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Dependence of Transgene Expression and the Relative Buffering Capacity of Dextran-Grafted Polyethylenimine

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Abstract: Branched polyethylenimine (PEI) is a cationic polymer capable of forming self-assembly complexes with DNA to become a highly efficient agent used in gene delivery. Conjugation through the primary amines of PEI is a most commonly used approach further to enable the targeting delivery or to improve the stability of the DNA−polymer complexes. An understanding of how the conjugation affects the transfection mechanisms can help in the design of efficient polycationic vectors. In order to investigate the effects of conjugation, folate and the dextrans of molecular weight 1500 (dex-1500) and 10 000 (dex-10000) were used to prepare three different types of PEI conjugates: dextran−PEI, folate−PEI, and folate−dextran−PEI, which were subsequently employed to form complexes with DNA. These conjugates were found to cause less cytotoxicity than the unmodified PEI as revealed by the MTT method, and to be able to deliver an approximate amount of ethidium monoazide labeled plasmid into the cells. The efficiencies of green fluorescent protein (GFP) expression mediated by these conjugates, however, were less efficient than those mediated by the unmodified PEI. A titration experiment suggested that conjugation through the primary amines of PEI resulted in the loss of relative buffering capacity, a major factor aiding the release of plasmid from the endosomes, presumably because the conjugated molecules hindered the protonation of the PEI conjugates. When a quantitative relationship between relative buffering capacity and transfection efficiency was examined, a threshold of relative buffering capacity, around 50% of the unmodified PEI, was noted to be required for minimal detection of GFP positive cells. In addition, the cytotoxicity could be also related to the relative buffering capacity in an approximately linear trend. It is thus concluded that the severe loss of relative buffering capacity by conjugation might be attributed to the inefficiency of transgene expression mediated by the dextran−PEI conjugates.

Keywords: Transfection; delivery; conjugation; nonviral vector; cellular mechanism

Introduction

Cationic polymer is a major type of synthetic vector used in gene delivery.[1,2] The positive charges of the polymers generally come from the amine groups, which play an important role in determining the vector efficacy. The amine groups participate in the formation of DNA−polymer complexes, the cellular entry of the complexes, and the intracellular processing of the complexes. In addition, the

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amine groups allow a way to chemically modify the vectors by conjugating other molecules onto the polymers.

Among the cationic polymers, such as polyethylenimine (PEI),[3,4] dendrimer,[5] polylysine,[6] and chitosan,[7] PEI has been shown to be a most efficient agent for transfection. Branched PEI consists of 25% primary, 50% secondary, and 25% tertiary amines on average. The high density of amine groups enables PEI to form tighter and smaller self-assembly complexes with DNA through charge interactions.[8] The positive surface charges of the complexes allow the attachment of the complexes onto the negatively charged proteoglycans of cell membranes,[9] and the subsequent entry into the cytosol via endocytosis. Inside the cytosol, the amine groups can further act as a proton-sponge to induce the influx of chloride ions into the endosomes,[3,10] thereby facilitating the release of DNA from the endosomes to get into the cytosol.[11−13] Once the released naked DNA or DNA−polymer complexes enter the nucleus, transcription begins.

Modifying the amine structures is usually employed to further promote the transfection efficiency mediated by DNA−polymer complexes or to enhance the stability of DNA−polymer complexes.[14,15] A commonly used approach is to attach a pendant group onto the primary amines of the cationic polymers. Polylysine conjugated with polyhistidine on its ϵ-amine groups has been shown to be more efficient than the unmodified polylysine.[16,17] The conjugated polylysine enhances the efficiency of transgene expression because the imidazole groups of polyhistidine can provide higher buffering capacity to more efficiently facilitate the endosomal release of DNA. In order to stabilize the DNA−polymer complexes, cationic polymers can be conjugated with the biocompatible polymers of no charge, such as polyethylene glycol[15,18−20] and dextran,[21,22] to decrease the charge effects of the salts and proteins present in the extracellular environments. These stabilized vectors, however, generally less efficiently deliver plasmid into the cells for transgene expression because such modifications decrease the interactions of the vectors and the extracellular environments as well as the interactions with the cells. Targeted delivery is then used to restore the transgene expression by conjugating ligands onto either the primary amines of the cationic polymers or the distal ends of the previously conjugated polymers,[23−25] such as the use of RGD peptide,[26] mannose,[27] transferrin,[20] and folate[18,28,29] for targeting en-

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dothelial cells, dendritic cells, hepatocytes, and folate-overexpression carcinoma, respectively.

