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Reducible DNA nanoparticles enhance *in vitro* gene transfer via an extracellular mechanism

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Abstract

We developed polylysine based DNA nanoparticles (DNA NPs) that contain disulfide linkage in the carrier and demonstrated that this reducible DNA NP enhances *in vitro* gene transfer via an extracellular mechanism. Polylysine was conjugated through an N-terminal cysteine to a polyethylene glycol chain (PEG) by either a disulfide bond (SS) or a thioether bond (CS), and the resulting PEG–peptide conjugates were used to compact plasmid DNA into reducible SS-DNA NPs or non-reducible CS-DNA NPs with identical physical properties. SS-DNA NPs mediated more than 10-fold higher *in vitro* gene transfer. Others have suggested that disulfide bonds in synthetic gene carriers undergo cleavage in the reducing environment inside the cell, allowing increased intracellular DNA release. In this study, however, both higher cellular uptake of SS-DNA NPs and inhibition of SS-DNA NP mediated *in vitro* gene transfer by blocking extracellular free thiols suggested an extracellular mechanism. DePEGylation of SS-DNA NPs by extracellular thiols caused aggregation which might lead to higher cellular uptake and higher transgene expression. A series of SS-DNA NPs prepared with stabilized disulfide bonds survived the extracellular environment without aggregation but lost the superior gene transfer ability, indicating that, in our system, intracellular mechanisms are not involved. These results provided further insight into the mechanisms of *in vitro* gene transfer enhancement by introducing reducible linkages, contributing to the rational design of more efficient non-viral gene delivery systems.

Keywords

DNA nanoparticles; Disulfide linkage; Cellular uptake; DePEGylation; Extracellular mechanism

1. Introduction

With simple and defined chemical structures, polyethylene glycol (PEG)–polycation block copolymers form complexes with DNA that are water soluble, colloiddally stable, non-toxic and effective in transfecting various cell types, thus representing a promising non-viral vector [1–3]. A specific formulation of PEG–polycation block copolymer that consists of 10 kDa PEG conjugated to a 30-mer polylysine through an N-terminal cysteine (CK₃₀) condenses plasmid DNA into DNA nanoparticles (DNA NPs) [4]. These particles deliver transgenes through a non-degradative pathway by interacting with the cell surface nucleolin [5] and transfect airway epithelium [6], ocular tissues [7] and central nervous system [8] *in*

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Conflict of interest

Dr. Pamela Davis holds equity in Copernicus Therapeutics, Inc. which is developing DNA nanoparticles for clinical use.

vivo with minimal adverse effect. Further enhancement of its gene transfer efficiency could improve their utility in clinical applications.

For a synthetic vector to efficiently deliver a transgene to the nucleus of target cells, many extracellular and intracellular barriers have to be overcome. In addition to cell surface binding, endosomal escape and nuclear translocation, it has been shown that vector unpacking is also a potential barrier for polyplex based gene delivery [9]. In order to promote vector unpacking without causing premature DNA release, a smart system that can respond to an intracellular cue is preferred. For this purpose, disulfide linkage has been successfully exploited to enhance the gene transfer efficiency of various vector systems [10–18]. It is hypothesized that while the disulfide linkages are stable in extracellular oxidizing environment, they undergo rapid cleavage due to the presence of high concentrations of glutathione and thioredoxin reductases inside the cells, which in turn triggers the disassembly of DNA complexes intracellularly and improves transgene expression. Since DNA NPs used in previous studies did not contain disulfide linkage, in this study we tested whether introducing a disulfide linkage further enhances gene transfer efficiency of DNA NPs. We found that replacing the non-reducible thioether bond between the PEG and CK₃₀ with a disulfide bond preserved the overall structure of DNA NP but markedly enhanced its gene transfer ability in cell culture.

Although introduction of disulfide linkage has been associated with enhanced gene transfer in almost all the vector systems tested so far [10–18], the site of disulfide bond reduction has not been confirmed and the detailed mechanism remains elusive. Two groups [2,16] have previously introduced disulfide linkage to PEG–polycation block copolymer based delivery systems. Although enhanced gene transfer was reported in both studies, different mechanisms were suggested. Takae et al. [16] introduced disulfide linkage between PEG and a poly (aspartamide) based polymer and attributed the enhanced gene transfer to an intracellular mechanism, based on the observation that cellular uptake of DNA was unaltered. Their findings were consistent with their hypothesis that high concentration of intracellular free thiols triggers the release of DNA leading to enhanced gene transfer. Kwok et al. [2] replaced a thioether bond linking a PEG chain and a short peptide Cys–Try–Lys₁₈ with a disulfide bond, and reported a higher cellular uptake for the reducible PEG–peptide DNA condensate, indicating that extracellular mechanisms might be involved. In support of their findings, Feener et al. [19] showed that disulfide bond reduction of a reducible peptide probe started at the cell surface. A later study found that cell surface protein disulfide isomerase (PDI) was involved in this reductive function of the plasma membrane [20]. Taken together with the findings that PDI is also involved with cellular entry of several viruses [21–23], an extracellular mechanism may exist at the plasma membrane for enhanced gene transfer mediated by disulfide containing non-viral gene transfer agents.

Here, we evaluated both extracellular and intracellular mechanisms for enhanced gene transfer by reducible DNA NPs. Instead of the intracellular mechanism or the plasma membrane associated extracellular mechanism proposed above, we identified an extracellular mechanism that involves the aggregation of reducible DNA NPs induced by extracellular excreted thiols. Stabilizing the disulfide bond inhibited the extracellular mechanism, but also abolished gene transfer enhancement, indicating that intracellular mechanisms are not involved.

2. Materials and methods

2.1. Materials

CH₃O–PEG–orthopyridyl disulfide (mPEG–OPSS MW 10 kDa) and CH₃O–PEG–maleimide (mPEG–MAL MW 10 kDa) were purchased from Laysan Bio (Arab, AL).

