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Effect of cell membrane thiols and reduction-triggered disassembly on transfection activity of bio-reducible polyplexes

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Abstract

Bio-reducible polyplexes are promising vectors for delivery of nucleic acids due to low toxicity and favorable transfection activity. The often improved transfection is usually explained by enhanced intracellular reductive disassembly of the polyplexes. This study evaluated the effect of enhanced reductive disassembly on transfection activity of plasmid DNA and antisense oligonucleotide (AON) polyplexes based on a series of bio-reducible poly(amido amine)s (PAA). We found that the presence of disulfide bonds in PAA had no effect on nucleic acid binding, hydrodynamic size and zeta potential of polyplexes. Increasing the disulfide content in PAA increased susceptibility to reduction-triggered DNA and AON release from the polyplexes. Increasing the disulfide content in PAA increased DNA transfection but had no effect on AON transfection. Plasma membrane protein thiols played a key role in the observed enhancement of DNA transfection. The presence of disulfide bonds in PAA had no significant effect on the rate of intracellular DNA clearance, suggesting that enhanced intracellular disassembly of the bio-reducible polyplexes is not a major contributing factor to the improved transfection activity.

Keywords

Polyplexes; Gene delivery; Transfection; Reducible polycations

1. Introduction

Polyplexes of nucleic acids and bio-reducible polycations have demonstrated promise as delivery vectors of a variety of potential nucleic acid therapeutics (Manickam et al., 2010; Namgung et al., 2010; Rahbek et al., 2008). The bio-reducible polyplexes take advantage of the strong reducing intracellular environment in which the disulfide-containing polycations are readily broken down by thiol–disulfide exchange reactions. The intracellular degradation is then responsible for decreased toxicity of bio-reducible polycations and is often credited also with increased transfection activity of due to easier DNA release. The reducing intracellular environment is primarily maintained by small redox molecules like glutathione (GSH) either with or without the help of redox enzymes (Donoghue and Hogg, 2002; Gilbert, 1997; Jiang et al., 1999). Recent study has surprisingly indicated that free cysteine thiols present in intracellular proteins are active components of cellular redox buffer and that they may represent a pool of active reducing species that is even larger than the pool of low molecular weight thiols like GSH (Hansen et al., 2009).

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Improved transfection activity of bioreducible polyplexes is an almost universal finding reported with various different types of bioreducible polycations, including polypeptides, polymethacrylates, and poly(amido amine)s (PAA) (Chen et al., 2006; Christensen et al., 2006; Lin et al., 2006; Manickam and Oupicky, 2006; Piest et al., 2008; You et al., 2007). The increased transfection activity is believed to be the consequence of the decreased affinity between DNA and the bioreducible polycations after their intracellular degradation. The reduced affinity is expected to enhance transcriptional availability of DNA due to faster and easier polyplex disassembly (Christensen et al., 2006; Read et al., 2003). This notion is supported by at least some available evidence, which suggest that artificial changes in intracellular GSH levels can change transfection activity of bioreducible polyplexes, although the effect is often small. In general, increasing intracellular GSH by incubation with a membrane-permeable derivatives of GSH leads to an increase in transfection of bioreducible polyplexes (Christensen et al., 2006; Neu et al., 2007; Read et al., 2003). Some reports, however, found the opposite effect as increased GSH lowered transfection (Read et al., 2005). Similar conflicting evidence is available from studies in which intracellular GSH is depleted by inhibiting its *de novo* synthesis. In most cases, a small decrease in transfection is observed after inhibiting GSH synthesis with inhibitor of γ -glutamylcysteine synthetase, buthionine sulfoximine (Christensen et al., 2006; Manickam et al., 2010; Neu et al., 2007; Read et al., 2003, 2005). The opposite effect of GSH inhibition on transfection of bioreducible polyplexes was reported in a study that showed increased transfection after inhibition of GSH biosynthesis (Hoon Jeong et al., 2007). These results suggest that the notion of a straightforward relationship between intracellular reducing potential, enhanced disassembly, and transfection activity of bioreducible polyplexes could be an oversimplified view.

In the present study, we evaluated how enhanced reductive disassembly affects transfection activity of plasmid DNA and antisense oligonucleotides (AON) polyplexes based on a series of bioreducible polycations with increasing content of disulfide bonds. We hypothesized that the molecular weight of the used nucleic acid would strongly influence activity of bioreducible polyplexes because of the differences in binding affinity with polycations.

2. Materials and methods

2.1. Materials

N,N'-hexamethylenebisacrylamide (HMBA) and *N,N'*-cystaminebisacrylamide (CBA) were obtained from Polysciences, Inc. (Warrington, PA). 1-(2-Aminoethyl) piperazine (AEPZ) was obtained from Acros Organics (Fisher Scientific, Pittsburgh, PA). Plasmid DNA, gWiz high-expression luciferase (gWiz-Luc) containing luciferase reporter gene was from Aldevron (Fargo, ND). Luciferase antisense oligonucleotides (AON) (5'-AACCGCTTCCCCGACTTCC-3') and its scrambled control (5'-CCAATGTCAAGCACTTCCGTT-3') with phosphorothioate linkages were custom synthesized by Midland Certified Reagent Company (Midland, TX). 1,2-Dioleoyl-3-trimethylammonium-propane chloride (DOTAP) was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). YOYO[®]-1 iodide, Dulbecco's Modified Eagle Medium (DMEM), Dulbecco's Phosphate Buffered Saline (PBS), Fetal Bovine Serum (FBS) and non-essential amino acids (NEAA) were from Invitrogen (Carlsbad, CA). All other reagents and chemicals were obtained from Fisher Scientific or VWR International unless otherwise noted.