Conjugation through primary amines is a very commonly used method to modify polycationic vectors for gene delivery. Nevertheless, how the conjugation affects the cellular mechanisms of transgene expression in a quantitative way has so far received little attention. In this study, folate was used as a model ligand to investigate how different types of conjugation affected the transgene expression mediated by polyethylenimine. We used folate and the dextrans of molecular weight 1500 and 10 000 to modify PEI, and prepared three different types of PEI conjugates: dextran−PEI, folate−PEI, and folate−dextran−PEI, each with various degrees of conjugation. These conjugates were used to form DNA−polymer complexes for transfection. Cellular mechanisms, from the cellular entry to the endosomal release, were investigated by the correlations among the physicochemical properties of the DNA−polymer complexes, the relative buffering capacity of each conjugate, the cellular cytotoxicity, the amount of intracellular plasmid, and the efficiency of transgene expression. The physicochemical properties were indicated by the sizes and the ζ potentials of the complexes. The relative buffering capacity of each conjugate was estimated as the amount of proton required for a pH change from 8.0 to 5.5 by titration, and the cellular cytotoxicity was examined using the MTT method. The efficiency of transgene expression and the amount of intracellular plasmid were monitored using green fluorescent protein (GFP) and ethidium monoazide (EMA) labeled plasmid, respectively. Flow cytometry was used to measure the number of cells expressing GFP and the fluorescence intensity of EMA-labeled plasmid.

Experimental Section

Materials. Polyethylenimine (PEI, MW 25 000), sodium cyanoborohydride, 2-chloroethylamine, dimethyl sulfoxide (DMSO), and sodium periodate were obtained from Aldrich (Milwaukee, WI). Dextran of molecular weight 10 000, denoted by dex-10000, and dextran of molecular weight 1500, denoted by dex-1500, were from Fluka Chemie (Buchs, Switzerland). Folate, thiazolyl blue tetrazolium bromide (MTT), Dulbecco's modified Eagle medium (DMEM), and folate-deficient Dulbecco's modified Eagle medium were purchased from Sigma (St. Louis, MO). Dicyclohexylcarbodiimide (DCC), 2,4,6-trinitrobenzene sulfonic acid (TNBS), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) were from Pierce (Rockford, IL). Ethidium monoazide (EMA) was purchased from Molecular Probes (Eugen, OR). All chemicals were used as received. Water was deionized by a Milli-Q water purification system (Bedford, MA). The pEGFP-C1 vector containing a mutated green fluorescent protein gene driven by a cytomegalovirus promoter was from Clontech (Palo Alto, CA). Dialysis membranes with different molecular weight cutoffs (MWCO) were from Spectrum (Los Angeles, CA). Fetal bovine serum and antibiotics of penicillin−streptomycin−amphotericin were from Hyclone (Logan, UT).

Plasmid Amplification and Labeling. The pEGFP-C1 vector was amplified in *Escherichia coli* and purified as previously described. The purified plasmid was covalently labeled with EMA, which could provide a stable fluorescent reporter of the bound DNA. The labeling of plasmid with EMA by photoactivation was carried out as previously described.[30] Briefly, the mixture of EMA and plasmid was exposed to white light for 30 min. The unreacted EMA was removed by extraction with saturated *n*-butanol and dialysis against 2 L of distilled water six times using Spectra/Por membrane (MWCO = 15 000) in the dark.

Preparation of Folate−Dextran−PEI Conjugate. (1) Amination of Dextran. Dextran (1.0 g) was dissolved in a solution of 2.2 g of NaOH in 10 mL of water. The mixture was incubated at 30 °C for 30 min. A weight of 2.6 g of 2-chloroethylamine was added to the above mixture. The reaction was performed at 30 °C with constant shaking. The extent of reaction was controlled by different reaction times: 12 and 18 h for dex-1500, and 12 and 24 h for dex-10000. After reaction, the reacted dextran was purified by repeated precipitation with a 2-fold volume of methanol until the color became whitish before lyophilization.

(2) Oxidization of Amino−Dextran. The vicinal hydroxyl groups of the above amino−dextran (0.6 g of dex-1500 or 2.0 g of dex-10000 in 20 mL of water) were oxidized to an aldehyde group in the presence of sodium periodate (0.4 g).[31] The reaction was carried out at 25 °C with constant shaking for 8 h in the dark. The oxidized amino−dextran was lyophilized after repeated precipitation with methanol as described above.