CH₃O-PEG-NH₂ (mPEG-NH₂ MW 10 kDa) was purchased from Rapp Polymere (Tübingen, Germany). pCMV-Luc plasmid DNA was purified from bacteria culture using EndoFree plasmid giga kit from Qiagen (Santa Clara, CA). CK₃₀, K₃₀ and 30-mer polylysine with an N-terminal penicillamine (PenK₃₀) were synthesized at the Molecular Biotechnology Core of the Cleveland Clinic Lerner Research Institute (Cleveland, OH). DNase I and reaction buffer were provided by the New England Biolabs (Ipswich, MA). 5, 5'-Dithio-bis-(2-nitrobenzoic acid) (DTNB) (as Ellman's reagent) and 4-succinimidylloxycarbonyl- α -methyl- α -[2-pyridyldithio]toluene (SMPT) were purchased from Pierce (Rockford, IL). YOYO-1 dimeric cyanine dye, tetramethylrhodamine (TMR) labeled dextran (neutral, MW 70 kDa) and LysoTracker Green DND-26 were purchased from Molecular Probes/Invitrogen (Eugene, OR). Uranyl acetate was purchased from Polysciences (Warrington, PA). All other reagents were purchased from Sigma (St. Louis, MO) unless specified otherwise.

2.2. Synthesis of PEGylated polylysine

PEGylated CK₃₀ with thioether linkage (PEG-CS-CK₃₀) was prepared as described [4], except that both CK₃₀ and mPEG-MAL were dissolved in 50 mM sodium phosphate (pH 7.0) with 5 mM EDTA. PEGylated CK₃₀ with disulfide linkage (PEG-SS-CK₃₀) was prepared in a similar way, except that a 2:1 molar ratio of mPEG-OPSS to CK₃₀ was used. The reaction mixture was incubated overnight at room temperature on a rotating wheel. PEGylated polylysine was then purified on a HiTrap SP Sepharose™ Fast Flow ion exchange column (GE Healthcare Life Science, Piscataway, NJ) equilibrated with 50 mM sodium phosphate (pH 7.0). The peptides were eluted using a NaCl step gradient, desalted, washed with 20 mM ammonium acetate and lyophilized.

2.3. Labeling of DNA plasmid

For flow cytometric analysis of cellular uptake, DNA plasmids were labeled with YOYO-1. 0.444 mg/ml pCMV-Luc DNA in 20 mM HEPES buffer (pH 7.4) was added to equal volume of YOYO-1 diluted in the same buffer. The concentration of YOYO-1 was adjusted to give a dye to DNA base pair ratio of 1:900. The reaction mixture was incubated in 50 °C water bath in the dark for 2 h. For fluorescence imaging studies, pCMV-Luc DNA plasmids were labeled with TMR using Label IT Tracker intracellular nucleic acid localization kit (Mirus, Madison, WI) as described [5].

2.4. Formulation of DNA NP

For unlabeled DNA NPs, 900 μ l of 0.222 mg/ml pCMV-Luc in sterile water was added in 100 μ l aliquots to a vortexing solution of 100 μ l of 0.4 mM PEGylated CK₃₀ in sterile water with 2 min vortexing between additions and 30 min vortexing after the final addition of DNA plasmid. The DNA concentration in the final solution was 0.2 mg/ml, and the end point N/P ratio was 2:1. The DNA NPs were then purified and concentrated in saline using a Vivaspinn 20 (100 k MWCO) centrifugal filter (Sartorius, Göttingen, Germany). For non-PEGylated DNA NPs (K₃₀), 0.4 mM K₃₀ was used and the DNA NPs (K₃₀) were stored in water to avoid salt induced aggregation. For YOYO-1 labeled DNA NPs, the compaction was performed with the same procedure but in 20 mM HEPES (pH 7.4). All PEGylated DNA NPs used in this study met or exceeded a series of qualification parameters as previously described [4].

2.5. Zeta potential measurements

The zeta potentials of DNA NPs (100–200 μ g DNA/ml in 1 mM NaCl) were measured using a NICOMP zeta potential/particle sizer 380 ZLS (Santa Barbara, CA). Carboxylated latex

microspheres (Brookhaven Instruments Corporation, Holtsville, NY) were used to validate the zeta potential measurements.

2.6. DNase I resistance assay

DNA NPs were incubated with increasing concentrations of DNase I in 1×DNase I reaction buffer at 37 °C for 1 h. 0.5 M EDTA was added to the mixture to quench the reaction, followed by treatment with equal volume of 2.5% trypsin at 37 °C for 1 h. The recovered DNA was purified with one phenol/chloroform extraction and analyzed by 1% agarose gel electrophoresis.

2.7. Cell culture

HeLa and HuH7 cells were grown in Dulbecco's modification of Eagle's medium (DMEM) (Cellgro/Mediatech, Manassas, VA) and RPMI 1640 medium (Gibco/Invitrogen, Carlsbad, CA), respectively, supplemented with 10% fetal bovine serum (FBS) (Gibco/Invitrogen), 100 units/ml penicillin and 100 µg/ml streptomycin (Cellgro/Mediatech). The cells were cultured at 37 °C in 5% CO₂.

2.8. Flow cytometry

HeLa and HuH7 cells were seeded 24 h prior to transfection in 6-well plates at initial density of 5×10^5 cells/well. After 24 h, the medium in each well was replaced with 0.5 ml serum-free medium containing 5 µg, 10 µg or 20 µg YOYO-1 labeled DNA NPs. After 12 h incubation, the DNA NPs were removed. The cells were then detached by trypsinization, washed once with 1×DPBS and resuspended in HBSS supplemented with 2% FBS. Flow cytometry was performed using a LSR I flow cytometer (BD Bioscience, San Jose, CA). YOYO-1 was excited at 488 nm (Ar laser) and the emission was detected using a 530/28-nm band-pass filter. The total cell population was appropriately gated by forward and side scattering to exclude dead cells. A total of 7000 events was collected per sample.

