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Gene delivery through the use of a hyaluronate-associated intracellularly degradable cross-linked polyethyleneimine

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Abstract

For a non-viral gene delivery system to be clinically effective, it should be non-toxic, compatible with biological components, and highly efficient in gene transfection. With this goal in mind, we investigated the gene delivery efficiency of a ternary complex consisting of DNA, an intracellularly degradable polycation, and sodium hyaluronate (DPH complex). Here, we report that the DPH ternary complex achieved significantly higher transfection efficiency than other polymer systems, especially in the presence of serum. The high transfection efficiency and serum tolerance of DPH are attributed to a unique interplay between CLPEI and HA, which leads to (i) the improved stability of DNA in the extracellular environment and at the early stage of intracellular trafficking and (ii) timely dissociation of the DNA-polymer complex. This study reinforces findings of earlier studies that emphasized each step as a bottleneck for efficient gene delivery; yet, it is the first to show that it is possible to overcome these obstacles simultaneously by taking advantage of two distinctive approaches.

Keywords

Non-viral gene delivery; crosslinked polyethyleneimine; sodium hyaluronate; extra- and intracellular stability; DNA unpacking

1. Introduction

Gene therapy has widely been explored as a promising therapeutic tool for treating genetic disorders as well as cancers. Modified viruses represent the most popular approach, having been used in two-thirds of clinical trials performed to date [1]. However, there are significant concerns about the safety of viral vectors, such as the potential for unwanted immune responses [2] and aberrant gene expression [3,4], which prompted parallel pursuit for non-viral vectors.

Most non-viral vectors are made of cationic lipids and polymers. Among them, polyethyleneimine (PEI) is one of the most widely used polymers. PEI forms a complex with DNA, whose size is suitable for endocytic uptake by non-phagocytic cells [5–7]. Once in the

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cells, PEI induces the so-called “proton sponge effect”, which allows for the complex to escape from endo-/lysosomal trafficking pathway [8]. PEI has long been considered a benchmark polymeric vector; however, there are several unsolved issues in using this polymer for therapeutic applications *in vivo*.

First, most PEI's that are efficient in gene delivery are cytotoxic [9]. It is believed that its cationic charges facilitate endocytosis through negatively charged cell surface, but such interactions also compromise the cell membrane and cause significant cell death [10]. Additionally, the internalized PEI causes apoptotic cell death by forming pores in the mitochondrial membrane [11]. Second, gene transfection efficiency of the DNA-PEI system diminishes significantly in the presence of biological components, which makes it unsuitable for systemic application. In addition, DNA-PEI complexes tend to aggregate in the presence of salts and/or serum proteins and are readily cleared by the immune system [12]. Third, PEI as well as other polymeric vectors are, in general, much less efficient than viral vectors [13].

In an effort to develop alternative non-viral vectors that are non-toxic and effective *in vivo*, a number of groups have synthesized new cationic polymers [14,15], modified existing polymers [16,17], or blended with more biocompatible polymers [18,19]. Earlier studies reported that addition of polysaccharide-based polyanions, such as alginate [20] or hyaluronate (HA) [21], improved the gene transfection efficiency of the DNA-PEI complex in the presence of serum. The main role of the polyanionic coating was to protect the complexes from undesirable interactions with serum proteins. The latter study also suggested that HA enhanced the gene transfection by loosening the complex, thereby facilitating the access of the gene transcription machinery [21]. In line with this observation, other studies found that polycations with disulfide linkages, which could degrade in the reductive intracellular environment and release DNA more easily, achieved higher gene expression than their uncleavable counterparts [22–24]. Among these polymers are polyrotaxane modified with disulfide linkage [22] and disulfide-crosslinked low molecular weight (MW) linear PEI (CLPEI) [23–25].

In light of these findings, we hypothesized that combined application of a polyanion (e.g., HA) and an intracellularly degradable polycation would further enhance the gene transfection efficiency, especially in the presence of serum. First, HA would decrease the positive charge of the polycation, thereby reducing interactions with serum proteins. Second, once taken up by cells, HA and CLPEI would synergistically promote the intracellular unpacking of the DNA-polymer complex. Here, we prepared a ternary complex consisting of plasmid DNA, CLPEI, and HA (Fig. 1A), and compared the level of *in vitro* gene expression with that of other carriers in the presence of various concentrations of serum. We also investigated the mechanism by which the DPH complex achieved relatively high level of gene transfection in the presence of serum.

2. Materials and Methods

2.1. Materials

Linear PEI's (MW: 2.5kDa and 25kDa) were purchased from Polysciences. Branched PEI (MW: 25kDa), dithiobis(succinimidyl propionate) (DSP), and Sephadex G25 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), calf serum (CS), LipofectamineTM2000 were purchased from Invitrogen (Carlsbad, CA, USA). Sodium hyaluronate was obtained from Lifecore Biomedical (Chaska, MN, USA). Label IT[®] Nucleic Acid Labeling Kits, CyTM3 and CyTM5 were purchased from Mirus Bio Corporation (Madison, WI, USA). All other chemicals were purchased from Aldrich and used without further purification.