(3) Conjugation of Folate to the Oxidized Amino−Dextran. Folate (0.083 g) was treated with an equal molar

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amount of EDC in 12 mL of DMSO at 25 °C for 1 h before the addition of 25 mL of sodium phosphate buffer (20 mM, pH 7.4) containing either 0.6 g of dex-1500 or 2.0 g of dex-10000. The reaction was carried out at 30 °C with constant shaking for 24 h in the dark. After reaction, the mixture was concentrated by rotary evaporation followed by repeated methanol precipitation as described above.

(4) Conjugation of Folate−Amino−Dextran to Polyethylenimine. The desired amount of folate−amino−dextran was reacted with 100 mg of polyethylenimine in the presence of 320 mg of sodium cyanoborohydride in 20 mL of borate buffer (100 mM, pH 8.0). The reaction was carried out at 45 °C with constant shaking for 48 h. After reaction, the mixture was dialyzed against 2 L of distilled water six times using Spectra/Por membrane (MWCO = 15000) followed by lyophilization.

Preparation of Folate−PEI Conjugate. The desired amount of folate (105, 315, or 425 mg) was mixed with a solution containing PEI (400 mg), pyridine (1 mL), and an equal molar amount of DCC in 20 mL of DMSO.[18] The reaction was carried out at 30 °C for 24 h with constant shaking. The conjugated PEI was purified by dialysis against 2 L of distilled water six times using Spectra/Por membrane (MWCO = 15000) followed by lyophilization.

Preparation of Dextran−PEI Conjugate. PEI (1.0 g) was mixed with the desired amount of dextran plus 160 mg of sodium cyanoborohydride in 25 mL of borate buffer (100 mM, pH 8.0).[4] The reaction mixture was incubated at 45 °C for 48 h with constant shaking. The PEI conjugate was lyophilized after the removal of sodium cyanoborohydride and the unreacted dextran by dialysis.

Determination of the Amount of Primary Amine on Each PEI Conjugate. The amount of primary amine remaining on each PEI conjugate was determined by TNBS. Briefly, 0.5 mL of each sample (1 mg/mL) was mixed with 0.25 mL of TNBS in 0.1 M sodium bicarbonate buffer (pH 8.5). The mixture was incubated at 37 °C for 2 h followed by the addition of 0.25 mL of 10% SDS and 0.125 mL of 1 N HCl. The absorbance of each sample was measured at 335 nm by a spectrophotometer (Jasco, Tokyo, Japan). The degree of grafting was calculated by the difference in the amount of primary amine on unmodified PEI and that on each conjugate.[32]

Determination of the Amount of Folate on the PEI Conjugate. Folate was measured at its characteristic absorbance of 368 nm.[18] The amount of folate on the PEI conjugate was determined according to the calibration curve constructed by unreacted folate.

Titration of the PEI Conjugates. Unmodified PEI and the PEI conjugates were diluted to a final concentration of 0.1 mg/mL with 0.1 N NaCl. The solution was adjusted to pH 8.0 before titration proceeded. An aliquot of 25 mL of 0.1 N HCl was successively added into 30 mL of 0.1 mg/mL polymer solution, and the changes in pH were monitored by a pH meter (MP220; Mettler Toledo, Greifensee, Switzerland).

Measurements of the Size and ζ Potential of the DNA−Polymer Complexes. A Zetamaster system (Malvern, Malvern, U.K.) was used to measure the sizes and ζ potentials of the DNA−polymer complexes prepared with either unmodified PEI or each conjugate as previously described. Briefly, the desired amount of each cationic polymer was mixed with 40 μg of DNA at an *N*/*P* ratio of 9, where the *N*/*P* ratio was used to indicate the charge ratio of the cationic polymer to DNA with *N* representing the number of amines in the polymer and *P* representing the number of phosphates in the DNA. The sizes and ζ potentials of the DNA−polymer complexes were examined using a salt-free buffer (20 mM Hepes, 5.2% glucose, pH 7.0) and DMEM without phenol red.

Cell Culture. KB cells, a human epidermal carcinoma cell line, were maintained at 37 °C, 5% CO_2, and 100% humidity in folate-deficient Dulbecco's modified Eagle medium supplemented with 1% antibiotics of penicillin−streptomycin−amphotericin and 10% fetal bovine serum. Cells were seeded onto 35 mm Petri dishes at 20 000 cells/cm^2 16 h before transfection.