2.2. Synthesis and characterization of PAA

A series of five linear PAA containing different disulfide content was synthesized by Michael addition of triamine AEPZ and different molar ratio of reducible CBA and non-

reducible HMBA (Scheme 1) following a published method (Lin et al., 2006; Manickam et al., 2010). Calculated amounts of HMBA, CBA and AEPZ were dissolved in methanol/water (7/3 v/v) and the polymerization was allowed to proceed in the dark at 37 °C for 72 h to form the linear polymers (Hong et al., 2007). The reaction mixtures were then diluted with water, acidified to pH 4 with HCl, and purified using centrifugal membrane filters (molecular weight cut-off 3 kDa). The concentrates were lyophilized and the composition of the polymers was analyzed by ¹H NMR. Absence of branched structures in the linear PAA was confirmed by ¹³C NMR. The following CBA:HMBA molar ratios were used in the polymerization: 0:1, 1:3, 1:1, 3:1 and 1:0, which resulted in a series of PAA with CBA content of 0, 25, 50, 75 and 100%. Weight – (M_w) and number – (M_n) average molecular weights of the polymers were determined by GPC using Shimadzu LC-10ADVP liquid chromatograph equipped with a multiangle light scattering detector and an interferometric refractometer (Wyatt Technology, Santa Barbara, CA). Sodium acetate (300 mM, pH 4.5) was used as an eluent at a flow rate of 1.0 mL/min and temperature of 35 °C.

2.3. Ethidium bromide and OliGreen exclusion assay

The ability of PAA to condense DNA was determined by ethidium bromide (EtBr) exclusion assay by measuring the changes in EtBr/DNA fluorescence. DNA solution at a concentration of 20 µg/mL was mixed with EtBr (1 µg/mL), fluorescence was measured and set to 100% using an excitation wavelength of 540 nm and an emission wavelength of 590 nm. Fluorescence readings were taken following a stepwise addition of the polycation solution, and the condensation curve for each polycation was constructed. The ability of PAA to condense AON was determined in a similar way by measuring changes in OliGreen/AON fluorescence, using 490 nm excitation and 520 nm emission.

2.4. Preparation and characterization of DNA and AON polyplexes

gWiz-Luc DNA solution at a concentration 20 µg/mL was prepared in 30 mM sodium acetate buffer (pH 5.0). PAA/DNA polyplexes were formed by adding predetermined volume of PAA to achieve the desired N/P ratio and mixed by vigorous vortexing for 10 s. Polyplexes were further allowed to stand for 30 min prior to use. AON polyplexes were prepared by following the same protocol, except that AON was heat-denatured for 10 min at 65 °C and snap-cooled on ice prior to dilution in the buffer. The determination of hydrodynamic diameters and zeta potentials of both polyplexes was performed following previously published method (Manickam et al., 2005). Results were expressed as mean ± standard deviation (S.D.) of 10 experimental runs each.

2.5. Agarose gel electrophoresis

Reduction-triggered disassembly of the polyplexes was examined by agarose gel electrophoresis using a previously described protocol (Read et al., 2003). Briefly, DNA polyplexes were formed as described above and incubated under indicated conditions. Samples were loaded onto a 0.8% agarose gel containing 0.5 µg/mL EtBr and run for 75 min at 120 V in 0.5× Tris/Borate/EDTA (TBE) running buffer. The gel was visualized under UV. AON polyplexes were analyzed on a 2% agarose gel containing EtBr for 30 min at 80 V under conditions mentioned above.

2.6. Cell culture

Mouse mammary carcinoma cell line D2F2 was a kind gift from Dr. Wei-Zen Wei, Karmanos Cancer Institute (Detroit, MI). The cells were maintained in DMEM supplemented with 4 mM L-glutamine, 0.1 mM NEAA and 10% FBS at 37 °C in 5% CO₂ atmosphere.

2.7. Transfection of DNA and AON polyplexes

All transfection experiments were conducted in 48-well plates during logarithmic growth phase following a previously published protocol (Manickam et al., 2010). Cells were seeded at a density of 40,000 cells/well 24 h prior to transfection. On a day of transfection, the cells were incubated with the polyplexes (DNA dose: 0.4 μ g/well) in 150 μ L of serum-free media. After 3 h incubation, polyplexes were completely removed and the cells were cultured in complete culture medium for 24 h prior to measuring luciferase expression. The medium was then discarded and the cells were lysed in 100 μ L of 0.5 $\times$ cell culture lysis buffer (Promega, Madison, WI) for 30 min. To measure the luciferase content, 100 μ L of 0.5 mM luciferin solution was automatically injected into each well containing 20 μ L of cell lysate and the luminescence was integrated over 10 s using BioTek Synergy 2 Microplate Reader. Total cellular protein in the cell lysate was determined by the bicinchoninic acid (BCA) protein assay using calibration curve constructed with standard bovine serum albumin solutions (Pierce, Rockford, IL). For cell surface thiol blocking, the cells were pre-incubated with 1 mM of DTNB in DMEM for 30 min. The DTNB solution was removed before conducting the transfection experiments as above. Transfection activity was expressed as RLU/mg cellular protein $\pm$ S.D. of quadruplicate samples. For AON transfections, the cells were transfected with 0.4 μ g luciferase DNA per well using DOTAP liposomes (N/P ratio 16) 24 h before transfected with AON polyplexes (0.8 μ g/well). The transfection efficiency was expressed as % knock-down compared with control scrambled AON.