2.2. Synthesis and characterization of disulfide-crosslinked low molecular weight linear polyethyleneimine (CLPEI)

CLPEI was synthesized according to the scheme modified from the literature [24]. Briefly, LPEI (2.5kDa) 500 mg was dissolved in phosphate-buffered saline (PBS, pH 7.2) at 50°C, to which DSP 72 mg in 400 μ L dimethyl sulfoxide was slowly added under stirring. The reaction mixture was kept at room temperature for 24 hours. CLPEI was purified using Sephadex G25 column and dialyzed against deionized (DI) water (Spectra/Por 7 MWCO: 1000), followed by lyophilization. The resulting light yellow solid was dissolved in 1 N HCl, which was further purified by precipitation in acetone. The final product was lyophilized and stored at -20°C. For analysis, the product was dissolved in DI water and precipitated with concentrated aqueous sodium hydroxide. The white gel-like residue was washed with water until the supernatant became neutral. The purified amine bases were dried at 70°C under reduced pressure. The resulting product was analyzed by ^1H NMR (Bruker ARX300) and elemental analysis (Columbia Analytical Services, Tucson, Az).

2.3. Preparation of pEGFP-C1 and pBR322 plasmids

pEGFP-C1 (4.7 kb) and pBR322 (4.36 kb) plasmids were propagated in *Escherichia coli* and then extracted and purified using the Plasmid Midi Kit (Qiagen, Valencia, CA). The DNA concentration was determined by measuring UV absorbance at 260 nm. DNA aliquots were stored at -20°C. The pEGFP-C1 plasmid was used as a reporter gene for evaluation of gene transfection efficiency. The pBR322 plasmid was used as a mock plasmid for monitoring endocytosis, intracellular trafficking, and FRET imaging of the DNA-polymer complexes. The pBR322 plasmid was labeled with Cy3, Cy5, or both using the Label IT[®] kit according to the manufacturer's protocol.

2.4. Cell culture

NIH/3T3 mouse fibroblasts and M109 murine lung cancer cells were obtained from American Type Culture Collection (Rockville, MD) and propagated to confluence in T-75 flasks (Corning Costar, Cambridge, MA) in 15 ml of DMEM medium supplemented with 100 IU/mL penicillin and 100 μ g/mL streptomycin, and 10% calf serum (CS, for fibroblasts) or 10% fetal bovine serum (FBS, for M109 cells). Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ and harvested from flasks with 0.25% trypsin/0.03% EDTA.

2.5. Agarose gel retardation assay

DNA-binding capability of CLPEI was evaluated using the agarose gel retardation assay and compared with that of the starting material, linear PEI (MW: 2.5kDa). The DNA-polymer complexes were prepared at various N/P ratios by mixing solutions of polymer and DNA in different ratios followed by incubation at room temperature for 30 min. The DNA-polymer complexes were analyzed by agarose gel (0.8%) electrophoresis.

2.6. Formation and characterization of DNA-CLPEI-HA (DPH) ternary complexes

Our preliminary experiment with binary complexes of DNA and CLPEI (DP) showed that the highest transfection efficiency was obtained at the N/P ratio of 40/1 (data not shown). To form a DPH ternary complex, a DP complex at the N/P ratio of 40/1 was first prepared by mixing solutions of DNA and CLPEI at an appropriate ratio in HEPES buffered saline (HEPES 10 mM, pH 7.2) and incubating the mixture at room temperature for 30 min. Subsequently, the DP complex was added to a solution of HA (35kDa, unless specified otherwise) in HEPES buffered saline to achieve a HA/DNA ratio (w/w) of 0.5 to 10 and incubated at room temperature for 10 min. Sizes and zeta potentials of DP and DPH complexes were determined with the Nano-ZS90 Zetasizer (Malvern Instruments Ltd., Worcestershire, UK). The DNA-polymer complexes were diluted with PBS prior to the measurement.

2.7. In-vitro gene transfection

Binary complexes of DNA and CLPEI (DP) and ternary complexes of DNA, CLPEI, and HA (DPH) were prepared using pEGFP-C1 as a reporter gene. The typical N/P ratio of DNA to CLPEI was 40/1, and the typical w/w ratio of HA to DNA in the DPH complex was 1/1, unless specified otherwise. For comparison, a DNA complex with Lipofectamine™2000 (Lipo2K) were prepared according to the manufacture's protocol. A binary complex (DB or DL) of DNA and branched PEI (25kDa) or linear PEI (25kDa) and its ternary complex with HA (DBH or DLH) were prepared at the N/P ratio of 20/1 and the HA/DNA w/w ratio of 1/1. Transfection medium was prepared by diluting the DNA-polymer complexes with DMEM medium, to which CS or FBS was added to 10% or 50%. NIH/3T3 and M109 cells were seeded at a density of 20,000 cells per well and 30,000 cells per well in 24-well plates, respectively, and grown to reach 70% confluence. The culture medium was replaced with 300 μ L of the transfection medium containing DL, DB, DP, DPH, or Lipo2K complex equivalent to 0.5 μ g DNA, followed by 24 hour-incubation at 37°C. The transfection medium was then replaced with the fresh complete DMEM medium (10% serum), and the cells were allowed to grow for 48 hours. Finally, the cells were washed with PBS and lysed with the cell culture lysis buffer (Promega) and the fluorescent intensity of the lysed cell suspension was measured with Tecan Spectrafluor Plus microplate reader (Ex: 485 nm; Em: 530 nm). The gene transfection efficiency was expressed as the relative fluorescence intensity.

