over time. Even at this DTT concentration, these polyplexes remained stable for a considerably longer period as compared to the PAA^{0m} based polyplexes, i.e. polyplex size remained almost constant for 1.5 h for the moderately hindered PAA^{2m} and 8 h for the severely hindered PAA^{4m} polyplexes. Full swelling was observed after 3 h and 15 h, respectively. Swelling of the polyplexes is related to the degradation of the polymers, since the number of multivalent interactions between polymer and DNA that compact the particle is decreasing. These results demonstrate rapid degradation of PAA^{0m}, which is in good accordance with previous findings [70], while PAA^{2m} and PAA^{4m} show increasing stability as the number of hindering methyl groups increases. This confirms that polyplex stability can be tuned by incorporating steric hindrance around the disulfide bond.

The degradation behavior of the polyplexes was further investigated by gel electrophoresis, using heparin and DTT. In cells, decomplexation not only occurs through polymer degradation, but polyplexes can also undergo polyanion exchange [44]. Dissociation under the influence of various heparin concentrations was therefore investigated, results are presented in Fig. S3. No notable differences in pDNA binding strength were observed with an increasing number of methyl groups adjacent to the disulfide bond. To examine decomplexation due to polymer degradation, polyplexes were exposed to different DTT concentrations for 30 min prior to electrophoresis, and pDNA release was monitored. Electrophoresis results are shown in Fig. 3. PAA^{0m} polyplexes released their pDNA after exposure to 1 mM DTT and higher, while the PAA^{2m} polyplexes retained their pDNA up to concentrations of 10 mM DTT. PAA^{4m} based polyplexes remained intact in the entire DTT concentration range. These polyplexes remained stable even at concentrations of 1 M DTT; dissociation was only observed with the exceedingly strong reductant hydrazine. Since reduced polymer fragments are unable to form stable complexes with pDNA, the pDNA release is a direct result of polymer degradation. The gel electrophoresis results are in accordance with the results obtained from the DLS measurements, and further confirm that the incorporation of sterically hindering groups adjacent to the disulfide bond increases polyplex stability.

3.3. Cell viability

Polymers with tunable reducibility have great promise as gene delivery vectors, since they enable intracellular delivery and release of their genetic cargo. To explore the potential of these polymers for gene delivery, their cytotoxicity was first evaluated. Polyplexes were prepared by mixing the synthesized polymers with plasmid encoding for Green Fluorescent Protein (GFP) in several weight ratios. Branched PEI (25 kDa, w/w 1.25, N:P 10:1) polyplexes were used as a control, since bPEI is known to be an efficient cationic polymer for gene delivery. African green monkey kidney (COS-7) cells were exposed to polyplexes, and cytotoxicity was quantified through MTT assays (Fig. 4A and Fig. S4).

MTT assays revealed that cells show >80% viability after treatment with PAA^{0m} polyplexes, only at a very high polymer ratio of 100 w/w a decrease to 77% viability was observed. PAA^{2m} polyplexes were non-toxic at 6.25 and 12.5 w/w polymer/pDNA ratios, showing significantly higher viability than that of the bPEI polyplexes. However, at higher ratios increasing toxicity was observed for the PAA^{2m} polyplexes. Similarly, PAA^{4m} polyplexes showed toxicity at most ratios, increasing at higher ratios. All PAAs exhibited increased cell viability when transfection was performed in the presence of 10% serum (Fig. S4). These MTT assays indicate moderately increasing toxicity as the number of methyl groups in the polymers increases. Elevated toxicity can be expected, especially for the PAA^{4m} polyplexes, since their stability is dramatically increased compared to the PAA^{0m} polyplexes and intact polymers exert more cytotoxicity than degraded polymer fragments. Furthermore, the addition of two or four methyl groups in each monomer renders the polymers significantly more hydrophobic, which may also contribute to the decreased cell viability, since hydrophobicity is a known cause for cytotoxicity [71]. Overall, it can be concluded that, in contrast to 25 kDa bPEI, both the PAA^{0m} and PAA^{2m} polyplexes show no or only low cytotoxicity, making them highly suitable for gene delivery.