2.8. Cell uptake by flow cytometry

The cellular association of the fluorescently-labeled polyplexes was measured using flow cytometry. Cells were plated in a 24-well plate 24 h before transfection. Polyplexes were formed using DNA labeled with YOYO-1 by mixing at a ratio of 1 dye molecule per 100 bp of DNA. After 3 h of transfection, cells were washed with PBS, detached, and resuspended in 200 μ L PBS. 10,000 events per sample were counted on FACS. Histogram plots were constructed using the CellQuest software. The mean fluorescence intensity from each sample was obtained and the cellular uptake was quantified as average mean fluorescence intensity $\pm$ SD of triplicate samples.

2.9. Kinetics of intracellular plasmid DNA clearance

Intracellular content of the gWiz-Luc plasmid DNA at different time points after incubation was measured using real-time PCR (RT-PCR, ABI Prism[®] 7300). The following probe and primers were designed in BioSearch online RealTimeDesign[™] software: FAM-BHQ probe TCAGGATTACAAGATTCAAAGTGCGCT, forward primer GAAGAGCTGTTTCTGAGG, reverse primer CGAAGAAGGAGAA-TAGGGT. The 18S rDNA probe and the master mix were purchased from Eurogentec (San Diego, CA). Cells were incubated with DNA polyplexes for 3 h under conditions used in the transfection studies. At predetermined times after incubation, the cells were washed twice with warm PBS and lysed with 100 μ L of RT-PCR lysis buffer for 1 h at 37 °C. The lysate (25 μ L) was transferred to a deep-well plate and diluted with 475 μ L of sterile water. The lysate was then incubated for 15 min in 95 °C water bath to inactivate proteinase K. During the RT-PCR cycle, 5 μ L of DNA template, 75 nM probe and 600 nM primer pairs were added. To determine 18S rRNA, 125 nM probe and 600 nM primer pairs were added to the template. The PCR cycle was run for 2 min at 50 °C, 10 min at 95 °C, 40 cycles for 15 s at 95 °C, and 1 min at 60 °C. The number of plasmid DNA copies was normalized to 18S rRNA and calculated as 2^n , where $n = (Ct_{18S\ rRNA} - Ct_{luc})$.

3. Results

3.1. Synthesis and characterization of PAA

We synthesized a series of five linear poly(amido amine)s with different disulfide content by controlling the feed molar ratio of reducible (CBA) and non-reducible (HMBA) bisacrylamide monomers. The weight-average molecular weight (M_w) and polydispersity index (PDI) of the five synthesized PAAs were determined by GPC and are listed in Table 1. The synthesized PAA had similar M_w ranging from 5700 to 7400 and polydispersity indices ranging from 1.1 to 2.0. The ratio of CBA-to-HMBA in the polymers was similar to the feed ratio.

3.2. Preparation of AON and DNA polyplexes

OliGreen is an ultra-sensitive fluorescent nucleic acid dye that binds oligonucleotides and single-stranded DNA. Its fluorescence corresponds to free unbound oligonucleotides in solution. The fluorescence intensity of OliGreen/AON decreased linearly with increasing N/P ratio (Fig. 1a). Full complexation of AON was achieved with all five PAA at N/P > 0.8 as indicated by no further fluorescence decrease beyond this ratio. EtBr exclusion assay was carried out to test the ability of PAA to condense plasmid DNA (Fig. 1b). The condensation curves for all PAA displayed typical sigmoidal shape characteristic of DNA condensation. N/P ratios above 1.8 were required to fully condense the DNA. Fig. 1 documents that there were no significant differences in DNA and AON complexation ability among the five PAA, suggesting that the presence of disulfide bonds in the polycations does not affect their binding to nucleic acids.

3.3. Particle size and zeta-potential of AON and DNA polyplexes

Size and zeta potential directly influence cellular uptake and transfection activity of polyplexes. Hydrodynamic size and zeta-potential of polyplexes were measured before every *in vitro* test. We have selected polyplexes formulated with about a 4-fold excess of polycation relative to the minimum amount needed to fully condense the corresponding nucleic acid as determined from Fig. 1. Thus, DNA polyplexes were prepared at N/P = 8 and AON polyplexes at N/P = 4 (Table 2). The sizes of PAA/DNA polyplexes showed no dependence on the disulfide content in PAA as they all fell into a narrow range of 96–102 nm. The sizes of all PAA/AON polyplexes (average size 64 ± 11 nm) were significantly smaller than the sizes of DNA polyplexes (average size 100 ± 5 nm), with the smallest size (53 nm) observed for polyplexes formulated with PAA with the highest disulfide content (PAA-100). All polyplexes, regardless of the type of nucleic acid, were positively charged with zeta potential ranging from 22 to 37 mV. The average zeta potential of DNA polyplexes (30.0 mV) was higher than the average zeta potential of AON polyplexes (26 mV).