To observe the effect of extracellular HA, the cells were transfected in the medium supplemented with 50–500 μ g/mL of HA in addition to 10% serum. When the effect of intracellular reduction potential on the gene transfection efficiency was investigated, the transfection experiment was performed after treating the cells with glutathione-monomethylester (GSH-MME) or buthionine sulfoxamine (BSO). To observe the effect of the increased intracellular reduction potential, NIH/3T3 cells were transfected with DNA-polymer complexes in the medium containing 0, 5, and 20 mM GSH-MME and 10% serum. To observe the response to the decreased intracellular reduction potential, NIH/3T3 cells were grown in the medium containing 2 mM BSO for 24 hours prior to transfection. Subsequently, the medium was replaced with the transfection medium (10% serum) containing various DNA-polymer complexes.

2.8. Cytotoxicity assay

The cytotoxicity of CLPEI and a mixture of CLPEI and HA were evaluated with the MTT assay. NIH/3T3 and M109 cells were seeded in 96-well plates at an initial density of 8,000 cells per well and 6,000 cells per well in 200 μ L of the complete medium. After 24 hours, the medium was replaced with 200 μ L of fresh complete medium, to which Lipofectamine™2000, BPEI, CLPEI, or a mixture of CLPEI and HA was added to achieve polymer concentrations from 1 μ g/mL to 50 μ g/mL. After 24 hours of incubation, the (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) (MTT) reagent and the stop solution were added according to the Promega protocol for the CellTiter 96® Non-Radioactive Cell Proliferation Assay. The cell viability was quantified by measuring the UV absorbance at 560 nm with a Tecan microplate reader. The measured absorbance was normalized to the absorbance of non-treated control cells.

2.9. Endocytosis and intracellular trafficking of DNA-polymer complexes

Cellular uptake and intracellular location of DNA-polymer complexes were monitored by fluorescence-activated cell sorting (FACS) and confocal microscopy, respectively. To avoid interference of green fluorescent protein, pBR322 plasmid with a comparable molecular weight was used in lieu of pEGFP-C1 plasmid. The plasmid was labeled with Cy3 or Cy5 for fluorescence detection.

For FACS, NIH/3T3 cells were cultured in a 6-well plate at the cell density of 500,000 cell per well in 2 mL medium and allowed to grow for 24 hours. The DNA-polymer complexes were prepared with Cy5-labeled pBR322 plasmid and added to the cells. After 3 hours, 6 hours, or 24 hours of incubation with the DNA-polymer complexes, the cells were washed with PBS twice, trypsinized with 0.25% trypsin-EDTA, washed with PBS containing 3% FBS, and fixed with 4% formaldehyde. The fixed cells were washed once and resuspended in PBS and stored at 4°C prior to the FACS measurement. In all FACS analysis, cell debris and aggregates were excluded by setting a gate on the plot of side-scattered light (SSC) vs. forward-scattered light (FSC). The cell fluorescence intensity was measured with FACS (Cytomics™ FC 500) equipped with Helium-Neon ion laser (633nm) and FL4 = 675 nm band path. A total of 20,000 gated cells were analyzed using WinMDI software (Joe Trotter, Scripps Institute). Non-treated cells were analyzed in parallel as a negative control.

For intracellular trafficking of the DNA-polymer complexes, NIH/3T3 cells were plated in a 35-mm dish with a glass window (MatTek) at a density of 100,000 cells in 2 mL of culture medium. The DNA-polymer complexes were prepared with Cy3-labeled pBR322 plasmid. After overnight culture, the medium was replaced with 2 mL of transfection medium containing the DNA-polymer complexes, and the cells were incubated with the DNA-polymer complexes for 6 hours. The transfection medium was then replaced with the fresh complete medium. The cells were imaged with the confocal laser scanning microscope (Olympus X81) operated with Fluoview software (Olympus, Japan). The DNA-polymer complexes were excited using a 488-nm laser, and the emission signal was read from 560 to 630 nm and expressed in red. LysoTracker® was added half hour before the observation at the concentration of 150 nM and excited using a 488-nm laser. Its emission was read from 500 to 550 nm and expressed in green. The nuclei were stained with DRAQ5 (5 µM, AXORA LLC, San Diego, CA, USA) and excited using a 633-nm laser, and its emission was read from 650 to 750 nm and expressed in blue. Images were processed with the NIH ImageJ software (Version 1.36b, National Institutes of Health, USA).

2.10. Stability of the DNA-polymer complexes in the presence of deoxyribonuclease (DNase), serum and dithiothreitol (DTT)

To evaluate the stability of DNA-polymer complexes in the presence of DNase and serum, DNA-polymer complexes were incubated with 166 U/mL DNase for 15 min or 50% CS for 1 hour, followed by the addition of EDTA buffer. The resulting mixture was optionally incubated with 4 mg/mL heparin for 2 hours prior to the agarose gel electrophoresis. To estimate the stability of DPH complexes in the extra- and intracellular environment, the complexes were incubated with 50% CS with or without 10 mM DTT for 3 hours. After quenching the enzyme activity by addition of EDTA buffer, the reaction mixture was loaded on 0.8% agarose gel and electrophoresed at a constant voltage (120 V). Agarose gels were stained with ethidium bromide and scanned with Gel documentation system (BioRad, Gel DOC XR). The gel intensity was quantified with BioRad Quantity One software (version 4.6.1).