Transfection. The transfection reagent was prepared by mixing 2 μg of plasmid with the desired conjugate at various *N*/*P* ratios (4, 6, 9, 12) in 0.2 mL of folate-deficient DMEM. The mixture was allowed to stand at room temperature for 15−30 min, and then 0.8 mL of folate-deficient DMEM was added to make the transfection reagents. For transgene expression, cells were cultured with the transfection reagents for 6 h and then replaced with culture medium for an additional 18 h. For the measurement of plasmid delivery, EMA-labeled plasmid instead of unlabeled plasmid was used in the preparation of transfection reagent at the *N*/*P* ratio of 9. The cells were incubated with the EMA-labeled plasmid for 6 h at 37 °C and 4 °C to determine the amount of plasmid that was associated with the cells and that adhered onto the cell surfaces, respectively.

Cytotoxicity Assay by MTT. The transfected cells at the *N*/*P* ratio of 9 were washed twice with 1 mL of phosphate-buffered saline (PBS; pH 7.2). Then, an aliquot of 1 mL of PBS solution containing MTT (0.5 mg/mL) was added to the cultured cells. After 4 h incubation, DMSO was used to solubilize the colored formazan product, and the absorbance at 540 nm was measured.

Flow Cytometric Analysis. The transfected cells were harvested with PBS containing 0.15% trypsin/1 mM EDTA. After the cells were detached, an equal volume of medium was added, and the cells were fixed in the presence of 1% paraformaldehyde for 10 min at room temperature. The fixed cells were then washed with 3 mL of PBS containing 1% paraformaldehyde three times. The final pellet was resuspended in PBS containing 1% paraformaldehyde and stored at 4 °C for flow cytometric analysis. For each sample, 20 000 events were collected by a flow cytometer (FACS Calibur; BD Biosciences, San Jose, CA) equipped with an argon ion

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Table 1. The Estimated Degrees of Conjugation on Each PEI Conjugate

	degree of conjugation			
	no. of molecules			
dextran MW	dextran	folate	denotation	symbol
1500	8.0	0	P-8LD	A
	17.4	0	P-17LD	B
	7.2	6.7	P-7LD-7FA	C
	8.4	30.5	P-8LD-31FA	D
	19.8	20.6	P-20LD-21FA	E
10000	0.7	0	P-1HD	F
	10.6	0	P-11HD	G
	18.9	0	P-19HD	H
	0.7	1.3	P-1HD-1FA	I
	8.7	20.9	P-9HD-21FA	J
	8.9	34.8	P-9HD-35FA	K
	15.9	38.1	P-16HD-38FA	L
none	0	10.3	P-10FA	M
	0	25.3	P-25FA	N
	0	34.8	P-35FA	O
unmodified	0	0	PEI	P

laser and a red diode laser. The analysis of the transfected cells was performed by CellQuest software (BD Biosciences, San Jose, CA) as previously reported.[30]

Results and Discussion

Preparation of PEI Conjugates. Three different types of conjugates were prepared in this study as listed in Table 1. Dextran and folate reacted directly with PEI to produce dextran−PEI and folate−PEI conjugates, respectively. Dextran was conjugated onto PEI by a reductive reaction between the aldehyde group of dextran and the amine group of PEI. Two levels of dex-1500 conjugation and three levels of dex-10000 conjugation were generated: P-8LD and P-17LD; P-1HD, P-11HD, and P-19HD. The former two denotations represented the conjugates containing around 8 and 17 molecules of dex-1500 per PEI, respectively, and the latter three denotations represented the conjugates containing around 1, 11, and 19 molecules of dex-10000 per PEI, respectively. Folate was conjugated by an amide bond between the carboxyl group of folate and the amine group of PEI. Three levels of folate conjugation were synthesized: P-10FA, P-25FA, and P-35FA, which contained around 10, 25, and 35 molecules of folate per PEI, respectively.

For preparation of the folate−dextran−PEI conjugate, dextran was first modified with 2-chloroethylamine because 2-chloroethylamine could react with either the hydroxyl or the aldehyde group of dextran. After amination, one molecule of the dex-1500 and dex-10000 contained 1.5 and 7 amino groups on average, respectively. A further treatment of the amino−dextran with periodate resulted in 1.3−2.5 and 5−6 molecules of reactive aldehyde per dex-1500 and dex-10000 molecule, respectively. Folate was then conjugated onto the amino end of the oxidized amino−dextran via an amide bond. Subsequently the aldehyde groups of the folate−dextran molecules reacted with the primary amines of PEI.