3.4. Reduction-triggered disassembly of PAA polyplexes

Reduction-triggered disassembly of the PAA polyplexes was evaluated from differences in DNA and AON release patterns in the oxidizing and reducing conditions using agarose gel electrophoresis (Fig. 2). In addition to treatment with 20 mM GSH, the polyplexes were also incubated with 0.75 M NaCl to further facilitate disassembly and release of the nucleic acids. Incubation with 20 mM GSH resulted in loosening and partial dissociation of the polyplexes, documented by a partial release of both AON and DNA. Release behavior of polyplexes of both nucleic acids showed a clear dependence on the disulfide content in PAA. Partial AON release was observed at lower disulfide content in PAA (PAA-50) than DNA release (PAA-75). Addition of NaCl enhanced not only the amount of released nucleic acid but also resulted in free nucleic acids observed at lower disulfide contents in PAA.

Despite the combined action of GSH and NaCl, neither DNA nor AON were released from non-reducible PAA-0 polyplexes. Quantitative analysis of the release of AON and DNA from PAA polyplexes incubated with 20 mM GSH and 0.75 M NaCl is shown in Fig. 3. Data for both AON and DNA polyplexes confirm that increasing the disulfide content leads to increased amount of released nucleic acid. PAA-100 polyplexes of DNA were more susceptible to the GSH/NaCl treatment than AON polyplexes, with almost 80% of released DNA vs. 68% of released AON. Overall, both types of nucleic acids showed similar release behavior.

3.5. Effect of reduction on stability of polyplexes against polyelectrolyte exchange

Exchange reactions with negatively charged intracellular proteins, including actin and tubulin, have been shown to be responsible for intracellular release of plasmid DNA from PEI polyplexes (Iida et al., 2007). The effect of PAA disulfide reduction on the resistance of the polyplexes against polyelectrolyte exchange with a competing polyanion heparin was evaluated by agarose gel electrophoresis (Fig. 4). In oxidizing conditions, DNA polyplexes show first signs of destabilization at a heparin concentration of 30 $\mu\text{g/ml}$ while AON polyplexes start to disassemble at a heparin concentration of 20 $\mu\text{g/ml}$. Similar behavior was observed for both AON and DNA polyplexes regardless of disulfide content. In reducing conditions of 20 mM GSH, the threshold heparin concentration required to destabilize the polyplexes and release the nucleic acids decreased with increasing disulfide content in PAA.

3.6. Stability of PAA polyplexes in serum

Systemic delivery of polyplexes through intravenous injection requires that they are stable in the presence of serum. The stability of both DNA and AON polyplexes in the presence of serum was investigated using agarose gel electrophoresis (Fig. 5). No DNA or AON release was observed after incubation with serum, indicating no significant cleavage of the disulfide bonds. By contrast, free DNA and AON bind with serum components as indicated by a smear on the gel (Fig. 5, lane 5). Although no free nucleic acid was observed, the increased fluorescence intensity in the wells in the presence of serum suggested that interactions with serum components result in some loosening and structural changes of the polyplexes that facilitate binding of EtBr.

3.7. Transfection activity of AON and DNA polyplexes

The effect of disulfide content on transfection activity of the AON and DNA polyplexes was investigated in D2F2 murine mammary carcinoma cells (Fig. 6). All PAA/AON polyplexes showed similar knock-down levels with no statistically significant (one-way ANOVA) effect of PAA disulfide content observed (Fig. 6a). In contrast, one-way ANOVA with Bonferroni multiple comparison test, revealed that PAA-100 polyplexes had significantly higher transfection activity than all the other polyplexes with lower disulfide content. PAA-100 polyplexes showed a 152-fold higher transfection than PAA-0/DNA and a 6-fold higher transfection compared with control PEI/DNA polyplexes.

3.8. Cell uptake of PAA/DNA polyplexes

Because we observed no effect of the presence of disulfide bonds on AON transfection, we focused further studies on DNA polyplexes only. To determine the reasons behind the dependence of DNA transfection on disulfide content in PAA, we first measured cellular uptake of PAA/DNA polyplexes (Fig. 7). The uptake was measured at the end of the 3 h incubation period by quantifying the fluorescence intensity of polyplexes formulated with DNA that was non-covalently labeled with YOYO-1. The results show about 2-fold higher DNA uptake with PAA-100/DNA polyplexes when compared with the other four PAA/DNA polyplexes.

3.9. Intracellular clearance of plasmid DNA after transfection with PAA polyplexes

Cell uptake shown in Fig. 7 is only a snapshot at a single time point and one that is unable to distinguish between intact (viable) and degraded (nonviable) DNA. The rate of intracellular plasmid DNA clearance is also an important determinant of transfection efficiency. Intracellular clearance of plasmid DNA has been connected to disassembly rates of polyplexes and thus we investigated the kinetics of intracellular clearance of plasmid DNA delivered by PAA (Fig. 8). D2F2 cells were incubated with the polyplexes for 3 h and then harvested at 0, 6, 12, 18, 24, and 48 h after the incubation. The amount of plasmid DNA in whole cell lysate at the different time points was quantified with RT-PCR. The results from this independent experiment confirmed findings from Fig. 7 that PAA-100/DNA polyplexes exhibit the highest initial cell uptake. We found that intracellular plasmid DNA content declined rapidly in the first 18 h after incubation. Analysis of the data using first-order kinetics ($\ln[\text{DNA}]$ vs. time) revealed no statistically significant trend as the rate constants of the plasmid DNA clearance were similar regardless of the disulfide content in PAA.