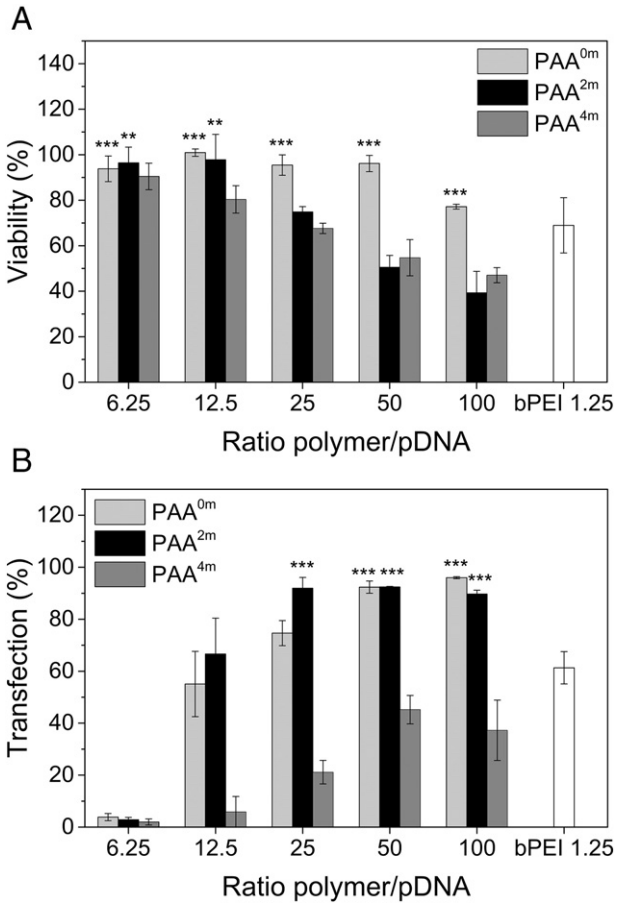


Fig. 4. In vitro results (see also Fig. S4) of (A) the cell viability of COS-7 cells exposed to PAA^{0m}, PAA^{2m} and PAA^{4m} polyplexes containing GFP pDNA at different polymer/pDNA ratios (1 μg pDNA/well) for 1 h in serum-free medium. (B) Transfection efficiency as measured by FACS. Asterisks indicate significant improvement relative to 25 kDa bPEI, $^{**}p < 0.01$ and $^{***}p < 0.001$ ($n = 3$).

3.4. Transfection efficiency

To assess the potential of these polymers for gene therapy, gene transfection efficiency on COS-7 cells was investigated using GFP-encoding pDNA. The percentage of transfected cells was quantified with FACS, the results are presented in Fig. 4B. PAA^{0m} polyplexes gave high transfection efficiencies – up to 96% – which were significantly higher at the two highest ratios compared to the control bPEI polyplexes. PAA^{2m} polyplexes show already very high efficiency of 92% at 25 w/w polymer/pDNA ratio, which remains almost constant at higher ratios. A moderate increase in transfection efficiency is observed with PAA^{2m} compared to PAA^{0m}, which can be attributed to the increase in polyplex stability but also to the addition of hydrophobic methyl groups, since hydrophobicity is reported to be a favorable factor for gene delivery [72,73]. However, transfection efficiencies with the PAA^{4m} polyplexes did not surpass 42%, which is most likely an effect of their high stability. The (extraordinary) stability of PAA^{4m} can prevent intracellular disassembly of the polyplexes, which leads to less free pDNA available for transcription and translation. For all polymers, addition of 10% serum to the medium during transfection reduced their transfection efficiency (Fig. S4). Taking into account the cytotoxicity, PAA^{0m} and PAA^{2m} emerge as promising polymers for gene delivery, performing better than bPEI in terms of both cell viability and transfection efficiency.

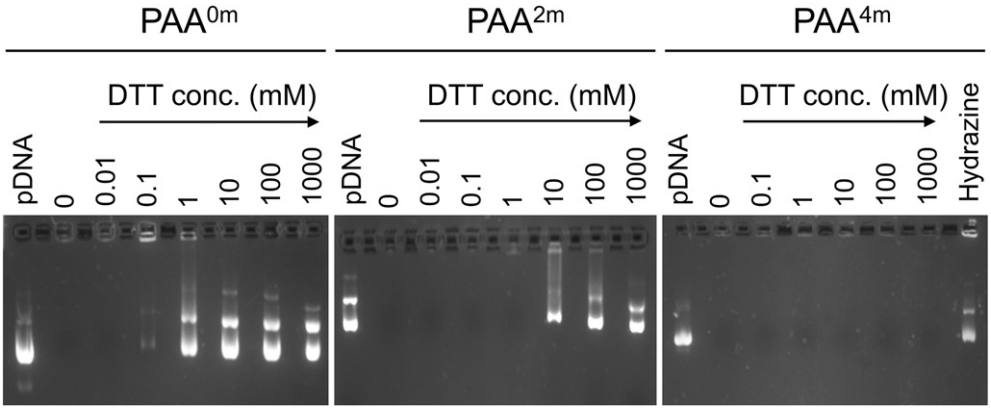


Fig. 3. Gel electrophoresis results of 50 w/w polyplexes, after treatment with increasing concentrations of DTT during 30 min. Polyplexes based on PAA^{4m} were also incubated with hydrazine (1 M) for 30 min.