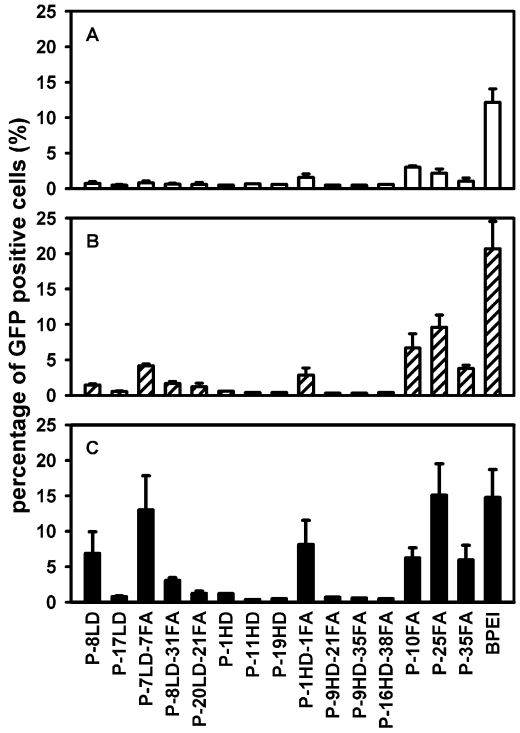

Figure 1. Transfection efficiencies of the PEI conjugates at different *N*/*P* ratios. The DNA−polymer complexes were prepared with the PEI conjugates as listed in Table 1, and then used to transfect cells. The transfection efficiency is indicated by the percentage of GFP positive cells measured by flow cytometry. The efficiencies of various conjugates were examined for the complexes formed at *N*/*P* ratios of 6 (panel A), 9 (panel B), and 12 (panel C) (mean ± SD; n = three independent transfection experiments).

By this way, several folate−dextran−PEI conjugates were synthesized: P-7LD-7FA, P-8LD-31FA, P-20LD-21FA, P-1HD-1FA, P-9HD-21FA, P-9HD-35FA, and P-16HD-38FA, each of which possessed folate on the conjugated dextran.

Transfection Mediated by DNA−Polymer Complexes. After the cells were incubated with various DNA−polymer complexes, flow cytometry was used to quantitate the fraction of the cells expressing GFP, which was set as an indicator of transfection efficiency in this study. The transfection efficiencies of certain polymers were found to exhibit a function of the *N*/*P* ratio. At the *N*/*P* ratio of 4, the GFP positive cells were very scarce under a fluorescence microscope for all the polymers, and were hardly detected by the flow cytometer (data not shown). When the *N*/*P* ratio was raised to 6, GFP positive cells could be detected mainly for the folate−PEI conjugates and unmodified PEI, the former of which showed very minimal efficiencies compared with the latter (Figure 1A). An increase of the *N*/*P* ratio to 9 enabled some polymers, such as P-8LD, P-7LD-7FA, P-8LD-31FA, and P-1HD-1FA, to produce some detectable GFP positive cells (Figure 1B). At this charge ratio, the efficiencies of the PEI−folate conjugates and unmodified PEI were also increased. When the *N*/*P* ratio was increased to 12, the transfection efficiencies were further enhanced for the

conjugates capable of producing GFP positive cells at the *N*/*P* ratio of 9 except for the unmodified PEI (Figure 1C).

An optimal degree of folate conjugation seemingly existed to improve the transfection efficiency. A moderate degree of additional folate on either the dextran−PEI conjugates or unmodified PEI could enhance the transfection efficiency, but the efficiency dropped when the degree of folate conjugation was further increased; for example, the efficiencies of P-7LD-7FA and P-25FA were the highest ones among the group P-8LD, P-7LD-7FA, and P-8LD-31FA and the group P-10FA, P-25FA, and P-35FA, respectively. The conjugates with dex-10000, however, failed to produce any detectable level of GFP positive cells at different *N*/*P* ratios except for the PEI conjugated with a very minimal level of dex-10000 and folate (P-1HD and P-1HD-1FA). Unmodified PEI was the most effective transfection agent at all the *N*/*P* ratios tested in this study, suggesting that the conjugation diminished the transfection effectiveness of PEI. In an effort to understand what factors altered the transfection effectiveness of PEI after conjugation, we investigated the physicochemical properties of the DNA−polymer complexes, the level of intracellular plasmid, and the relative buffering capacity of the PEI conjugates.