3.10. Influence of cell surface thiols on transfection activity of PAA/ DNA polyplexes

To further investigate the role of PAA disulfide bonds in facilitating enhanced cell uptake and transfection activity, we studied the effect of plasma membrane protein thiols on transfection activity of PAA polyplexes (Fig. 9). D2F2 cells were treated for 30 min before transfection with cell impermeable nonspecific thiol blocker DTNB to block external plasma membrane protein thiols. We then measured the effect of the thiol blocking on transfection activity and expressed the results as a relative transfection, calculated as the ratio of luciferase expression in DTNB-treated vs. untreated cells. As shown in Fig. 9, non-reducible polyplexes (PAA-0) showed a 27% decrease in transfection after DTNB pretreatment (i.e., relative transfection 0.73). In contrast, all bio-reducible PAA polyplexes exhibited significant decrease in transfection. Polyplexes prepared with PAA-100 exhibited the largest decrease (97.6%) after blocking the plasma membrane surface thiols. As a result of the DTNB treatment, absolute levels of luciferase expression of all five PAA/DNA polyplexes were similar regardless of the disulfide content in PAA.

4. Discussion

Bio-reducible polycations have been widely investigated as delivery vectors of nucleic acids because of their favorable toxicity profile and the possibility to preferentially restrict release of the delivered nucleic acids to intracellular locations such as cytoplasm and nucleus. Both the decreased toxicity and controlled intracellular release are believed to be caused by degradation of disulfide-containing polycations mediated by reduced GSH abundantly present in the cytoplasm (Vercauteren et al., 2011). Multiple studies indeed reported decreased toxicity and increased transfection of bio-reducible polyplexes compared with non-bio-reducible controls. While reduced toxicity of bio-reducible polycations is a clearly established and a nearly-universal finding, improved transfection activity of bio-reducible polyplexes is not. This raises a question about the importance of the presumed increased intracellular disassembly of bio-reducible polyplexes for transfection. Our recent study suggested that whether the presence of disulfides in polyplexes benefits their transfection activity depends strongly on the cell line and the type of the used nucleic acid (e.g., plasmid DNA vs. mRNA) (Manickam et al., 2010). The goal of the present study was to systematically investigate how decreasing stability against disassembly affects transfection activity of plasmid DNA and AON polyplexes.

Bio-reducible PAA received significant attention because of their easy synthesis and access to structurally diverse polycations (Brumbach et al., 2010; Chen et al., 2009; Lin et al., 2007; Piest and Engbersen, 2010). In this study, we have used PAA based on a piperazine

triamine AEPZ because of previous reports that confirm their good transfection activity (Blacklock et al., 2009; Lin et al., 2006; Manickam et al., 2010). Although AEPZ may function potentially as a trifunctional monomer in the polymerization, the higher reactivity of the secondary amine in AEPZ compared with the primary amine allows synthesis of linear polymers by a simple control of reaction stoichiometry (Lin et al., 2006). Independence of the physicochemical properties of polyplexes on disulfide content (Fig. 1, Table 2) allowed us to minimize, although not fully eliminate, any potential confounding effects on the studied biological behavior of the polyplexes that were not directly related to the presence of disulfides.

Previously, we have reported on the mechanism of reduction-triggered disassembly and release of DNA from bioreducible PAA/ DNA polyplexes using atomic force microscopy in situ (Wan et al., 2009). Here, we focused on the influence of disulfide content and nucleic acid size on the reduction-triggered disassembly behavior. Despite greatly different sizes of AON and plasmid DNA, we observed only a small effect of the nucleic acid size on the disassembly behavior. Contrary to our expectations, AON polyplexes of PAA-25 were more resistant to reduction-triggered disassembly than similar DNA polyplexes. The relative stability of AON polyplexes is even more pronounced when one considers the fact that the AON polyplexes were prepared at lower N/P ratio (4) than the DNA polyplexes (N/P 8). We suggest that the stability is in part caused by AON forming more tightly packed polyplexes, as indicated by overall smaller hydrodynamic sizes and zeta potentials than corresponding DNA polyplexes.

Having established that both AON and DNA polyplexes display a range of disulfide-dependent differences in their sensitivity to disassembly, we have evaluated how such differences affect transfection activity (Fig. 6). We found that despite the similarities in the disassembly behavior, bioreducible PAA only enhanced transfection of DNA, while providing no benefits for AON. The pattern of DNA transfection dependence on disulfide content in PAA was similar to what was reported for the same PAA previously (Lin et al., 2006). Our data clearly show that intracellular degradation of PAA plays no significant role in AON delivery and indirectly suggest that disassembly is not a barrier to effective AON delivery by polyplexes. These results also suggest that cell-free *in vitro* assays like the heparin- and GSH-induced disassembly are poor predictors of polyplex behavior in cells.