2.11. Förster resonance energy transfer (FRET) imaging

FRET imaging was performed to investigate the intracellular dissociation of the DPH complexes. The pBR322 plasmid was dual-labeled with Cy3 and Cy5 using the Label IT® kit. According to the manufacturer's protocol, the average density of fluorescent dyes is one dye molecule per 170 DNA base pairs. DPH complexes were prepared using the dual-labeled pBR322. NIH/3T3 cells were plated in a 35-mm dish with a glass window (MatTek) at a density of 100,000 cells per dish and incubated for 24 hours. Transfection medium containing the DPH complexes was then added to cells at the concentration of 4 µg DNA per well. After 3 hours of incubation, the transfection medium was removed by aspiration, and the cells were washed twice with the fresh complete medium. The emission spectra of the DPH complexes were

acquired with a confocal laser scanning microscope (Olympus X81) using the lambda function of the Fluoview software (Olympus, Japan). Excited with a 543 nm laser, the emission signals were collected stepwise from 550 to 750 nm at the step length of 5 nm and at the bandwidth of 5 nm.

2.12. Statistical analysis

All data were expressed as averages with standard deviations. First, ANOVA was used to determine statistical difference among the groups, and then pair-wise comparison was made using the Student t-test. A p-value of <0.05 on a 2-tailed test was considered statistically significant.

3. Results

3.1. Characterization of CLPEI and the DNA-polymer complexes

CLPEI was synthesized according to the method modified from the literature [24]. According to the elemental analysis, every two linear PEI molecules were crosslinked via 1.18 disulfide bonds. As shown in the agarose gel retardation assay (Supplementary Fig. S1), CLPEI formed a complex with DNA at a lower N/P ratio than the starting material, LPEI 2.5kDa. This result implies that MW of the CLPEI was higher than LPEI 2.5kDa, the starting material, as a result of crosslinking via DSP. Earlier studies show that a PEI with a relatively higher MW forms a DNA/polymer complex at a lower N/P ratio [10,26].

A binary complex of DNA and CLPEI (DP complex) was 108.2 ± 10.3 nm in diameter at the N/P ratio of 40/1, according to the dynamic light scattering measurement, and its zeta potential was 14.9 ± 0.6 mV (Supplementary Fig. S2). Upon addition of HA, the zeta potential of the DPH complexes decreased from 14.9 mV to -12 mV in proportion to the amount of HA. The size of DPH complex increased with the increase of HA, reaching maximum at the neutral zeta potential, most likely due to aggregation of the neutralized complexes. Further addition of HA at a HA/DNA w/w ratio of 50/1 resulted in aggregate formation despite the negative surface charge, which is attributable to entanglement of excessive HAs. Particle size and zeta potential of the DPH complex with a HA/DNA w/w ratio of 1/1 and the N/P ratio of 40/1, which was used in most transfection studies, were 458.9 ± 0.7 nm and 5.0 ± 0.3 mV, respectively. Due to the interference of serum proteins, particle size in media containing serum was not determined; thus, it is not known whether the particle size would further change in the presence of serum.

3.2. In-vitro gene transfection efficiency

The gene transfection efficiency of the DP and DPH complex was compared with those of other binary or ternary systems (DL, DLH, DB, and DBH) based on BPEI (25kDa) or LPEI (25kDa), or LipofectamineTM2000, in 10% or 50% serum. Ten percent was chosen as it is a typical serum concentration in most *in-vitro* cell culture studies, and 50% serum content was used to make it comparable to the serum content in the blood [27]. In the absence of serum, the DP complex showed relatively high transfection efficiency as compared to other binary complexes (DL or DB) in the NIH/3T3 fibroblast cells (Fig. 1B, Supplementary Fig. S3), which is consistent with the literature [24]. However, when the DP complex was applied in the medium containing serum, the transfection efficiency was significantly reduced, especially in 50% serum. Other binary systems (DL, DB) also showed negligible gene transfection in 50% serum.

For the complexes containing BPEI or LPEI, formation of ternary complexes with HA did not improve the gene transfection in the presence of 50% serum. While the DBH complex showed significant transfection in 10% serum, it had no effect in 50% serum. The DLH complex was also not effective in 50% serum. On the other hand, the NIH/3T3 cells transfected with the

DPH ternary complex showed consistently high GFP expression at all levels of serum (Fig. 1B, Supplementary Fig. S3). LipofectamineTM2000, a commercial gold standard transfection reagent, showed lower transfection efficiency than DPH complex in the serum-free medium, but it had comparable transfection to that of the DPH complex in 10 or 50% serum. The DPH complexes were similarly effective in the M109 cell line (Fig. 1C), but the serum tolerance of the DPH complexes was not observed at the HA/DNA w/w ratio exceeding 5/1. Unlike in the NIH/3T3 cells, LipofectamineTM2000 showed negligible gene transfection in the M109 cells in the presence of 50% serum.