Physicochemical Properties of the DNA−Polymer Complexes in the Salt-Free and DMEM Buffers. For the measurements of the physiochemical properties of the complexes, the DNA−polymer complexes were prepared at the *N*/*P* ratio of 9 in the salt-free and DMEM buffers, respectively. The sizes of the complexes were found to be below 200 nm in the salt-free buffer when PEI was conjugated with either dex-1500 or folate (Figure 2A). But when the above complexes were prepared in the DMEM buffer, the sizes became larger than 1500 nm. On the other hand, the sizes of the complexes prepared with PEI conjugated with dex-10000 exhibited slightly larger sizes (200−400 nm) in the salt-free buffer (Figure 2A). The sizes of these complexes, however, remained at the approximate magnitude (<400 nm) when the DMEM buffer was used, indicating that the conjugation of dex-10000 could retard the salt-induced aggregation of complexes and thereby stabilized the complexes. These observations were consistent with a previous report,[22] which demonstrated that the conjugation of large dextran could better stabilize the DNA−polymer complexes than the conjugation of small dextran.

The conjugation of folate seemed to affect the ζ potentials of the complexes prepared in the DMEM buffer. For the approximate degrees of dextran conjugation, the polymers containing folate showed larger negative values of ζ potential in comparisons with those containing dextran alone; for example P-8LD-31FA vs P-8LD, P-20LD-21FA vs P-17LD, P-9HD-21FA vs P-11HD, and P-16HD-38FA vs P-19HD (Figure 2B). These observations might reflect the nature of folate conjugation, which were on the outer conjugated dextran of the complexes. The surface charges of the complexes were also more negative when dex-10000 was used than when dex-1500 was used, suggesting that dex-10000 had a longer connection to the PEI and provided a better charge shield.

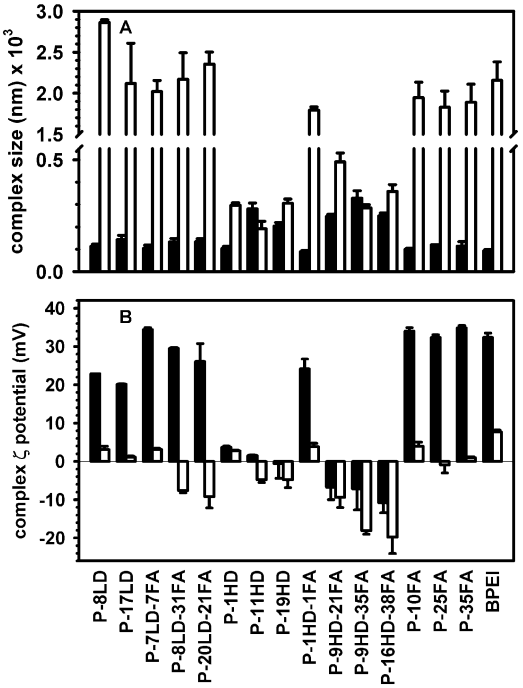

Figure 2. Sizes and ζ potentials of the DNA−polymer complexes in the DMEM and salt-free buffers. The synthesized PEI conjugates were used to form complexes with DNA in the presence of salt-free buffer (solid bars) and DMEM buffer (hollow bars). The sizes and ζ potentials of the complexes are displayed in panel A and panel B, respectively (mean ± SD; n = three independent complex preparations).

Entry of Plasmid Delivered by the DNA−Polymer Complexes. The cellular entry of DNA−polymer complexes was via endocytosis, which did not occur at the reduced temperature of 4 °C. At 4 °C the plasmid remained only bound on the cell membranes. The amounts of membrane-bound complexes were at the same level for all the polymers ($p < 0.1$) (Figure 3A). The intracellular amounts of the delivered plasmid could be estimated by the differences in the mean fluorescence intensities of the cells incubated with EMA-labeled plasmid at 4 °C and those at 37 °C (Figure 3B). In this study, flow cytometry was used to measure the changes in the cellular fluorescence intensity of each individual cell after the cells were incubated with the EMA-labeled plasmid. Although the conjugation of folate was attempted to enable the targeted delivery of plasmid into the cells, the intracellular amounts of plasmid delivered by various polymers were of the same level ($p < 0.1$) irrespective of the grafting degrees and of the types of conjugation.