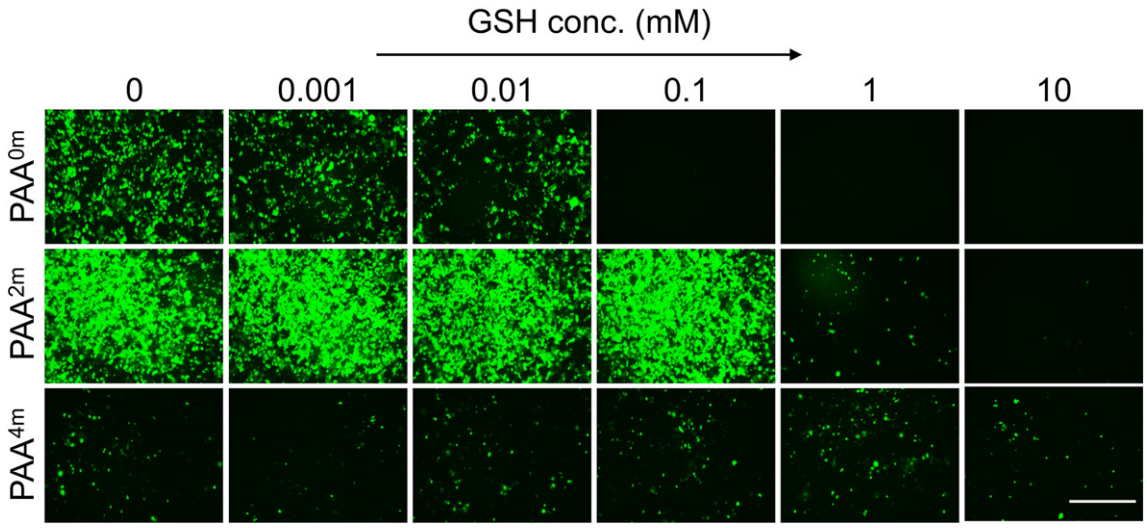


Fig. 5. Transfection of COS-7 cells in the presence of GSH-containing medium, by polyplexes of the PAAs and GFP pDNA at 25 w/w ratio. Scale bar is 1 mm.

Preventing degradation of the polyplexes before they reach the cytoplasm is a key factor for successful transfection. To determine at which GSH concentrations (the most prominent intracellular reducing agent) the polyplexes remain stable and functional, the cell culture medium was supplemented with GSH during transfection experiments. The degradation that would normally take place intracellularly is now induced in the medium through artificially high glutathione levels. When extracellular polyplex degradation occurs faster than cellular uptake, the transfection efficiency should be significantly affected, characterized by a loss in GFP expression. The results of COS-7 transfections with GFP-pDNA polyplexes at 25 w/w ratio under increasing GSH concentrations from 0 to 10 mM as visualized by fluorescence microscopy are given in Fig. 5. MTT assays of these transfection experiments revealed that the added GSH concentrations in the cell culture medium did not have significant influence on the cytotoxicity of the polyplexes (Fig. S5).

As shown in Fig. 5, PAA^{0m} polyplexes exhibited efficient transfection at GSH concentrations 0.01 mM and lower. However, at GSH concentrations of 0.1 mM and higher, GFP-positive cells were no longer present. For the PAA^{2m} polyplexes an increased GFP intensity was observed, and the decrease in GFP production occurred at an approximately 100-fold higher GSH concentration. Transfection with the PAA^{4m} polyplexes resulted in lower GFP production and almost no effect of GSH concentrations in de medium was observed. The diminished GFP production upon increasing GSH concentration for PAA^{0m} and PAA^{2m} is not due to a decrease of viable cells; rather it implies polyplex dissociation before cell internalization. GSH-induced disulfide cleavage of the polymer leads to the formation of short polymer fragments that are unable to bind and transport the pDNA, resulting in premature polyplex disassembly. The cell transfection images are in good accordance with the DLS and gel electrophoresis data, once more confirming that increasing the steric hindrance around the disulfide bond increases the stability of the polyplexes.

These data suggest that PAA^{2m} is an excellent polymeric gene vector, remaining stable in reducing conditions similar to plasma (GSH concentrations ~ 0.002 mM), while fully degrading intracellularly (GSH concentrations ~ 1–20 mM) [42,43]. Of course, the optimal stability of a gene carrier depends on its application. For example, elevated GSH levels were measured in different types of tumors, both intra- and extracellularly [74]. These elevated levels provide protection against oxidative stress and cytotoxic compounds, and are due to GSH release by dying cells, while the high cell proliferation, characteristic for tumors, is associated with increased reducing strength [75–77]. The Michael-type polyaddition employed in this study may also be used to prepare polymers containing varying degrees of sterically-hindered disulfides, which allows fine-tuning the stability of these polymers, and their resulting polyplexes, to exploit the specific GSH levels found in different tumors.