2.9. In vitro transfection

HeLa and HuH7 cells were seeded 24 h prior to transfection in 24-well plates at initial density of 8×10^4 cells/well and 1.2×10^5 cells/well, respectively. At the time of transfection, the medium in each well was replaced with 200 µl of fresh serum-free medium containing DNA NPs (1 µg pDNA/well). After incubation for the indicated times at 37 °C, the cells were washed once with 1×DPBS and incubated in 1 ml of fresh complete medium for additional 42 h. The luciferase gene activity was then evaluated using the Luciferase Assay System (Promega, Madison, WI) and a Torpax Optocomp I luminometer (MGM Instruments, Hamden, CT). The results were expressed as relative luminescence units (RLU) normalized to total cellular protein content determined by DC protein assay kit (BioRad, Hercules, CA). For DTNB inhibition experiments, the cells were pretreated with DTNB directly dissolved in serum-free medium at indicated concentrations for 30 min, followed by incubation with fresh serum-free medium containing DNA NPs (1 µg pDNA/200 µl/well) in the presence of DTNB for 1 h 45 min. After incubation for additional 42 h in complete media, luciferase assay were conducted as described above.

2.10. Cytotoxicity assay

HuH7 and HeLa cells were seeded at initial density of 10^4 cells/well in microplates (tissue culture grade, 96 wells). After 24 h incubation, the medium was aspirated and 100 µl of fresh serum-free medium containing DTNB at indicated concentrations was added to each well. The cytotoxicity of DTNB was evaluated using the Cell Proliferation Kit I (MTT) (Roche, Indianapolis, IN) according to the manufacturer's instruction.

2.11. Sedimentation assay

For DNA NPs in saline, sedimentation assay was conducted as described [6] with DNA concentration determined by OD260. For DNA NPs in culture medium, the method was modified because OD260 measurement was interfered by the components of culture medium. Briefly, after incubation with fresh or conditioned culture medium, 200 μ l of DNA solution was centrifuged at 6000 rpm for 1 min. DNA in the supernatant before and after centrifugation was recovered as described for DNase I resistance assay. Sedimentation was quantified by comparing the band volume of the sample before and after centrifugation.

2.12. Quantifications of thiols in culture supernatants

Extracellular thiols were quantified using Ellman's Reagent according to the manufacturer's instruction. Briefly, the conditioned media of HuH7 or HeLa cells (200 μ l per well in 24-well plates) were centrifuged at 10,000 rpm for 2 min at 4 °C. The supernatants were diluted with 2.55 ml of Reaction Buffer (100 mM sodium phosphate, (pH 8.0) containing 1 mM EDTA) and then mixed with 50 μ l of Ellman's Reagent Solution (4 mg/ml DTNB in Reaction Buffer). After incubation at room temperature for 15 min, 200 μ l of the reaction mixture was transferred to a microplate and the absorbance at 412 nm was read with a VersaMax tunable microplate reader (Molecular Device, Sunnyvale, CA). The concentration of thiols was determined by comparison to a cysteine standard.

2.13. PEG release assay

HuH7 and HeLa cells were cultured in 24-well plate and incubated with DNA NPs as described in the section of *in vitro* transfection. After incubation for the indicated times at 37 °C, the media were centrifuged at 10,000 rpm for 2 min at 4 °C. The supernatants were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). To reveal the released PEG, the gel was soaked in 5% barium chloride solution for 10 min, rinsed with distilled water and stained with 0.1 N iodine solution for 10–15 min. DNA NPs were also incubated with fresh serum-free medium in the presence or absence of cysteine in 37 °C water bath as controls.

2.14. Transmission electron microscopy (TEM)

DNA NPs were diluted in water or saline to a final concentration of 5 ng/ μ l. 10 μ l of diluted DNA NPs was applied to a formvar-carbon coated grid (Ted Pella, Redding, CA). For DNA NPs in cell culture, HuH7 and HeLa cells were cultured in 24-well plate and incubated with DNA NPs as described in the section of *in vitro* transfection. After incubation for the indicated times at 37 °C, 10 μ l of the medium containing DNA NPs was applied directly to a grid. The grids were then washed in HPLC grade water and stained with 1% uranyl acetate in water. Images were obtained using a JEM-1200 EX transmission electron microscope (JEOL, Tokyo, Japan).

2.15. Disulfide bond stabilization

One methyl group and one benzene ring (PhM) were introduced to the carbon α to the OPSS group on the PEG chain by reacting mPEG-NH₂ with the cross linker SMPT. Briefly, 0.2 ml of 20 mM SMPT in acetonitrile was added dropwise to a vortexing solution of 0.8 ml of 1.25 mM mPEG-NH₂ in 50 mM sodium phosphate (pH 7.0). After 2 h incubation at room temperature, the reaction was quenched by adding 50 μ l of 1 M Tris-HCl (pH 7.9). PEG modified with SMPT (PEGPhM-OPSS) was purified on a PD-10 desalting column (GE Healthcare Life Science) and eluted with 50 mM sodium phosphate (pH 7.0) containing 5 mM EDTA. More than 95% of the PEG was modified by SMPT as determined by quantifying the pyridine-2-thione reaction using a spectrophotometer after incubation of the purified PEGPhM-OPSS with excessive 2-mercaptoethanol (2-ME). Two methyl groups

were introduced to the carbon α to the sulfhydryl group in the CK₃₀ by replacing the cysteine residue with a penicillamine using a method adopted from Kwok et al. [13] except that only one Pen residue was incorporated. Stabilized PEGylated polylysine PEGPhM-SS-CK₃₀, PEG-SS-PenK₃₀ and PEGPhM-SS-PenK₃₀ were prepared and purified as described in the Synthesis of PEGylated polylysine section, except that a higher molar ratio of PEG to polylysine (3:1 to 4:1) and a higher pH (8.0) were used to favor the formation of the hindered disulfide bonds.

2.16. Acid-urea polyacrylamide gel electrophoresis (AU-PAGE)

AU-PAGE was performed according to a method previously described [24] with minor modification. Briefly, 12% polyacrylamide gels containing 5 M urea and 5% acetic acid were pre-electrophoresed at 150 V for 1 h. Samples were solubilized in 5% acetic acid and diluted 2:1 with sample buffer (9 M urea in 5% acetic acid containing methylene blue as the tracking dye).