Given that the consistently high gene transfection in 50% serum was only seen in the DPH complex with an optimal HA/DNA ratio, it was expected that the serum-tolerant transfection capability of the DPH complex would be due to the interplay between CLPEI and HA. The following experiments were performed to investigate the mechanism by which the DPH complex achieved the high gene transfection efficiency at all levels of serum.

3.3. Cytotoxicity of the DNA carriers

To examine if the DPH had an exceptional safety profile, cytotoxicity of various DNA carriers was evaluated using the MTT assay. As shown in Figs. 2A and 2B, the carrier part of DPH system (a mixture of CLPEI and HA) was not toxic for both NIH/3T3 and M109 cell lines at its working concentration. CLPEI was similarly non-toxic in both cell lines. It was suggested that the low toxicity of CLPEI was due to the intracellular degradability of the polymer [23]. Inside the cells, CLPEI degrades to lower MW linear PEI (LPEI), which is relatively non-toxic, owing to the low MW and the secondary amine structure of the LPEI [9, 23, 28]. LipofectamineTM2000 was also non-toxic to NIH/3T3 fibroblasts but was slightly toxic to M109 cells at its working concentration. BPEI was relatively more toxic in both cell lines as compared to other carriers, but both cells maintained >80% viability at the concentration that BPEI was typically used. Despite some difference, most DNA carriers were not significantly toxic, showing that the cytotoxicity alone did not explain the high transfection efficiency and serum tolerance of the DPH complex.

3.4. Endocytosis of DNA-polymer complexes

To investigate if the high DPH transfection efficiency was a result of HA receptor-mediated endocytosis, the transfection experiment was performed in the medium supplemented with HA. The transfection efficiency in the NIH/3T3 cells was not inhibited by the HA in the medium (Supplementary Fig. S4), suggesting that the high transfection efficiency of DPH complex was not due to the privileged endocytosis.

Flow cytometric observation of the endocytosis of DNA-polymer complexes was consistent with the previous result. As early as in 3 hours, 70–100% of the cells treated with the DB, DBH, DP, and DPH complexes were Cy5-positive (Fig. 2C). The fractions of cells taking up the ternary complexes (DBH, DPH) were lower than those of binary counterparts (DB, DP) (Fig. 2C). These results show that the transfection efficiency did not correlate with endocytosis of the complex, and that the higher transfection efficiency of the DPH complex was not due to the enhanced cellular uptake of the complex. While individual cells transfected with Lipo2K showed more intense fluorescence as compared to those with DB, DBH, DP, and DPH complexes (Fig. 2D, Fig. 2E), the fraction of Cy5-positive cells was <70% at 3 hours. Interestingly, the Cy5-positive fraction of Lipo2K-transfected cells decreased to 45% after 24 hours. In contrast, the cells internalizing DB, DBH, DP and DPH complexes maintained the level of fluorescence for 24 hours.

3.5. Intracellular trafficking of DNA-polymer complexes

Intracellular fates of the DNA-polymer complexes were compared using confocal laser scanning microscopy. As shown in Fig. 2E, most DNA-polymer complexes including DB, DP, DPH and Lipo2K (red fluorescence due to the Cy5-labeled DNA) were not colocalized with lysosomes (green fluorescence) in as early as 6 hours, showing that all the complexes had already moved from endo-/lysosomes to the cytosol in 6 hours. Therefore, the difference in transfection efficiency was not due to the efficiency of endosomal escape.

3.6. Stability of the DNA-polymer complexes in the presence of serum

Most serum proteins such as albumin and globulins are negatively charged at the physiological pH; thus, interaction between the protein and the DNA-polymer complexes may destabilize the complexes [29]. Moreover, serum contains various nucleases, which can further degrade the DNA exposed on the surface of the carriers [30]. To test if the DPH complex has higher stability in the presence of some serum components, plasmid DNA, DB, DBH, DP, and DPH complexes were treated with 166 U/mL of DNase and/or 4 mg/mL of heparin. Here, heparin was used on behalf of negatively charged serum proteins [25].

Degradation of plasmid DNA by DNase with or without heparin was evidenced by the faint smear of DNA down the lane (Fig. 3A). DNA in the DB and DBH complexes was well protected against DNase. The position of DNA smear indicated that DNA degradation under heparin treatment was less than that of free plasmid, but it was evident that neither complex protected DNA from heparin treatment completely.

In contrast, DNA in the DP and DPH complexes was more resistant to the treatment with DNase and heparin. Only a small degree of DNA in the well (loose enough to allow ethidium bromide intercalation) was noticeable, and intensities of DNA smear bands of DP and DPH were significantly smaller than those of DB and DBH treated in the same conditions (Fig. 3A). Since DPH appeared to be more stable than DP, the two complexes were further compared after treatment with 50% CS, a condition where DPH showed transfection but DP did not. As shown in Fig. 3B, DPH displayed less intense smear than DP in 50% CS ($p < 0.05$). These results suggest that the high transfection efficiency of DPH may be attributable, at least partly, to the resistance to the serum-mediated decomplexation and DNA degradation.