4. Conclusions

Bioreducible poly(amido amine)s (PAAs) with varying degrees of steric hindrance around the disulfide bond were synthesized through introduction of adjacent methyl groups. The polymers become increasingly less susceptible to bioreduction (e.g. cleavage by glutathione attack) as steric hindrance near the disulfide bond increases. Tuning the stability of the PAAs, and therefore the polyplexes, allows precise control over DNA release. The moderately hindered PAA^{2m} exhibited excellent properties: remaining stable at extracellular GSH concentrations, while readily degrading at intracellular GSH concentrations. Moreover, this polymer significantly outperformed a commonly used cationic polymer for gene delivery, 25 kDa branched PEI. At lower N:P ratios, the PAA^{2m}-based polyplexes have significantly lower toxicity than bPEI polyplexes, and superior transfection efficiency was observed at higher N:P ratios. These results highlight the excellent potential of these polymers for gene therapy. Furthermore, this approach – using steric hindrance to adjust the stability of bioreducible PAAs – may be employed to optimize PAA stability for specific applications such as intra-tumoral gene delivery.

Acknowledgments

The funding of this work by the provinces of Overijssel and Gelderland, as well as the project consortium by the Center for Medical Imaging – North East Netherlands (CMI-NEN) is gratefully acknowledged. The authors acknowledge Dr. Karin Roelofs for scientific discussions.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2016.08.021.

References

[1] K.M. Boycott, M.R. Vanstone, D.E. Bulman, A.E. MacKenzie, Rare-disease genetics in the era of next-generation sequencing: discovery to translation, Nat. Rev. Genet. 14 (2013) 681–691, http://dx.doi.org/10.1038/nrg3555.
[2] S.E. McCandless, J.W. Brunger, S.B. Cassidy, The burden of genetic disease on inpatient care in a children's hospital, Am. J. Hum. Genet. 74 (2004) 121–127, http://dx.doi.org/10.1086/381053.

[3] A. Biffi, E. Montini, L. Lorioli, M. Cesani, F. Fumagalli, T. Plati, et al., Lentiviral hematopoietic stem cell gene therapy benefits metachromatic leukodystrophy, Science (80-) 341 (2013) 1233158, http://dx.doi.org/10.1126/science.1233158 (1–11).

[4] S. Hacein-Bey-Abina, J. Hauer, A. Lim, C. Picard, G.P. Wang, C.C. Berry, et al., Efficacy of gene therapy for X-linked severe combined immunodeficiency, N. Engl. J. Med. 363 (2010) 355–364, http://dx.doi.org/10.1056/NEJMoa1000164.

[5] S.O. Freytag, H. Stricker, M. Lu, M. Elshaikh, I. Aref, D. Pradhan, et al., Prospective randomized phase 2 trial of intensity modulated radiation therapy with or without oncolytic adenovirus-mediated cytotoxic gene therapy in intermediate-risk prostate cancer, Int. J. Radiat. Oncol. 89 (2014) 268–276, http://dx.doi.org/10.1016/j.ijrobp.2014.02.034.

[6] M. Izquierdo, Short interfering RNAs as a tool for cancer gene therapy, Cancer Gene Ther. 12 (2005) 217–227, http://dx.doi.org/10.1038/sj.cgt.7700791.

[7] H.-P. Kiem, K.R. Jerome, S.G. Deeks, J.M. McCune, Hematopoietic-stem-cell-based gene therapy for HIV disease, Cell Stem Cell 10 (2012) 137–147, http://dx.doi.org/10.1016/j.stem.2011.12.015.

[8] J.-M. Escoffre, J. Teissié, M.-P. Rols, Gene transfer: how can the biological barriers be overcome? J. Membr. Biol. 236 (2010) 61–74, http://dx.doi.org/10.1007/s00232-010-9275-0.

[9] C.E. Thomas, A. Ehrhardt, M.A. Kay, Progress and problems with the use of viral vectors for gene therapy, Nat. Rev. Genet. 4 (2003) 346–358, http://dx.doi.org/10.1038/nrg1066.

[10] M.A. Mintzer, E.E. Simanek, Nonviral vectors for gene delivery, Chem. Rev. 109 (2009) 259–302, http://dx.doi.org/10.1021/cr800409e.

[11] J.C. Burns, T. Friedmann, W. Driever, M. Burrascano, J.K. Yee, Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells, Proc. Natl. Acad. Sci. 90 (1993) 8033–8037, http://dx.doi.org/10.1073/pnas.90.17.8033.

[12] C.S. Manno, V.R. Arruda, G.F. Pierce, B. Glader, M. Ragni, J. Rasko, et al., Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response, Nat. Med. 12 (2006) 342–347, http://dx.doi.org/10.1038/nm1358.