The intracellular amount of plasmid has been reported to be a critical factor in determining the level of transgene expression.[33] The complexes of a large size have been shown

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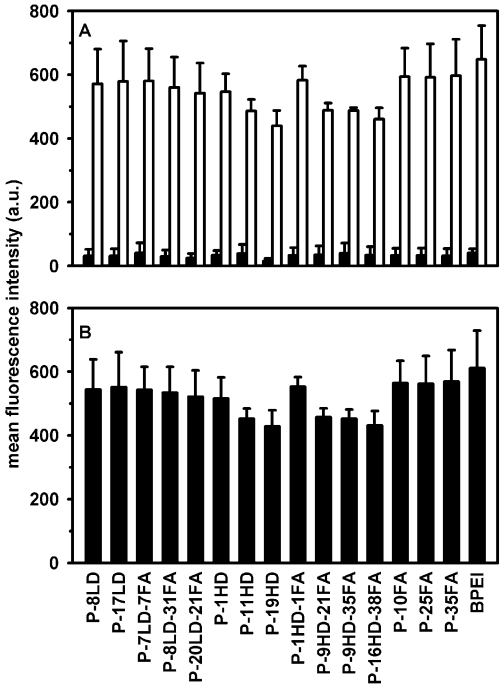

Figure 3. Mean fluorescence intensities of the cells after incubation with the DNA−polymer complexes containing EMA-labeled plasmid. Flow cytometry was used to analyze the distribution of the fluorescence intensities of the cells incubated with the DNA−polymer complexes containing EMA-labeled plasmid. The mean values of the fluorescence distributions were used to indicate the cellular amounts of plasmid at 4 °C (panel A, solid bars) and 37 °C (panel A, hollow bars). The differences in the fluorescence intensities at 4 °C and 37 °C represent the intracellular amounts of the EMA-labeled plasmid delivered by the conjugates (panel B) (mean ± SD; n = three independent transfection experiments).

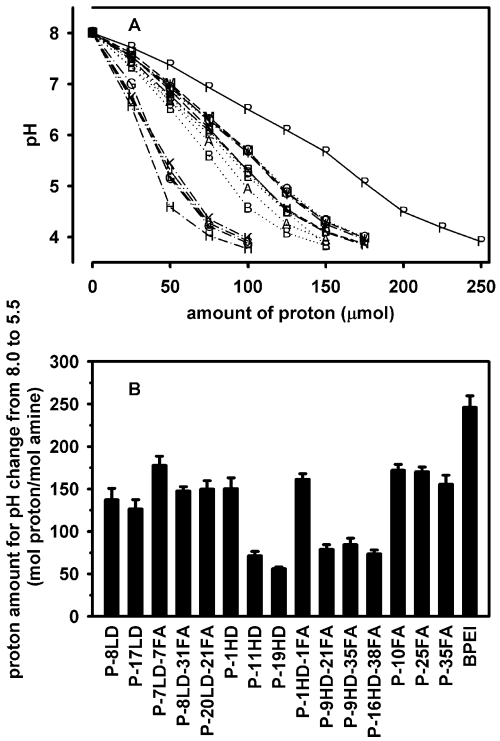

Figure 4. Titration curves of the PEI conjugates. Panel A displays a typical titration curve of each conjugate titrated with 0.1 N HCl. The letters in panel A refer to the conjugates listed in Table 1. The solid line (—) represents the unmodified PEI, the dotted lines (···) represent either the dex-1500−PEI or folate−dex-1500−PEI conjugates, the dashed−dotted lines (— · —) represent either the dex-10000−PEI or folate−dex-10000−PEI conjugates, and the dashed lines (— — —) represent the folate−PEI conjugates. Panel B displays the amounts of proton required to result in a change in pH from 8.0 to 5.5 for each conjugate (mean ± SD; n = three independent titration experiments).

to increase the level of transgene expression by delivering more plasmid into the cells,[34] and the complexes of a high surface charge have been also shown to promote the cellular entry of plasmid. In this study, however, neither the larger sizes nor the higher ζ potentials resulted in higher amounts of intracellular plasmid. In an effort to assess whether the conjugates with folate favored the receptor−endocytosis pathway, various concentrations of free folate were added into the culture medium (folate-deficient DMEM), and then the transfection efficiencies were compared. The results revealed that the transgene expression was enhanced at 1 mM of free folate for all three different types of PEI conjugates as well as unmodified PEI. The transfection efficiency was decreased by further increases in the folate concentration. The observations were consistent with the previous findings[35] that showed that low folate concentrations could enhance the transfection efficiency mediated by PEI. These results suggested that it remained unclear which pathway the DNA−polymer complexes bearing folate took to enter the cells by the competitive binding assay, and that the approximately equal levels of intracellular plasmid might be presumably due to the saturation of the endocytic entry rate.