3.7. Intracellular unpacking of DNA-polymer complexes

Since we originally hypothesized that HA would facilitate DNA unpacking triggered by the intracellular CLPEI degradation, we expected that DPH transfection would be affected by the level of intracellular GSH level that causes CLPEI degradation. To test this, DPH transfection was evaluated in the cells treated with GSH-MME or BSO. GSH-MME is a precursor for intracellular GSH synthesis and rapidly internalized by cells to form GSH [31,32]. Treatment with BSO, an inhibitor of γ -glutamylcysteine synthetase, results in depletion of intracellular GSH [33]. Fig. 4A shows that the transfection by the DPH complex increased significantly with increasing GSH-MME, and depletion of intracellular GSH by BSO led to inhibition of DPH transfection (Fig. 4B). This result shows that CLPEI degradation did play a role in DPH transfection, presumably through the efficient unpacking of DNA.

Much like DPH, DP transfection was completely inhibited upon treatment with BSO. On the other hand, neither DB nor DP was affected by the increase of GSH-MME concentration. The lack of difference in DP transfection at higher GSH-MME concentrations was consistent with the previous report [23] but surprising, given that the DPH (which shared the same degradable component, CLPEI) showed significant difference in such conditions. A potential interpretation is that the unpacking of DP complex was completed before the unpacking-dependent cellular events started to occur, irrespective of the GSH level, whereas HA continued

to protect the endocytosed DPH and delayed the DNA unpacking from the DPH so that the extent of DNA unpacking could be reflected on the gene expression.

To observe the kinetics of intracellular unpacking of the DNA-polymer complexes, the endocytosed complexes were observed by Förster Resonance Energy Transfer (FRET) imaging over 24 hours. DNA was dual-labeled with Cy3 and Cy5, which emit FRET signals only in the proximity (<10 nm) [34], and encapsulated in DB, DP, and DPH complexes. Three and 24 hours of transfection with these complexes, fluorescent signals (i.e., Cy3 and FRET (Cy5) emissions induced by 543-nm laser) were located in the cells and their intensities were measured. Surprisingly, the DPH complex had the highest ratio of FRET (Cy5) signal to Cy3 signal ($I_{660\text{-nm}}/I_{565\text{-nm}}$), which represents tightness of the complex, at 3 hour post-transfection, followed by the DP and DB complex (Figs. 4C, 4D). The difference disappeared in 24 hours. This result suggests that the DPH maintained a stable complex in the relatively early phase of transfection and then allowed DNA to unpack in the later stages.

3.8. Effect of molecular weight of HA on DPH transfection

Given the stability of DPH in DNase and 50% serum (Fig. 3) and tightness of the complex in the early stage of transfection (Figs. 4C, 4D), we hypothesized that the main effect of combined use of HA and CLPEI would be to protect DNA in the extracellular environment as well as during the early phase of intracellular trafficking by providing a diffusion barrier to hostile enzymes. If this is the case, the protective effect may depend on the molecular weight of HA, which would be reflected on the transfection efficiency. To test this, DPH complexes were prepared with different MW HAs (4.7kDa and 35kDa) maintaining the HA/DNA ratio (w/w) at 1/1 to compare their transfection efficiencies. As expected, there was significant difference between the two MW HAs (Supplementary Fig. S5A). The 4.7kDa HA had negligible effect on the DPH transfection.

To further confirm the hypothesis, DPH complexes with different MW HAs (4.7kDa, 35kDa, and 360kDa) were incubated in 50% serum (mimicking extracellular environment) or 50% serum plus 10 mM DTT (mimicking intracellular environment) for 3 hours, and DNA stability was monitored by agarose gel electrophoresis. The stability of DPH complexes increased with the molecular weight of HA (Supplementary Fig. S5B). While there was no statistical difference between DP and DPH (HA: 4.7kDa) in the smear band intensity, DPH (HA: 360kDa) was significantly different from DP in both conditions (paired t-test, $p < 0.05$).

4. Discussion

For a non-viral gene delivery system to be clinically effective, it should be non-toxic, compatible with biological components, and highly efficient in gene transfection. With this goal in mind, we investigated the gene delivery efficiency of a ternary complex consisting of DNA, an intracellularly degradable polycation, and sodium hyaluronate (DPH complex). Previously, Breunig *et al.* found that the disulfide-crosslinked low molecular weight PEI (CLPEI) existed as a high MW PEI in the extracellular environment but readily degraded to low MW PEI in the reductive intracellular environment [23,24]. As a result, the DNA-CLPEI complex retained advantages of both PEI's: high transfection efficiency of high MW PEI and low intracellular toxicity. Additional advantage of CLPEI was thought to be the enhanced intracellular release of nucleic acid [24], which had been noted as a limiting step for non-viral gene delivery in an earlier study [35]. In another study, Ito *et al.* suggested that addition of sodium hyaluronate (HA) could improve transfection efficiency of DNA-PEI complex by forming a protective coating against blood components and loosening the complex to facilitate access of the gene transcription machinery [21]. Therefore, we anticipated that the combined use of HA and CLPEI would further improve the gene delivery by synergistically facilitating the intracellular DNA unpacking, in addition to their respective benefits. Interestingly, the

results of this study suggest that the high transfection efficiency and serum tolerance of the DPH complex were achieved by the unique interplay between HA and CLPEI, which improved the stability of DNA and allowed for timely unpacking of the DNA.

The DPH ternary complex achieved high gene transfection efficiency, which was much higher than any other binary or ternary complexes and at least comparable, if not superior, to that of LipofectamineTM2000, the current gold standard commercial transfection agent (Fig. 1B, 1C). Most notably, the high transfection efficiency of the optimal DPH complex was not negatively influenced by the presence of serum, unlike other complexes.