[13] U.P. Dave, Gene therapy insertional mutagenesis insights, Science (80-) 303 (2004) 333, http://dx.doi.org/10.1126/science.1091667.

[14] D.W. Pack, A.S. Hoffman, S. Pun, P.S. Stayton, Design and development of polymers for gene delivery, Nat. Rev. Drug Discov. 4 (2005) 581–593, http://dx.doi.org/10.1038/nrd1775.

[15] J.D. Hood, Tumor regression by targeted gene delivery to the neovasculature, Science (80-) 296 (2002) 2404–2407, http://dx.doi.org/10.1126/science.1070200.

[16] R. Kircheis, A. Kichler, G. Wallner, M. Kursa, M. Ogris, T. Felzmann, et al., Coupling of cell-binding ligands to polyethylenimine for targeted gene delivery, Gene Ther. 4 (1997) 409–418, http://dx.doi.org/10.1038/sj.gt.3300418.

[17] N.C. Bellocq, S.H. Pun, G.S. Jensen, M.E. Davis, Transferrin-containing, cyclodextrin polymer-based particles for tumor-targeted Gene delivery, Bioconjug. Chem. 14 (2003) 1122–1132, http://dx.doi.org/10.1021/bc034125f.

[18] J.P. Behr, Synthetic gene-transfer vectors, Acc. Chem. Res. 26 (1993) 274–278, http://dx.doi.org/10.1021/ar00029a008.

[19] M.M. Lufino, P.A. Edser, R. Wade-Martins, Advances in high-capacity extrachromosomal vector technology: episomal maintenance, vector delivery, and transgene expression, Mol. Ther. 16 (2008) 1525–1538, http://dx.doi.org/10.1038/mt.2008.156.

[20] T. Niidome, L. Huang, Gene therapy progress and prospects: nonviral vectors, Gene Ther. 9 (2002) 1647–1652, http://dx.doi.org/10.1038/sj.gt.3301923.

[21] T. Park, J. Jeong, S. Kim, Current status of polymeric gene delivery systems, Adv. Drug Deliv. Rev. 58 (2006) 467–486, http://dx.doi.org/10.1016/j.addr.2006.03.007.

[22] A. Aied, U. Greiser, A. Pandit, W. Wang, Polymer gene delivery: overcoming the obstacles, Drug Discov. Today 18 (2013) 1090–1098, http://dx.doi.org/10.1016/j.drudis.2013.06.014.

[23] O. Boussif, F. Lezoualc'h, M.A. Zanta, M.D. Mergny, D. Scherman, B. Demeneix, et al., A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine, Proc. Natl. Acad. Sci. 92 (1995) 7297–7301, http://dx.doi.org/10.1073/pnas.92.16.7297.

[24] D.Y. Kwoh, C.C. Coffin, C.P. Lollo, J. Jovenal, M.G. Banaszczyk, P. Mullen, et al., Stabilization of poly-L-lysine/DNA polyplexes for in vivo gene delivery to the liver, Biochim. Biophys. Acta Gene Struct. Expr. 1444 (1999) 171–190, http://dx.doi.org/10.1016/S0167-4781(98)00274-7.

[25] S. Agarwal, Y. Zhang, S. Maji, A. Greiner, PDMAEMA based gene delivery materials, Mater. Today. 15 (2012) 388–393, http://dx.doi.org/10.1016/S1369-7021(12)70165-7.

[26] C. Dufes, I. Uchegbu, A. Schatzlein, Dendrimers in gene delivery, Adv. Drug Deliv. Rev. 57 (2005) 2177–2202, http://dx.doi.org/10.1016/j.addr.2005.09.017.

[27] C.O. Mellet, J.M.G. Fernández, J.M. Benito, Cyclodextrin-based gene delivery systems, Chem. Soc. Rev. 40 (2011) 1586–1608, http://dx.doi.org/10.1039/C0CS00019A.

[28] D. Anderson, A. Akinc, N. Hossain, R. Langer, Structure/property studies of polymeric gene delivery using a library of poly(β-amino esters), Mol. Ther. 11 (2005) 426–434, http://dx.doi.org/10.1016/j.ymthe.2004.11.015.

[29] M. Piest, C. Lin, M.A. Mateos-Timoneda, M.C. Lok, W.E. Hennink, J. Feijen, et al., Novel poly(amido amine)s with bioreducible disulfide linkages in their diamino-units: structure effects and in vitro gene transfer properties, J. Control. Release 130 (2008) 38–45, http://dx.doi.org/10.1016/j.jconrel.2008.05.023.

[30] C. Lin, J.F.J. Engbersen, Effect of chemical functionalities in poly(amido amine)s for non-viral gene transfection, J. Control. Release 132 (2008) 267–272, http://dx.doi.org/10.1016/j.jconrel.2008.06.022.