We subsequently focused on elucidating the reasons for increased DNA transfection and found that the dependence of cell uptake on disulfide content in PAA mirrors well the transfection dependence (Fig. 7). It is known that physicochemical properties of polyplexes may affect their cell uptake. But because all the PAA/DNA polyplexes had similar physicochemical properties, we hypothesized that thiol–disulfide exchange between the disulfides in PAA/DNA polyplexes and reducing thiols in plasma membrane proteins could be responsible for the increased uptake and transfection of bioreducible DNA polyplexes. It has been reported that similar thiol–disulfide reactions on the cell surface are required for cell entry of viruses like HIV and play a role in cell entry of disulfide-containing peptides (Aubry et al., 2009; Jain et al., 2007; Markovic et al., 2004; Ryser et al., 1994). An early study also suggested the reducing cell surface thiols are involved in cell up-take of bioreducible lipoplexes (Kichler et al., 1995). By blocking the cellular surface thiols, we have indeed shown that transfection of disulfide-containing polyplexes is decreased to a much larger extent than transfection of non-reducible PAA polyplexes. In fact, blocking the surface thiols resulted in the bioreducible polyplexes losing their transfection advantage as all five tested PAA/DNA poly-plexes exhibited similar levels of transfection in cells with the cell surface thiols blocked. This evidence suggests that the thiol–disulfide reactions at the cell membrane play a key role in enhancing transfection of the bioreducible PAA/DNA polyplexes.

We predicted that if the hypothesis that the use of bio reducible PAA/DNA polyplexes enhances intracellular disassembly is correct, we should observe differences in intracellular clearance of the delivered plasmid DNA. This hypothesis is supported by previous publications that either found or suggested a relationship between the rate of polyplex disassembly, DNA degradation, and transfection activity (Chen et al., 2008; Varga et al., 2005). Models of intracellular trafficking of polyplexes also suggest that there is an optimum rate of polyplex disassembly that leads to maximum transfection activity. Importantly, the models predict that altering intracellular disassembly rates changes the relative role of enzymatic degradation of plasmid DNA in the intracellular trafficking and that as a result, altered DNA clearance rates should be observed. However, the kinetics of intracellular plasmid DNA clearance measured by RT-PCR showed clearance rates that were independent of the disulfide content in PAA. These experiments cannot answer whether the decrease in intracellular plasmid DNA content is caused by intracellular degradation, exocytosis, simple dilution due to cell division, or a combination of all three factors (Duncan and Gaspar, 2011). Regardless of the mechanism of plasmid DNA clearance, it is obvious that beyond cell uptake, bio reducible PAA used in this study do not alter to any significant extent intracellular bioavailability of DNA. This suggests to us that disassembly is not a major barrier for delivery of plasmid DNA with these PAA. We caution, however, against broad generalization of our results to other bio reducible polycations, especially those with higher molecular weights and higher affinities to DNA, in which cases disassembly may be a more prominent barrier.

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References

- Aubry S, Burlina F, Dupont E, Delaroche D, Joliot A, Lavielle S, Chassaing G, Sagan S. Cell-surface thiols affect cell entry of disulfide-conjugated peptides. *FASEB J.* 2009; 23:2956–2967. [PubMed: 19403512]
- Blacklock J, You YZ, Zhou QH, Mao G, Oupicky D. Gene delivery in vitro and in vivo from bio reducible multilayered polyelectrolyte films of plasmid DNA. *Biomaterials.* 2009; 30:939–950. [PubMed: 19013638]
- Brumbach JH, Lin C, Yockman J, Kim WJ, Blevins KS, Engbersen JFJ, Feijen J, Kim SW. Mixtures of poly(triethylenetetramine/cystamine bisacrylamide) and poly(triethylenetetramine/cystamine bisacrylamide)-g-poly(ethylene glycol) for improved gene delivery. *Bioconj Chem.* 2010; 21:1753–1761. [PubMed: 20882996]
- Chen CP, Kim JS, Steenblock E, Liu D, Rice KG. Gene transfer with poly-melittin peptides. *Bioconj Chem.* 2006; 17:1057–1062. [PubMed: 16848415]
- Chen HH, Ho YP, Jiang X, Mao HQ, Wang TH, Leong KW. Quantitative comparison of intracellular unpacking kinetics of polyplexes by a model constructed from quantum Dot-FRET. *Mol Ther.* 2008; 16:324–332. [PubMed: 18180773]
- Chen J, Wu C, Oupicky D. Bio reducible hyperbranched poly(amido amine)s for gene delivery. *Biomacromolecules.* 2009; 10:2921–2927. [PubMed: 19743843]
- Christensen LV, Chang CW, Kim WJ, Kim SW, Zhong ZY, Lin C, Engbersen JFJ, Feijen J. Reducible poly(amido ethylenimine)s designed for triggered intracellular gene delivery. *Bioconj Chem.* 2006; 17:1233–1240. [PubMed: 16984133]
- Donoghue N, Hogg PJ. Characterization of redox-active proteins on cell surface. *Methods Enzymol.* 2002; 348:76–86. [PubMed: 11885296]
- Duncan R, Gaspar R. Nanomedicine(s) under the microscope. *Mol Pharm.* 2011; 8:2101–2141. [PubMed: 21974749]
- Gilbert HF. Protein disulfide isomerase and assisted protein folding. *J Biol Chem.* 1997; 272:29399–29402. [PubMed: 9367991]