To identify critical attributes of the DPH complex that uniquely contributes to its high efficiency and serum tolerance, we performed a series of experiments that could probe the role (s) of the carrier components in key steps of gene delivery. Such steps included not only those specifically targeted in this study (cytotoxicity [36], extracellular protection [29], and intracellular unpacking of DNA [35]) but also other steps traditionally known to be important, such as cellular uptake and intracellular trafficking [37]. Of those, the DPH complex did not have significant advantages in cytotoxicity, cellular uptake, and intracellular trafficking, as compared to other systems with much lower transfection efficiency and serum tolerance.

On the other hand, the DPH complex had much higher stability in DNase and/or heparin than plasmid DNA, DB, and DBH complexes. Exposure to anionic serum proteins, soluble glycosaminoglycans, and extracellular matrix extract, which are prevalent in the extracellular environment, can cause premature decomplexation of the DNA-polymer complexes, resulting in significant reduction in the transfection efficiency [29,38]. The DPH complex showed the best stability among the tested in the presence of DNase and/or heparin. Notably, the DP complex also showed relatively good stability as compared to DB and DBH. The stability of DP complex may be explained by the relatively low density of protonated amines. At the respective N/P ratios (20/1 for DB and 40/1 for DP), the DB and DP complexes did not show dramatically different surface charges (11.9 mV for DB and 14.9 mV for DP). However, according to calculation, the net charge density of DB complex is 1.46 times higher than that of DP, which means that more protonated amines exist internally in DB than in DP. Upon exposure to the serum proteins, both complexes may undergo partial dissociation and rearrangement of the components, during which serum proteins and nucleases may be attracted to DB more avidly than DP due to the high charge density. The increased stability of the DPH complex could be explained in two ways. First, the negatively charged HA formed a layer of polyelectrolyte complex with cationic CLPEI on the surface of the DP complex. The HA/CLPEI layer locked DNA in the complex and prevented its premature release or degradation. Second, HA decreased the surface charge of the complex and reduced the electrostatic interaction with serum proteins and nucleases. This effect appears to be unique to a specific level of HA. Ito *et al.* reported that HA was one of the few polyanions that could coat the DNA-polycation complexes without disrupting the structures [21]. In addition, we observed that DNA-polymer complexes treated with heparin, a highly negatively charged glycosaminoglycan, were quickly dissociated to release DNA (Fig. 3), and inclusion of excessive HA (HA/DNA ratio >5) abolished DPH transfection at 50% serum (Fig. 1C). Therefore, both density and amount of negative charges of the polyanion seem to be important for optimal protection of the complex.

The most interesting finding of this study is that the protective effect of HA continued to play a role in the cells during the early phase of transfection. We examined the tightness of the endocytosed DNA-polymer complexes using FRET imaging at 3 and 24 hours after transfection. Contrary to our original hypothesis (that DPH would be the least compact due to the synergistic effect of CLPEI degradation and charge neutralization by HA), the DPH complex was the most compact in 3 hours, as evidenced by the highest ratio of FRET (Cy5)

signal to Cy3 signal. On the other hand, the difference among the complexes disappeared in 24 hours, and all three complexes showed relatively weak FRET signals.

The intracellular stability of the DPH complex was further supported by the comparison of DP and DPH complexes in their responses to the intracellular GSH level. Both DP and DPH were expected to show increasing transfection efficiencies with the additional intracellular GSH, which would facilitate the degradation of CLPEI (thereby DNA unpacking), in a concentration dependent manner. As expected, DPH transfection increased with the increase in the intracellular GSH (Fig. 4A). Interestingly, the DP complex did not show such dependence on the GSH level. A similar result was reported by Breunig *et al.* [23], in which the lack of difference was interpreted as sufficiency of the inherent GSH. If this is true, the difference between DP and DPH in their responses to the intracellular GSH level should be explained by the kinetics of DNA unpacking rather than the extent.

To explain this observation, we propose that there are two time points at which the degree of intracellular DNA unpacking is critical. The first point is the time by which DNA should be protected, and the second point is when the DNA should become available for transcription. In DP, most DNA is dissociated from the complex early on (prior to the first point) upon entry into the cells (Fig. 4D). Therefore, further increase in intracellular GSH level does not increase gene expression in the cells transfected with DP (Fig. 4A). Moreover, the extent of gene expression by DP is far lower than that by DPH (Figs. 1B, 1C, 4A, and 4B), because the released DNA is more likely to be subject to intracellular degradation. In contrast, DPH maintains the stability of DNA by delaying the diffusion of GSH through the HA/CLPEI surface layer and preventing the CLPEI degradation until the first point, and then dissociates to release DNA by the second point. The extent of gene expression would be proportional to the amount of intact DNA released between the two points, which would depend on the rate of CLPEI degradation during the interval. In the presence of HA/CLPEI layer as a diffusion barrier, the rate of CLPEI degradation would be proportional to the flux (J) of GSH, which increases with the increasing concentration of GSH (C), according to the Fickian diffusion model ($J = -D \cdot dC/dx$) (x is the thickness of layer; D the diffusion coefficient). Consequently, the gene expression by DPH increases significantly with increase in the intracellular GSH level (Fig. 4A).