2.17. Fluorescence imaging

HeLa and HuH7 cells were seeded 24 h prior to transfection in 35-mm glass-bottomed dishes (MatTek, Ashland, MA) at initial density of 2.5×10^5 cells/dish and transfected with 5 μ g of TMR labeled DNA NPs in 1 ml serum-free medium for 2 h and 5 h, respectively. As positive controls for lysosomal localization, the cells were incubated with 1 mg/ml TMR labeled dextran in 1 ml serum-free medium for 4 h. DNA NPs or dextran were then removed and the cells were stained with 100 nM LysoTracker Green in 2 ml serum-free medium for 30 min. The cells were washed three times with 1 $\times$ DPBS and incubated in 1 ml serum-free medium without phenol red before imaging. The cells were imaged live on a LSM 510 META confocal microscope (Carl Zeiss, Peabody, MA) with a 100 $\times$ objective at excitation wavelength of 488 nm (Ar laser) and 543 nm (HeNe laser) for LysoTracker Green and rhodamine, respectively.

3. Results

3.1. Reducible DNA NPs mediate superior in vitro gene transfer compared to non-reducible ones

To prepare DNA NPs containing different chemical linkages in their polycation carrier, reducible and non-reducible PEG substituted polylysine were synthesized. PEG chains (10 kDa) with different thiol reactive functional groups were conjugated to CK₃₀ forming PEG-SS-CK₃₀ (with a disulfide bond) or PEG-CS-CK₃₀ (with a thioether bond) (Fig. 1A). The PEG-peptide conjugates were then purified by ion exchange chromatography to separate the PEGylated polylysine from unreacted PEG, CK₃₀ and CK₃₀ dimers. To test the reducibility of the two conjugates, they were incubated with a reducing agent, 2-ME and the PEGylation state of the polylysine was then analyzed by AU-PAGE and Coomassie blue staining. DePEGylated polylysine was revealed only from 2-ME treated PEG-SS-CK₃₀, but not from PEG-CS-CK₃₀ (Fig. S1 of Appendix A), confirming that PEG-SS-CK₃₀ can be reduced by free thiols while PEG-CS-CK₃₀ cannot.

DNA plasmids carrying a luciferase reporter gene were then compacted by either PEG-SS-CK₃₀ or PEG-CS-CK₃₀ using a method described by Liu et al. [4] into DNA NPs that are either reducible (SS-DNA NPs) or non-reducible (CS-DNA NPs). As previously described for CS-DNA NPs [4], SS-DNA NPs were also tested by a panel of quality control assays including sedimentation assay, turbidity assay (data not shown), DNase I resistance assay (Fig. S2A of Appendix A) and TEM (Fig. 1B) and found to have similar colloidal and morphological properties to CS-DNA NPs. In addition, the zeta potentials of the DNA NPs were measured and no statistically significant difference was found between the two: $4.2 \pm$

1.6 mV for SS-DNA NPs and 3.8 ± 1.2 mV for CS-DNA NPs (mean $\pm$ SD of 5 measurements), suggesting that replacing the CS bond with an SS bond does not change the surface charge of the DNA NPs.

In vitro transfection of HeLa and HuH7 cells by SS- and CS-DNA NPs indicated that SS-DNA NPs mediated more than a log higher luciferase gene transfer than CS-DNA NPs in both cell lines (Fig. 1C). These results indicate that introducing a reducible linkage to the polycation carrier of DNA NP significantly enhances its *in vitro* gene transfer activity without changing the initial physical properties of the DNA NP.

3.2. SS-DNA NP shows higher cellular uptake suggesting an extracellular mechanism

To investigate the cellular mechanism for this enhanced transfection activity, we measured the cellular uptake of DNA. We hypothesized that similar cellular uptake of the two DNA NPs would suggest an intracellular mechanism while different cellular uptakes would indicate an extracellular mechanism. DNA plasmids were fluorescently labeled with a dimeric cyanine dye YOYO-1 which does not affect the subsequent formulation of DNA NPs as shown by sedimentation assay (data not shown), DNase I resistance assay and TEM (Fig. S2 of Appendix A). HeLa cells were transfected with YOYO-1 labeled SS- or CS-DNA NPs and the fluorescence signal from single cells was measured by flow cytometry. Fig. 2A and B showed that both SS- and CS-DNA NPs were taken up by the cells in a dose dependent manner, but the uptake of SS-DNA NP was significantly higher (Fig. 2C). Similar results were obtained using HuH7 cells (data not shown). These data suggest that an extracellular mechanism exists for the enhanced *in vitro* gene transfer mediated by SS-DNA NP.

3.3. Enhanced *in vitro* gene transfer mediated by SS-DNA NP is inhibited by DTNB

Free thiols can be found in the cell surface proteins; they are also secreted into the extracellular environment, though at a much lower level compared to intracellular thiols [25]. We therefore tested whether this extracellular mechanism is related to extracellular thiols. A cell impermeable disulfide compound, DTNB, was used to block extracellular free thiols. Cell viability assay showed that DTNB, at concentrations up to 5 mM, did not affect the metabolic activity of HeLa cells (Fig. 3A). HeLa cells were pretreated with DTNB and transfected with SS- or CS-DNA NPs in the presence of DTNB at concentrations indicated in the figure and the effect of DTNB on *in vitro* gene transfer was determined by luciferase assay. A dose dependent inhibition of *in vitro* gene transfer by DTNB was observed for SS-DNA NP, but not for CS-DNA NP (Fig. 3B). The same experiments were also conducted using HuH7 cells. Unlike HeLa cells, the viability of HuH7 cells was affected by DTNB at high concentrations (Fig. 3A). When the concentration was lowered to avoid cytotoxicity, DTNB failed to inhibit *in vitro* gene transfer to HuH7 cells mediated by either SS- or CS-DNA NPs (data not shown). From these data, we conclude that the mechanism by which SS-DNA NP enhances *in vitro* gene transfer is related to extracellular thiols and can be inhibited when extracellular thiols are effectively blocked by high concentration of DTNB. However, from this experiment alone, we cannot distinguish between two possible extracellular mechanisms: SS-DNA NP could react with free thiols either in culture medium or on the cell surface.