[31] C. Lin, C.-J. Blaauboer, M.M. Timoneda, M.C. Lok, M. van Steenbergen, W.E. Hennink, et al., Bioreducible poly(amido amine)s with oligoamine side chains: synthesis, characterization, and structural effects on gene delivery, J. Control. Release 126 (2008) 166–174, http://dx.doi.org/10.1016/j.jconrel.2007.11.012.

[32] F. Martello, M. Piest, J.F.J. Engbersen, P. Ferruti, Effects of branched or linear architecture of bioreducible poly(amido amine)s on their in vitro gene delivery properties, J. Control. Release 164 (2012) 372–379, http://dx.doi.org/10.1016/j.jconrel.2012.07.029.

[33] C. Lin, Z. Zhong, M.C. Lok, X. Jiang, W.E. Hennink, J. Feijen, et al., Novel bioreducible poly(amido amine)s for highly efficient gene delivery, Bioconjug. Chem. 18 (2007) 138–145, http://dx.doi.org/10.1021/bc060200l.

[34] D. Oupický, J. Li, Bioreducible polycations in nucleic acid delivery: past, present, and future trends, Macromol. Biosci. 14 (2014) 908–922, http://dx.doi.org/10.1002/mabi.201400061.

[35] P.M. Klein, E. Wagner, Bioreducible polycations as shuttles for therapeutic nucleic acid and protein transfection, Antioxid. Redox Signal. 21 (2014) 804–817, http://dx.doi.org/10.1089/ars.2013.5714.

[36] F. Meng, W.E. Hennink, Z. Zhong, Reduction-sensitive polymers and bioconjugates for biomedical applications, Biomaterials 30 (2009) 2180–2198, http://dx.doi.org/10.1016/j.biomaterials.2009.01.026.

[37] Y.S. Lee, S.W. Kim, Bioreducible polymers for therapeutic gene delivery, J. Control. Release 190 (2014) 424–439, http://dx.doi.org/10.1016/j.jconrel.2014.04.012.

[38] R. Cheng, F. Feng, F. Meng, C. Deng, J. Feijen, Z. Zhong, Glutathione-responsive nanovehicles as a promising platform for targeted intracellular drug and gene delivery, J. Control. Release 152 (2011) 2–12, http://dx.doi.org/10.1016/j.jconrel.2011.01.030.

[39] K. Miyata, Y. Kakizawa, N. Nishiyama, A. Harada, Y. Yamasaki, H. Koyama, et al., Block catiomer polyplexes with regulated densities of charge and disulfide cross-linking directed to enhance gene expression, J. Am. Chem. Soc. 126 (2004) 2355–2361, http://dx.doi.org/10.1021/ja0379666.

[40] S. Bauhuber, C. Hozsa, M. Breunig, A. Göpferich, Delivery of nucleic acids via disulfide-based carrier systems, Adv. Mater. 21 (2009) 3286–3306, http://dx.doi.org/10.1002/adma.200802453.

[41] L. Brülisauer, M.A. Gauthier, J.-C. Leroux, Disulfide-containing parenteral delivery systems and their redox-biological fate, J. Control. Release 195 (2014) 147–154, http://dx.doi.org/10.1016/j.jconrel.2014.06.012.

[42] D.S. Manickam, D. Oupický, Polyplex gene delivery modulated by redox potential gradients, J. Drug Target. 14 (2006) 519–526, http://dx.doi.org/10.1080/10611860600834409.

[43] D.P. Jones, J.L. Carlson, P.S. Samiec, P. Sternberg, V.C. Mody, R.L. Reed, et al., Glutathione measurement in human plasma, Clin. Chim. Acta 275 (1998) 175–184, http://dx.doi.org/10.1016/S0009-8981(98)00089-8.

[44] D. Oupický, R.C. Carlisle, L.W. Seymour, Triggered intracellular activation of disulfide crosslinked polyelectrolyte gene delivery complexes with extended systemic circulation in vivo, Gene Ther. 8 (2001) 713–724, http://dx.doi.org/10.1038/sj.gt.3301446.

[45] Y. Kakizawa, A. Harada, K. Kataoka, Glutathione-sensitive stabilization of block copolymer micelles composed of antisense DNA and thiolated poly(ethylene glycol)-block-poly(L-lysine): a potential carrier for systemic delivery of antisense DNA, Biomacromolecules 2 (2001) 491–497, http://dx.doi.org/10.1021/bm000142l.

[46] M.A. Gosselin, W. Guo, R.J. Lee, Efficient gene transfer using reversibly cross-linked low molecular weight polyethylenimine, Bioconjug. Chem. 12 (2001) 989–994, http://dx.doi.org/10.1021/bc0100455.