Comparison of DPH complexes with different MW HAs also supports the role of HA/CLPEI layer as a diffusion barrier. The DPH complex with 4.7kDa HA (an order of magnitude lower MW than typical one, 35kDa) was not much different from the DP complex in GFP expression, although it had the same amount of HA as the typical DPH complex (Fig. S5A). This implies that the diffusion barrier made of the low MW HA was not as effective as that of higher MW HA. Here, the MW of HA might have influenced the barrier effect by altering the diffusion coefficient (D) of GSH, serum proteins, or nucleases in the HA/CLPEI layer, where high MW HA results in relatively low D due to the high viscosity of the layer and vice versa. Stability of DPH complexes in 50% serum with or without DTT showed consistent dependence on HA MW (Fig. S5B), although the difference between different MW HA's was not as dramatic as in the gene transfection test. The relatively modest difference observed in the DNA stability test is probably due to the consumption of DTT in the interaction with serum proteins, which left an insufficient level of DTT for mimicking the intracellular reductive condition.

This study highlights that stability of DNA-polymer complexes in the extra- and intracellular environments and efficient intracellular unpacking of DNA are critical to achieving high gene transfection efficiency. The results reinforce findings of earlier studies, which emphasized each step as a bottleneck for efficient gene delivery [35,38,39]. Yet, this study is the first to show that it is possible to overcome these obstacles simultaneously by taking advantage of two distinctive approaches. Another important implication of this study is that HA is uniquely suited for this purpose: HA can decrease the cationic charge on the surface without disrupting the

complex structure and form a protective surface layer together with CLPEI that does not hamper DNA unpacking at a critical point. On the other hand, the decrease in surface charge appears to induce particle aggregation, which may still limit the systemic application of this system. An effort to reduce the particle size below 200 nm, which will be followed by *in-vivo* evaluation of this system, is on-going.

5. Conclusion

The DPH ternary complex achieved high gene transfection efficiency, which was much higher than any other binary or ternary complexes and at least comparable, if not superior, to that of Lipofectamine™2000. Most notably, the high transfection efficiency of the optimal DPH complex was not negatively influenced by the presence of serum, unlike other complexes. The DPH complex did not have significant advantages in cytotoxicity, cellular uptake, and intracellular trafficking, as compared to other systems with much lower transfection efficiency and serum tolerance. The high transfection efficiency and serum tolerance of the DPH complex are rather attributed to (i) the improved stability of DNA both in the extracellular environment and at the early stage of intracellular trafficking and (ii) timely dissociation of the DNA-polymer complex.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Acronyms

CLPEI	Disulfide-crosslinked low molecular weight linear polyethyleneimine
BPEI	Branched polyethyleneimine
LPEI	Linear polyethyleneimine
HA	Sodium hyaluronate
DP	Binary complex of DNA and CLPEI
DPH	Ternary complex of DNA, CLPEI, and HA
DB	Binary complex of DNA and BPEI
DBH	Ternary complex of DNA, BPEI, and HA
Lipo2K	

Lipofectamine™2000

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Figure 1.

(A) Schematic of DNA-CLPEI-HA (DPH) ternary complex. (B) Transfection efficiency of DNA-polymer complexes in NIH/3T3 fibroblasts. (C) Transfection efficiency of DPH complexes with different HA/DNA ratios in M109 cells. DL, DB, and DP: binary complexes of pEGFP and LPEI (linear PEI 25kDa), BPEI (branched PEI 25kDa), or CLPEI; DLH, DBH, and DPH: ternary complexes of pEGFP, corresponding polymers (LPEI, BPEI, and CLPEI), and HA; Lipo2K: LipofectamineTM2000. Results are presented as means and standard deviations of three experiments.

DNA; Blue DRAQ5-stained nuclei. The bottom row is the merged view of the above three images. Scale bar = 10 μ m.

Figure 3. Agarose gel electrophoresis of DNA-polymer complexes incubated in (A) DNase and/or heparin and (B) 50% CS and/or heparin. The bar graphs below the gel images compare the band intensities obtained by densitometry. Results are presented as means and standard deviations of three experiments. The bands in the dotted boxes were excluded from the analysis because they were artifacts from CS. The bottom tables show the pairs of (A) *Smear1* or (B) *Smear* (indicated on the gel images) band intensities compared by the paired t-test and the p-values.

Figure 4.

(A, B) Effect of the intracellular GSH level on transfection efficiencies of DNA-polymer complexes. NIH/3T3 fibroblasts were treated with (A) GSH-MME or (B) BSO to increase or decrease the intracellular GSH, respectively, in the presence of 10% serum. Results are presented as means and standard deviations of three experiments. (C) Representative FRET spectra of DNA-polymer complexes acquired at 3 hours and 24 hours after internalization of the complexes. pBR322 plasmid was dual-labeled with Cy3 and Cy5. (D) Ratio of FRET (Cy5) signal to Cy3 signal ($I_{660\text{-nm}}/I_{565\text{-nm}}$), which represents tightness of the complex, at 3- and 24-hour post-transfection. All results are presented as means and standard deviations of three experiments and compared by the t-test (*: $p < 0.05$; **: $p < 0.01$).