3.4. SS-DNA NP is destabilized by extracellular secreted thiols

To test the ability of culture medium to reduce SS-DNA NPs, we assessed the stability of both SS- and CS-DNA NPs in culture medium by sedimentation assay. This assay compares the amount of DNA in the supernatant before and after centrifugation. Colloidally unstable DNA NPs usually form aggregates which will be sedimented, resulting in the loss of DNA in the supernatant. DNA NPs were incubated with fresh or conditioned medium in test tubes

without direct cell contact, thus only the interactions between DNA NPs and the components in the medium were evaluated, excluding the potential effects of cell surface proteins. No sedimentation was observed for either SS- or CS-DNA NPs when fresh medium was used (data not shown). However, when fresh medium was replaced by conditioned medium, SS-DNA NP, but not CS-DNA NP, demonstrated significant levels of sedimentation (Fig. 4A). Interestingly, this process was faster in conditioned DMEM from HeLa cells than in conditioned RPMI 1640 from HuH7 cells (Fig. 4A). These results indicated that SS-DNA NP, but not CS-DNA NP, is destabilized by free thiols in the culture medium.

By Ellman's assay, we confirmed that free thiols are secreted into the extracellular environment by both HeLa and HuH7 cells, but at quite different levels (Fig. 4B). Thiols were rapidly secreted by HeLa cells at relatively high levels (up to 180 μ M) while HuH7 cells maintained a low level of extracellular thiols (about 30 μ M) with only gradual increase. This difference in thiol secretion explains the different time courses of SS-DNA NP sedimentation in conditioned media from the two cell lines and further correlates enhanced gene transfer by SS-DNA NP with extracellular thiol concentration.

3.5. DePEGylation of SS-DNA NP by extracellular thiols causes aggregation and condensation

Although the result from the sedimentation assay indicated aggregation of SS-DNA NP when incubated in conditioned media, it offered no direct insights into the colloidal and morphological change of SS-DNA NP in cell culture. We speculated that SS-DNA NPs are dePEGylated by extracellular secreted thiols and form aggregates in culture medium. To demonstrate directly extracellular dePEGylation of SS-DNA NPs, we measured the release of PEG from DNA NPs in culture medium with cell contact. HeLa and HuH7 cells were cultured and transfected with DNA NPs under the same conditions as in Fig. 1C. At different time points, culture media were collected and analyzed by SDS-PAGE followed by PEG specific iodine staining. Conjugated PEG was retained on the top of the gel with DNA complex while released PEG migrated into the gel. As shown in Fig. 5A, PEG was released from SS-DNA NPs, but not from CS-DNA NPs, in culture media of both HeLa and HuH7 cells. Notably, this release process also followed different time courses in the two cell lines, which correlated well with the level of extracellular thiols. In control experiments, PEG release was not observed from either of the DNA NPs incubated in fresh medium without cell contact. PEG release occurred from SS-DNA NPs when 4 mM cysteine was added to the medium (Fig. 5A), which confirmed the role of free thiols.

To monitor directly the aggregation of dePEGylated SS-DNA NPs, we examined the morphological change of DNA NPs over time by TEM. Media collected at different time points from transfected cell culture were directly used to prepare TEM samples. No change of morphology was observed for CS-DNA NP over time (Fig. 5B). In contrast, images of SS-DNA NP showed aggregation in culture media of both HeLa and HuH7 cells. Consistent with the time course of PEG release, aggregation of SS-DNA NP was detected within an hour in culture medium of HeLa cells but became evident only after three hours in culture medium of HuH7 cells (Fig. 5B). Taken together, this represents direct evidence that dePEGylated SS-DNA NPs form aggregates in extracellular environment.

We reasoned that if this extracellular mechanism is the major mechanism, the level of extracellular thiols should have a similar temporal effect on transgene expression as observed for the dePEGylation and aggregation of SS-DNA NPs. To test this hypothesis, we conducted *in vitro* gene transfer experiment as described above but varied the incubation time of DNA NP. In HeLa cells (Fig. 5D), with one hour incubation, transgene expression from SS-DNA NPs was already 42-fold compared to CS-DNA NPs and more than a log difference was maintained with 3 and 6 h incubation time. In contrast, in HuH7 cells, longer

incubation time (>3 h) was required for SS-DNA NPs to exhibit similar enhancement of gene transfer (Fig. 5C). Since the two cell lines were cultured in different media, we switched the medium conditions and repeated the *in vitro* transfection. Similar gene expression profiles were observed for both cell lines regardless of the media used (data not shown). In addition, Ellman's assay confirmed that thiol secretion was similar in each cell line, whatever the medium (data not shown). Taken together, transgene expression, in addition to dePEGylation and aggregation of SS-DNA NPs, correlates with the level of secreted thiols, lending further support to the thiol related extracellular mechanism.

To determine whether aggregation by means that are not related to extracellular thiols also enhances *in vitro* gene transfer, we tested non-PEGylated DNA NPs as a control. Non-PEGylated polylysine (K₃₀) compacts DNA plasmids into small spherical particles or short rods in water, which upon exposure to physiological solution (Fig. S3 of Appendix A), forms aggregates much larger than those produced by disulfide reduction. Both HeLa and HuH7 cells were transfected with PEGylated and non-PEGylated DNA NPs and luciferase activity assessed. Aggregation of DNA NP (K₃₀) resulted in increase of gene transfer in both cell lines, albeit to different extents (2 fold in HeLa cells and 8 fold in HuH7 cells) (Fig. 5E), but fell short of the enhancement mediated by SS-DNA NPs (20 fold in HeLa cells and 15 fold in HuH7 cells).