[47] A. Meister, M.E. Anderson, Glutathione, Annu. Rev. Biochem. 52 (1983) 711–760, http://dx.doi.org/10.1146/annurev.bi.52.070183.003431.

[48] N.L. Letvin, V.S. Goldmacher, J. Ritz, J.M. Yetz, S.F. Schlossman, J.M. Lambert, In vivo administration of lymphocyte-specific monoclonal antibodies in nonhuman primates. In vivo stability of disulfide-linked immunotoxin conjugates, J. Clin. Invest. 77 (1986) 977–984, http://dx.doi.org/10.1172/JCI112399.

[49] S.F. Carroll, S.L. Bernhard, D.A. Goff, R.J. Bauer, W. Leach, A.H.C. Kung, Enhanced stability in vitro and in vivo of immunoconjugates prepared with 5-methyl-2-iminothiolane, Bioconjug. Chem. 5 (1994) 248–256, http://dx.doi.org/10.1021/bc00027a010.

[50] P.E. Thorpe, P.M. Wallace, P.P. Knowles, M.G. Relf, A.N. Brown, G.J. Watson, et al., New coupling agents for the synthesis of immunotoxins containing a hindered disulfide bond with improved stability in vivo, Cancer Res. 47 (1987) 5924–5931http://www.ncbi.nlm.nih.gov/pubmed/3499221.

[51] S. Arpicco, F. Dosio, P. Brusa, P. Crosasso, L. Cattel, New coupling reagents for the preparation of disulfide cross-linked conjugates with increased stability, Bioconjug. Chem. 8 (1997) 327–337, http://dx.doi.org/10.1021/bc970025w.

[52] L. Greenfield, W. Bloch, M. Moreland, Thiol-containing crosslinking agent with enhanced steric hindrance, Bioconjug. Chem. 1 (1990) 400–410, http://dx.doi.org/10.1021/bc00006a006.

[53] W. Sun, P.B. Davis, Reducible DNA nanoparticles enhance in vitro gene transfer via an extracellular mechanism, J. Control. Release 146 (2010) 118–127, http://dx.doi.org/10.1016/j.jconrel.2010.04.031.

[54] C. Wu, S. Wang, L. Brülisauer, J.-C. Leroux, M.A. Gauthier, Broad control of disulfide stability through microenvironmental effects and analysis in complex redox environments, Biomacromolecules 14 (2013) 2383–2388, http://dx.doi.org/10.1021/bm400501c.

[55] L. Brülisauer, N. Kathriner, M. Prenrecaj, M.A. Gauthier, J.-C. Leroux, Tracking the bioreduction of disulfide-containing cationic dendrimers, Angew. Chem. Int. Ed. 51 (2012) 12454–12458, http://dx.doi.org/10.1002/anie.201207070.

[56] D. Pezzoli, M. Zanda, R. Chiesa, G. Candiani, The yin of exofacial protein sulfhydryls and the yang of intracellular glutathione in in vitro transfection with SS14 bioreducible lipoplexes, J. Control. Release 165 (2013) 44–53, http://dx.doi.org/10.1016/j.jconrel.2012.10.016.

[57] S. Aubry, F. Burlina, E. Dupont, D. Delaroche, A. Joliot, S. Lavielle, et al., Cell-surface thiols affect cell entry of disulfide-conjugated peptides, FASEB J. 23 (2009) 2956–2967, http://dx.doi.org/10.1096/fj.08-127563.

[58] J. Wan, S. Persiani, W.-C. Shen, Transcellular processing of disulfide-and thioether-linked peroxidase-polylysine conjugates in cultured MDCK epithelial cells, J. Cell. Physiol. 145 (1990) 9–15, http://dx.doi.org/10.1002/jcp.1041450103.