- Hansen RE, Roth D, Winther JR. Quantifying the global cellular thiol–disulfide status. *Proc Natl Acad Sci USA*. 2009; 106:422–427. [PubMed: 19122143]
- Hong CY, You YZ, Wu DC, Liu Y, Pan CY. Thermal control over the topology of cleavable polymers: from linear to hyperbranched structures. *J Am Chem Soc*. 2007; 129:5354–5355. [PubMed: 17417853]
- Hoon Jeong J, Christensen LV, Yockman JW, Zhong Z, Engbersen JF, Jong Kim W, Feijen J, Wan Kim S. Reducible poly(amido ethylenimine) directed to enhance RNA interference. *Biomaterials*. 2007; 28:1912–1917. [PubMed: 17218006]
- Iida T, Mori T, Katayama Y, Niidome T. Overall interaction of cytosolic proteins with the PEI/DNA complex. *J Controlled Release*. 2007; 118:364–369.
- Jain S, McGinnes LW, Morrison TG. Thiol/disulfide exchange is required for membrane fusion directed by the Newcastle disease virus fusion protein. *J Virol*. 2007; 81:2328–2339. [PubMed: 17151113]
- Jiang XM, Fitzgerald M, Grant CM, Hogg PJ. Redox control of exofacial protein thiols/disulfides by protein disulfide isomerase. *J Biol Chem*. 1999; 274:2416–2423. [PubMed: 9891011]
- Kichler A, Remy JS, Boussif O, Frisch B, Boeckler C, Behr JP, Schuber F. Efficient gene delivery with neutral complexes of lipospermine and thiol-reactive phospholipids. *Biochem Biophys Res Commun*. 1995; 209:444–450. [PubMed: 7733911]
- Lin C, Zhong Z, Lok MC, Jiang X, Hennink WE, Feijen J, Engbersen JF. Linear poly(amido amine)s with secondary and tertiary amino groups and variable amounts of disulfide linkages: synthesis and in vitro gene transfer properties. *J Controlled Release*. 2006; 116:130–137.
- Lin C, Zhong Z, Lok MC, Jiang X, Hennink WE, Feijen J, Engbersen JF. Novel bioreducible poly(amido amine)s for highly efficient gene delivery. *Bioconjug Chem*. 2007; 18:138–145. [PubMed: 17226966]
- Manickam DS, Bisht HS, Wan L, Mao G, Oupicky D. Influence of TAT-peptide polymerization on properties and transfection activity of TAT/DNA polyplexes. *J Controlled Release*. 2005; 102:293–306.
- Manickam DS, Li J, Putt DA, Zhou QH, Wu C, Lash LH, Oupicky D. Effect of innate glutathione levels on activity of redox-responsive gene delivery vectors. *J Controlled Release*. 2010; 141:77–84.
- Manickam DS, Oupicky D. Multiblock reducible copolypeptides containing histidine-rich and nuclear localization sequences for gene delivery. *Bioconjug Chem*. 2006; 17:1395–1403. [PubMed: 17105216]
- Markovic I, Stantchev TS, Fields KH, Tiffany LJ, Tomić M, Weiss CD, Broder CC, Strebel K, Clouse KA. Thiol/disulfide exchange is a prerequisite for CXCR4-tropic HIV-1 envelope-mediated T-cell fusion during viral entry. *Blood*. 2004; 103:1586–1594. [PubMed: 14592831]
- Namgung R, Brumbach JH, Jeong JH, Yockman JW, Kim SW, Lin C, Zhong ZY, Feijen J, Engbersen JFJ, Kim WJ. Dual bio-responsive gene delivery via reducible poly(amido amine) and surviving-inducible plasmid DNA. *Biotechnol Lett*. 2010; 32:755–764. [PubMed: 20155385]
- Neu M, Germershaus O, Mao S, Voigt KH, Behe M, Kissel T. Crosslinked nanocarriers based upon poly(ethylene imine) for systemic plasmid delivery: in vitro characterization and in vivo studies in mice. *J Controlled Release*. 2007; 118:370–380.
- Piest M, Engbersen JFJ. Effects of charge density and hydrophobicity of poly(amido amine)s for non-viral gene delivery. *J Controlled Release*. 2010; 148:83–90.
- Piest M, Lin C, Mateos-Timoneda MA, Lok MC, Hennink WE, Feijen J, Engbersen JFJ. Novel poly(amido amine)s with bioreducible disulfide linkages in their diamino-units: structure effects and in vitro gene transfer properties. *J Controlled Release*. 2008; 130:38–45.
- Rahbek UL, Howard KA, Oupicky D, Manickam DS, Dong M, Nielsen AF, Hansen TB, Besenbacher F, Kjems J. Intracellular siRNA and precursor miRNA trafficking using bioresponsive copolypeptides. *J Gene Med*. 2008; 10:81–93. [PubMed: 18000993]
- Read ML, Bremner KH, Oupicky D, Green NK, Searle PF, Seymour LW. Vectors based on reducible polycations facilitate intracellular release of nucleic acids. *J Gene Med*. 2003; 5:232–245. [PubMed: 12666189]