3.6. Stabilized SS-DNA NP can survive the extracellular environment but loses superior gene transfer ability

To further test whether intracellular mechanisms are involved, without the complication of extracellular mechanism, we prepared reducible DNA NPs that survive the extracellular environment by stabilizing the disulfide bond between PEG and polylysine. Steric hindrance to the nucleophilic sulfhydryl attack was introduced by adding methyl groups and a benzene ring to the carbons adjacent the disulfide bond. The cysteine residue in CK₃₀ was replaced with a penicillamine (Pen) during peptide synthesis to introduce two methyl groups to the carbon α to the sulfhydryl group on the peptide; one benzene ring and one methyl group (PhM) were introduced to the carbon α to the OPSS group on the PEG by reacting mPEG-NH₂ with a heterobifunctional sulfhydryl and amine-reactive crosslinker SMPT (Fig. 6A). PenK₃₀ and PEGPhM-OPSS were initially synthesized and purified. By cross-reacting CK₃₀ or PenK₃₀ with PEG-OPSS or PEGPhM-OPSS, PEGylated polylysine with one (PEGPhM-SS-CK₃₀), two (PEG-SS-PenK₃₀), or three (PEGPhM-SS-PenK₃₀) additional methyl groups were obtained (Fig. 6A).

Reduction by cysteine was used to test whether the PEGylated polylysines were progressively stabilized as more hindering groups were introduced. The PEG-peptide conjugates were incubated with increasing concentrations of cysteine and analyzed by AU-PAGE and Coomassie blue staining as described above. If the conjugate is reduced, dePEGylated polylysine will appear at the bottom of the gel. As shown in Fig. 6B (far right), PEG-peptide conjugates with thioether bond could not be reduced by cysteine. All the conjugates with disulfide bond could be reduced, but at different concentrations of cysteine. The more methyl groups introduced, the more stable the conjugate. Notably, unstabilized PEG-SS-CK₃₀ was reduced by 0.2 mM cysteine while all three methylated conjugates were stable at this concentration of cysteine which equals the highest level of extracellular thiols in HeLa cells (Fig. 4B). In addition, all three methylated polymers could be reduced by 1 to 4 mM cysteine, which is the level of intracellular thiols. These results suggest that modified PEGylated polylysine are stable in extracellular environment but can still be reduced inside the cell.

Stabilized PEGylated polylysines were then used to compact DNA plasmid into reducible DNA NPs with one (PhMSS-DNA NP), two (2MSS-DNA NP) or three (Ph3MSS-DNA NP)

methyl groups. The stability of methylated SS-DNA NPs was assessed with sedimentation assay, PEG release assay (data not shown) and TEM (Fig. S4 of Appendix A): all were stable in the extracellular environment after 1 h incubation with HeLa cells or 3 h incubation with HuH7 cells.

To test the effect of disulfide bond stabilization on the gene transfer ability of DNA NPs, *in vitro* transfection was performed as described above with 1 h and 3 h transfection of DNA NPs for HeLa and HuH7 cells, respectively. Luciferase assay showed that the reporter gene expression from all the stabilized SS-DNA NPs was reduced to the level similar to that of CS-DNA NPs (Fig. 7). No delayed increase of transgene expression was observed in any case when the incubation time after transfection was prolonged (Fig. S5 of Appendix A), thus ruling out the possibility that intracellular reduction of stabilized SS-DNA NPs has a stimulatory effect on transgene expression, but is so slow that longer post-transfection incubation is required in order for it to manifest. Taken together, inhibiting the extracellular mechanism by stabilizing SS-DNA NPs completely abolished the superior gene transfer ability of SS-DNA NPs, indicating that thiol related intracellular mechanism is not involved.

3.7. Intracellular fate of SS- and CS-DNA NPs

To test whether aggregated SS-DNA NPs enter the cells via a pathway different from that used by CS-DNA NPs we examined the intracellular distribution of DNA NPs and their colocalization with lysosomes. HeLa and HuH7 cells were transfected by rhodamine labeled DNA NPs, followed by incubation with LysoTracker Green, a cell-permeable fluorescent dye which selectively accumulates in acidic organelles. The colocalization of DNA NPs with lysosomes in live cells was examined by confocal fluorescence microscopy. As shown in Fig. 8, SS-DNA NPs showed more efficient cellular uptake compared to CS-DNA NPs, consistent with the results from flow cytometry. Neither SS- nor CS-DNA NPs showed significant colocalization with lysosomes in either of the two cell lines. As a positive control for lysosomal localization, cells were incubated with fluorescently labeled dextran (neutral, 70 k MW), a fluid phase macropinocytosis marker, known to sort to lysosomes [26,27]. In contrast to DNA NPs, the majority of dextran was colocalized with LysoTracker, showing yellow dots. These results indicated that both SS- and CS-DNA NPs, regardless of their difference in size, entered the cells via a non-degradative pathway or escaped from the lysosomes very efficiently.

4. Discussion

Disulfide linkages have been introduced to gene delivery vector systems based on peptides [2,11,13,14,17], polyamines including poly-ethylenimine (PEI) [12,15,18], other synthetic polymers [16] and lipids [10], but the sites at which the disulfide bonds are introduced vary. They fall into three groups: 1) disulfide bonds in the polycation backbone [12,15,17,18], 2) disulfide bonds between the polycation backbone and PEG [2,4,10,16], and 3) disulfide bonds cross-linking two polycation macromolecules formed during template polymerization [11,13,14]. For a comprehensive review on current advances in the development of reduction-sensitive non-viral delivery system, the reader is referred to two recent review articles [28,29]. To study DNA NPs that can be reduced, we placed the disulfide bond between the polylysine backbone and the PEG for several reasons. First, there is some evidence that the PEG shielding, while improving the colloidal stability of the DNA complex in extracellular environment, may hinder transgene expression [30,31], so rapid detachment of the PEG moiety may favor efficient gene transfer. Second, this chemical linkage replacement does not change the physical properties of the DNA NPs as we confirmed, but introducing the disulfide bonds at other sites can cause changes in the charge, shape or size of the DNA complexes [11,14] which may complicate the interpretation of the results. Lastly, the synthesis process is relatively simple. The thioether bond between PEG

and polylysine can be easily replaced with a disulfide bond by changing the functional group on the PEG. Consistent with other studies that introduced disulfide bonds between PEG and polycation [2,16], SS-DNA NPs mediated more than 10-fold higher transgene expression *in vitro* compared to CS-DNA NPs (Fig. 1C).