[59] H.J. Ryser, R. Mandel, F. Ghani, Cell Surface sulfhydryls are required for the cytotoxicity of diphtheria toxin but not of ricin in chinese hamster ovary cells, J. Biol. Chem. 266 (1991) 18439–18442http://www.ncbi.nlm.nih.gov/pubmed/1655751.
[60] G. Bulaj, T. Kortemme, D.P. Goldenberg, Ionization–reactivity relationships for cysteine thiols in polypeptides, Biochemistry 37 (1998) 8965–8972, http://dx.doi.org/10.1021/bi973101r.
[61] C. Wu, J.-C. Leroux, M.A. Gauthier, Twin disulfides for orthogonal disulfide pairing and the directed folding of multicyclic peptides, Nat. Chem. 4 (2012) 1044–1049, http://dx.doi.org/10.1038/nchem.1487.
[62] C. Wu, C. Belenda, J.-C. Leroux, M.A. Gauthier, Interplay of chemical microenvironment and redox environment on thiol-disulfide exchange kinetics, Chemistry (Easton) 17 (2011) 10064–10070, http://dx.doi.org/10.1002/chem.201101024.
[63] J. Zhang, Pharmaco attributes of dioleoylphosphatidylethanolamine/cholesterylhemisuccinate liposomes containing different types of cleavable lipopolymers, Pharmacol. Res. 49 (2004) 185–198, http://dx.doi.org/10.1016/j.phrs.2003.09.003.
[64] B.A. Kellogg, L. Garrett, Y. Kovtun, K.C. Lai, B. Leece, M. Miller, et al., Disulfide-linked antibody–maytansinoid conjugates: optimization of in vivo activity by varying the steric hindrance at carbon atoms adjacent to the disulfide linkage, Bioconjug. Chem. 22 (2011) 717–727, http://dx.doi.org/10.1021/bc100480a.
[65] P. Nadrah, U. Maver, A. Jemec, T. Tišler, M. Bele, G. Dražić, et al., Hindered disulfide bonds to regulate release rate of model drug from mesoporous silica, ACS Appl. Mater. Interfaces 5 (2013) 3908–3915, http://dx.doi.org/10.1021/am400604d.
[66] P.R. Hamann, L.M. Hinman, C.F. Beyer, D. Lindh, J. Upeslacis, D.A. Flowers, et al., An anti-CD33 Antibody–calicheamicin conjugate for treatment of acute myeloid leukemia. Choice of linker, Bioconjug. Chem. 13 (2002) 40–46, http://dx.doi.org/10.1021/bc0100206.
[67] M. Fujita, Y. Miyashita, N. Amir, Y. Kawamoto, K. Kanamori, K. Fujisawa, et al., Stereoselective formation and properties of mononuclear and polynuclear nickel(II) complexes with 1-amino-2-propanethiolate, Polyhedron 24 (2005) 1991–2001, http://dx.doi.org/10.1016/j.poly.2005.05.023.
[68] E. Emilitri, E. Ranucci, P. Ferruti, New poly(amidoamine)s containing disulfide linkages in their main chain, J. Polym. Sci. Part A Polym. Chem. 43 (2005) 1404–1416, http://dx.doi.org/10.1002/pola.20599.
[69] A. Alam, Y. Takaguchi, S. Tsuboi, Simple, extremely fast, and high-yielding oxidation of thiols to disulfides, Synth. Commun. 35 (2005) 1329–1333, http://dx.doi.org/10.1081/SCC-200057253.
[70] L.J. van der Aa, P. Vader, G. Storm, R.M. Schiffelers, J.F.J. Engbersen, Optimization of poly(amido amine)s as vectors for siRNA delivery, J. Control. Release 150 (2011) 177–186, http://dx.doi.org/10.1016/j.jconrel.2010.11.030.
[71] T. Atsumi, S. Fujisawa, K. Tonosaki, (Meth)acrylate monomer-induced cytotoxicity and intracellular Ca2+ mobilization in human salivary gland carcinoma cells and human gingival fibroblast cells related to monomer hydrophobicity, Biomaterials 27 (2006) 5794–5800, http://dx.doi.org/10.1016/j.biomaterials.2006.07.041.
[72] M. Piest, J.F.J. Engbersen, Effects of charge density and hydrophobicity of poly(amido amine)s for non-viral gene delivery, J. Control. Release 148 (2010) 83–90, http://dx.doi.org/10.1016/j.jconrel.2010.07.109.
[73] M. Kurisawa, M. Yokoyama, T. Okano, Transfection efficiency increases by incorporating hydrophobic monomer units into polymeric gene carriers, J. Control. Release 68 (2000) 1–8, http://dx.doi.org/10.1016/S0168-3659(00)00246-7.
[74] P. Kuppusamy, H. Li, G. Ilangovan, A.J. Cardounel, J.L. Zweier, K. Yamada, et al., Noninvasive imaging of tumor redox status and its modification by tissue glutathione levels, Cancer Res. 62 (2002) 307–312http://www.ncbi.nlm.nih.gov/pubmed/11782393.
[75] G.K. Balendiran, R. Dabur, D. Fraser, The role of glutathione in cancer, Cell Biochem. Funct. 22 (2004) 343–352, http://dx.doi.org/10.1002/cbf.1149.
[76] M. Steiner, I. Hartmann, E. Perrino, G. Casi, S. Brighton, I. Jelesarov, et al., Spacer length shapes drug release and therapeutic efficacy of traceless disulfide-linked ADCs targeting the tumor neovasculature, Chem. Sci. 4 (2013) 297–302, http://dx.doi.org/10.1039/C2SC21107F.
[77] J.M. Estrela, A. Ortega, E. Obrador, Glutathione in cancer biology and therapy, Crit. Rev. Clin. Lab. Sci. 43 (2006) 143–181, http://dx.doi.org/10.1080/10408360500523878.