- Read ML, Singh S, Ahmed Z, Stevenson M, Briggs SS, Oupicky D, Barrett LB, Spice R, Kendall M, Berry M, Preece JA, Logan A, Seymour LW. A versatile reducible polycation-based system for efficient delivery of a broad range of nucleic acids. *Nucleic Acids Res.* 2005; 33:e86. [PubMed: 15914665]
- Ryser HJ, Levy EM, Mandel R, DiSciullo GJ. Inhibition of human immunodeficiency virus infection by agents that interfere with thiol–disulfide interchange upon virus–receptor interaction. *Proc Natl Acad Sci USA.* 1994; 91:4559–4563. [PubMed: 8183947]
- Varga CM, Tedford NC, Thomas M, Klibanov AM, Griffith LG, Lauffenburger DA. Quantitative comparison of polyethylenimine formulations and adenoviral vectors in terms of intracellular gene delivery processes. *Gene Ther.* 2005; 12:1023–1032. [PubMed: 15815703]
- Vercauteren D, Piest M, van der Aa LJ, Al Soraj M, Jones AT, Engbersen JFJ, De Smedt SC, Braeckmans K. Flotillin-dependent endocytosis and a phagocytosis-like mechanism for cellular internalization of disulfide-based poly(amido amine)/DNA polyplexes. *Biomaterials.* 2011; 32:3072–3084. [PubMed: 21262529]
- Wan L, You Y, Zou Y, Oupicky D, Mao G. DNA release dynamics from bioreducible poly(amido amine) polyplexes. *J Phys Chem B.* 2009; 113:13735–13741. [PubMed: 19522487]
- You YZ, Manickam DS, Zhou QH, Oupicky D. Reducible poly(2-dimethylaminoethyl methacrylate): synthesis, cytotoxicity, and gene delivery activity. *J Controlled Release.* 2007; 122:217–225.

Fig. 1. Nucleic acid condensation by PAA. (a) OliGreen exclusion assay to assess AON condensation and (b) EtBr exclusion assay to assess DNA condensation.

Fig. 2. Reduction-triggered release of AON (top) and plasmid DNA (bottom) from PAA polyplexes. Polyplexes were prepared at N/P 4 (AON) and N/P 8 (DNA) and incubated with 20 mM GSH and 0.75 M NaCl.

Fig. 3. Quantification of the reduction-triggered release of AON (a) and plasmid DNA (b) from PAA polyplexes. Polyplexes prepared at N/P 4 (AON) and N/P 8 (DNA) were incubated with 20 mM GSH and 0.75 M NaCl and the amount of released nucleic acid quantified by analyzing the fluorescence intensity of the bands. % nucleic acid release equals the ratio of fluorescence intensity of released AON or DNA band to the fluorescence intensity of the free AON or DNA.

Fig. 4. Effect of disulfide reduction on susceptibility of PAA polyplexes to polyelectrolyte exchange reactions. AON (left) and plasmid DNA (right) polyplexes of PAA were treated with increasing concentration of heparin and/or 20 mM GSH. AON and DNA release were analyzed by agarose gel electrophoresis.

Fig. 5.

Effect of serum on stability of AON and DNA polyplexes. PAA polyplexes, free nucleic acids (NA) (AON, DNA), and RPMI medium were incubated with (+) or without (-) 10% FBS and the presence/content of free nucleic acids was analyzed by agarose gel electrophoresis.

Fig. 6. Effect of disulfide content on transfection activity of PAA polyplexes. D2F2 cells were transfected with (a) anti-luciferase AON polyplexes and (b) luciferase DNA polyplexes. Results are expressed as % knock-down of luciferase expression and RLU/mg protein, respectively (mean $\pm$ S.D., $n = 4$).

Fig. 7. Effect of disulfide content on cell uptake of PAA/DNA polyplexes. D2F2 cell uptake of DNA labeled with YOYO-1 was measured after 3 h incubation with PAA polyplexes using flow cytometry. Cell uptake is shown as mean relative fluorescence intensity (RFI) $\pm$ S.D. ($n = 6$).

Fig. 8. Intracellular clearance of plasmid DNA delivered by PAA. D2F2 cells were incubated with PAA/DNA polyplexes for 3 h. The cells were then harvested and lysed at different time points post-incubation and luciferase DNA was quantified and normalized to 18S rDNA by RT-PCR (mean number of plasmid DNA copies per 18S rDNA $\pm$ S.D., $n = 4$).

Fig. 9. Effect of cell surface thiols on inhibition of transfection of PAA/DNA polyplexes. D2F2 cells were pretreated with 1 mM DTNB for 30 min before transfection. The data represent relative transfection in RLU/mg protein in cells pretreated with DTNB vs. untreated cells.

Scheme 1.
Synthesis of PAA.

Table 1

Molecular weights of PAA.

Polymer	Reducible content (%) [*]		M_w	M_w/M_n
	In feed	In PAA		
PAA-0	0	0	7400	1.1
PAA-25	25	36	7100	1.1
PAA-50	50	57	6200	1.2
PAA-75	75	78	5700	1.9
PAA-100	100	100	5800	2.0

^{*} Relative content of CBA: $100 * [CBA] / ([CBA] + [HMBA])$.

Table 2

Hydrodynamic size and zeta potential of PAA polyplexes.

Polymer	DNA polyplexes ^a		AON polyplexes ^b	
	Size (nm)	Zeta-potential (mV)	Size (nm)	Zeta-potential (mV)
PAA-0	96 ± 2	26 ± 2	66 ± 3	27 ± 2
PAA-25	98 ± 10	30 ± 1	74 ± 2	22 ± 2
PAA-50	107 ± 1	27 ± 1	75 ± 2	26 ± 1
PAA-75	96 ± 5	28 ± 1	53 ± 2	29 ± 4
PAA-100	102 ± 1	37 ± 1	53 ± 1	25 ± 5

^aPrepared at N/P ratio 8.^bPrepared at N/P ratio 4.