In contrast to the rationale of exploiting the high redox potential difference between the oxidizing extracellular space and the reducing intracellular space as a trigger for intracellular DNA release, our data suggested an extracellular mechanism for the enhanced gene transfer mediated by reducible DNA NPs. The first evidence came from the detection of different cellular uptakes of SS- and CS-DNA NPs (Fig. 2C). Many studies on reducible gene transfer agents failed to measure or report cellular uptake of the gene transfer reagent; so extracellular mechanisms could have existed in those cases, but went undetected. The second key piece of evidence supporting extracellular mechanisms was the inhibition of SS-DNA NP mediated *in vitro* transfection by the cell impermeable reagent DTNB.

To test whether SS-DNA NPs react with secreted free thiols in the medium which can be blocked by DTNB, we first examined the stability of SS-DNA NPs in extracellular environment. In many other published studies, the stability of reducible gene transfer agents was also assessed, but only in cell free systems with added reducing agent either at very high concentration (10–45 mM) to show that DNA can be released under reducing conditions [12,14,16,17], or at very low concentration (10 μ M) to show the DNA complexes are stable under conditions considered comparable to extracellular environment [16]. This study directly tested conditioned media with Ellman's assay and found the extracellular secreted thiols reached levels of 30–180 μ M, much higher than 10 μ M. With multiple assays, we clearly showed that extracellular secreted free thiols caused dePEGylation and, instead of premature DNA release, aggregation of SS-DNA NPs. The different thiol secretion profiles of the two cell lines we tested provided a good model system to test our hypothesis. In both cell lines, the level of extracellular thiols correlated not only with dePEGylation and aggregation, but also with gene expression. However, disulfide cleavage may result in DNA release instead of aggregation for other reduction-sensitive vector systems. The outcome and even the dynamics of the process [17] are likely to be determined by numerous factors including the site of disulfide introduction, N/P ratio and the concentration of free thiols.

To test the possibility that SS-DNA NPs are reduced by cell surface PDI, which is also inhibited by DTNB [20], we further studied the effect of a PDI inhibitor, bacitracin, on *in vitro* gene transfer mediated by SS-DNA NPs. At concentrations non-toxic to the cells, bacitracin had no inhibitory effect on transgene expression (data not shown), indicating that PDI is not involved in SS-DNA NP mediated enhanced *in vitro* gene transfer.

Our data from non-PEGylated DNA NPs suggest that it is aggregation of DNA NPs, whether it is caused by disulfide reduction or other means, which directly causes the increase in gene transfer. To corroborate this conclusion, we prepared a series of partially PEGylated non-reducible DNA NPs by compacting DNA plasmids with mixed PEG-CS-CK₃₀ and K₃₀. TEM and *in vitro* transfection studies (Fig. S6 of Appendix A) indicated that independent of disulfide reduction, transgene expression correlated in a dose dependent manner with the extent of aggregation which was ultimately determined by the PEGylation ratio. Notably, with 1 h incubation time, SS-DNA NP mediated gene transfer was less efficient than partially PEGylated DNA NPs in HuH7 cells but more efficient than all of them in HeLa cells (Fig. S6B of Appendix A). This is explained because the aggregation of SS-DNA NP is determined by the accumulation of extracellular thiols which is in turn determined by thiol secretion rate of the cell lines and incubation time. Large aggregates of non-PEGylated DNA NPs were less efficient than SS-DNA NPs in *in vitro* gene transfer with 6 h incubation time (Fig. 5E), so an intermediate size may be optimal for cellular

uptake, given enough time. We speculate that the aggregation of dePEGylated or partially PEGylated DNA NPs results in changes of the hydrodynamic size and surface charge of the DNA NPs which may lead to enhanced physical concentration of DNA at the cell surface [32] and more efficient binding to cell surface negative charges.

To test directly whether intracellular mechanisms are involved, a series of progressively stabilized SS-DNA NPs were prepared to ensure that at least one of the stabilized SS-DNA NPs can survive the extracellular environment but still be reduced intracellularly. Our data suggested that at least PhMSS-DNA NP met both of the above criteria. In support of this conclusion, a previous study showed that a disulfide cross-linked immunotoxin conjugate which was also stabilized extracellularly by SMPT could be reduced inside the cell to release functional toxin [33], indicating that PhMSS-DNA NP could also be reduced intracellularly.

Although our data indicated that no thiol related intracellular mechanism is involved, it is still possible that different intracellular fates of particles with different sizes may affect gene expression. Ogris et al. [34] provided evidence that aggregated DNA/PEI complexes (>1000 nm), which mediated more efficient gene transfer than smaller particles (40 nm), are also more efficient in endosomal escape; incubation with chloroquine enhanced the gene expression of small complexes 10-fold but had no effect on large complexes. To test whether the same mechanism applies for aggregated SS-DNA NPs, we performed a similar experiment with chloroquine. In contrast to their results, no increase of transfection was observed in the presence of chloroquine for either SS-DNA NPs or CS-DNA NPs (data not shown). Previous studies in our lab [5], and by others [35,36], showed that non-reducible DNA NPs do not enter endolysosomal pathway. We confirmed this result by confocal fluorescence imaging and further showed that reducible DNA NPs were not colocalized with lysosomal markers either (Fig. 8). Those results explain why chloroquine had no effect on either of the DNA NPs.

In summary, we describe in detail an extracellular mechanism for enhanced gene transfer mediated by reducible DNA NPs. This is the first study to show the important role of extracellular secreted thiols in determining the stability of a reducible gene transfer agent and its transfection efficiency. Although reduction-sensitive vector systems with different formulations may be affected differently by extracellular or even intracellular thiols, extracellular mechanisms should be considered when introducing disulfide linkages to gene delivery systems as well as other drug delivery systems. We also established a strategy to enhance progressively the stability of disulfide bonds in synthetic polymers which may find application in other polymer based delivery systems. With stabilized reducible DNA NPs as controls, we showed that intracellular dePEGylation is not a rate limiting step for PEG-polycation block copolymer based gene delivery. The utility of reducible DNA NPs *in vivo* still requires further investigation. Judged by its working mechanism *in vitro*, it is probably more useful for local gene delivery in a confined compartment or in gene activated matrix.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.jconrel.2010.04.031.

Fig. 1. Reducible SS-DNA NPs mediate superior *in vitro* gene transfer as compared to non-reducible CS-DNA NPs. (A) Schematics depicting the preparation of SS- and CS-DNA NPs. (B) Electron micrographs of negatively stained SS- and CS-DNA NPs diluted in saline. Scale bar=200 nm. (C) Luciferase expression of SS- and CS-DNA NPs in HeLa and HuH7 cells. Cells were transfected with SS- or CS-DNA NPs for 6 h in serum-free media. Results are mean±SD of 8 replicates from 2 independent experiments. ** $P<0.01$, *** $P<0.001$.

Fig. 2. SS-DNA NPs show higher cellular uptake compared to CS-DNA NPs by flow cytometry. Flow cytometric analysis of uptake of YOYO-1 labeled CS-DNA NPs (A) or SS-DNA NPs (B) by HeLa cells at dosage (μg pDNA/well in 6-well plates) indicated in the figure. (C) Comparison of cellular uptake of CS- and SS-DNA NPs (10 μg pDNA/well).

Fig. 3.

Enhanced *in vitro* gene transfer mediated by SS-DNA NPs can be inhibited by DTNB. (A) Cytotoxicity of DTNB. Cell viability was measured by MTT assay and expressed in percentage of metabolic activity relative to untreated controls. The results shown are mean $\pm$ SD from 6 replicates. (B) Inhibition of *in vitro* gene transfer by DTNB. HeLa cells were incubated with increasing concentrations of DTNB before (for 30 min) and during transfection (for 1 h 45 min). No Trt controls, cells were transfected with DNA NPs in the absence of DTNB. Results are mean $\pm$ SD of 12 replicates from 4 independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 4.

SS-DNA NPs are destabilized by extracellular thiols. (A) Sedimentation assay of SS- and CS-DNA NPs incubated with conditioned media. SS- or CS-DNA NPs were incubated with HeLa or HuH7 conditioned media in 37 °C water bath for the indicated periods of time. Sedimentation was assayed as described in the methods and quantified as the percent of DNA recovered after centrifugation. Results are mean $\pm$ SD from 3 independent experiments. (B) Ellman's assay. Cells were grown under the same condition as in Fig. 1C, but without adding DNA NPs. At indicated time points, the concentration of total free thiols in the media was determined by Ellman's assay. Results are mean $\pm$ SD from 3 independent experiments.

Fig. 5.

DePEGylation of SS-DNA NPs by extracellular thiols causes aggregation of DNA which leads to higher transgene expression. In all the experiments, HeLa and HuH7 cells were cultured and transfected with DNA NPs under the same condition as in Fig. 1C, except for the time of DNA NP incubation, which was varied as indicated. (A) PEG released in the media from DNA NP transfected cell culture was analyzed by SDS-PAGE and stained with iodine. DNA NPs were also incubated in 37 °C water bath with fresh media with or without 4 mM cysteine as controls. (B) Aggregation of SS-DNA NPs detected by TEM. Media from DNA NP transfected cell culture were directly used to prepare TEM samples. Control samples were prepared with media from cell cultures that were not transfected with DNA NPs. Scale bar=200 nm. (C, D) The time of DNA NP incubation with HuH7 (C) and HeLa (D) cells was varied from 1 h to 6 h as indicated in the figure. Results are mean $\pm$ SD of 8 replicates from 2 independent experiments. (E) DNA plasmids were compacted with non-PEGylated polylysine (K₃₀) in water. The cells were incubated with DNA NPs for 6 h. The luciferase expression from CS-DNA NP transfected cells was set to arbitrary 1. Results are mean $\pm$ SD from 8 replicates of 2 independent experiments. ** $P < 0.01$, *** $P < 0.001$.

Fig. 6.

Preparation and characterization of PEGylated polylysine with stabilized disulfide bonds. (A) Schematics depicting the synthesis routes for PEGylated polylysines with progressively stabilized disulfide bonds. (B) Resistance of stabilized disulfide bonds to reduction by cysteine. Stabilized and unstabilized reducible PEGylated polylysines were incubated with increasing concentrations of cysteine as indicated in the figure at pH 7.0 in 37 °C water bath for 1 h. Non-reducible PEG-CS-CK₃₀ was also incubated with or without cysteine as controls. The PEGylation state of polylysine was determined by AU-PAGE and Coomassie blue staining. Ph, benzene ring. M, methyl group.

Fig. 7. Stabilized SS-DNA NPs lost the superior gene transfer ability. HeLa and HuH7 cells were cultured and transfected with unstabilized and stabilized SS-DNA NPs and CS-DNA NPs under the same condition as in Fig. 1C. The time of incubation with DNA NPs was 1 h for HeLa cells and 3 h for HuH7 cells. The luciferase expression from CS-DNA NP transfected cells was set to arbitrary 1. Results are mean $\pm$ SD from 9 replicates of 3 independent experiments.

Fig. 8.

Intracellular distribution of SS- and CS-DNA NPs. Rhodamine labeled CS- or SS-DNA NPs (red) were incubated with HeLa and HuH7 cells. As positive controls for lysosomal localization, the cells were incubated with rhodamine labeled dextran (neutral 70 k MW). After incubation with DNA NPs or dextran, lysosomes were stained with LysoTracker (green). Live cell confocal fluorescence microscopy was then performed to analyze the internalized DNA NPs for colocalization with lysosomes. Scale bar=10 μ m.