Titration of PEI Conjugates. Because the buffering capacity of polycationic vectors affected the extents of the DNA−polymer complexes released from the endosomes, titration was used to examine whether the conjugation affected the buffering capacity of PEI.[5] The polymers of high buffering capacity would undergo a small change in pH when the same amount of HCl was added into the polymer solutions during titration. Higher extents of changes in pH were found for the PEI conjugates than for the unmodified PEI (Figure 4A), indicating that the buffering capacity might be reduced after conjugation. Because the pH of the endosomes was maintained around 5.5−6 by ATP-driven

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pumps, the polymer would presumably undergo a pH change from 7.4 to around 5.5 when the DNA−polymer complexes were endocytosed into the cells. We assumed that the polydispersities of all the polymers were one, and then converted the amount of proton required for a change in pH from 8.0 to 5.5 during in vitro titration from the weight basis to be the basis of per millimole of total amine (Figure 4B). A high amount of the required proton in the pH change indicated that the polymer had strong buffering capacity and a high tendency to be protonated.

The decreases in the tendency to be protonated after conjugation seemed to depend on both the degrees of conjugation and the types of conjugated molecules (Figure 4B). Large molecules of no charge, such as dex-10000, caused the conjugate to be much less protonated even under a very minimal level of conjugation. Only one conjugated molecule of dex-10000 was sufficient to result in 35% of reduction in the required proton amount (P-1HD), and the dextran−PEI conjugate only required $^1/_5$ of the same proton amount of unmodified PEI to undergo a pH change from 8 to 5.5 when 19 molecules of dex-10000 were conjugated onto PEI (P-19HD). For the approximate degree of dex-1500 conjugation, P-17LD needed half of the proton amount of unmodified PEI to undergo the same pH change. On the other hand, a higher degree of folate conjugation (P-35FA) still required more than half of the proton amount. Besides, additional conjugation of folate onto dextran hardly further decreased the required amount of proton of the dextran−PEI conjugates, for example, P-8LD-31FA vs P-8LD, P-1HD vs P-1HD-1FA, and P-9HD-21FA vs P-9HD-35FA.

A previous report showed that the conjugation of either cyclodextrin or an alkyl chain onto PEI would lower the pK_a value of PEI mainly because the conjugated molecules could sterically restrict the protonation of PEI.[36] In this study we found that the PEI conjugates had lower extents of protonation than unmodified PEI when the same amount of proton was added. The finding was consistent with the observations of the previous report. We also noticed that the extent of protonation of the three types of PEI conjugates depended on the nature of the conjugated molecules, and exhibited a increasing order: dex-10000 < dex-1500 < folate, suggesting that the large molecule, dex-10000, might create high steric hindrances to decrease the extent of protonation of PEI. The decreases in the extent of protonation indicated the decreases in buffering capacity, which might cause insufficient disruption of the endosomes to aid the DNA release and resulted in the low efficiency of transgene expression. A previous study showed that the conjugation of dextran through the primary amine of linear PEI resulted in a very minimal level of transgene expression, possibly also due to the steric hindrance of protonation after conjugation.[22]

Measurements of Cellular Cytotoxicity. Low cell viability was found when the cells were transfected with PEI. Conjugation of either dex-10000 or dex-1500 could moderate the cellular cytotoxicity of the unmodified PEI (Figure 5). Such improvements in cytotoxicity were the same for various levels of either dex-1500 or dex-10000 conjugation ($p > 0.1$).

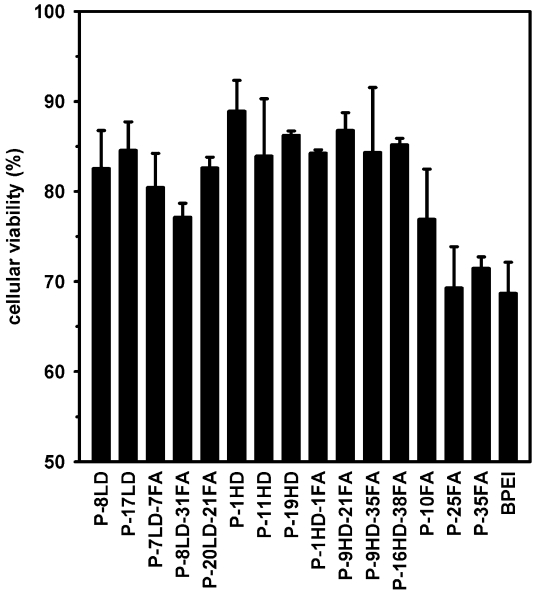

Figure 5. Cellular cytotoxicities of the DNA−polymer complexes. Cytotoxicity was assayed by the MTT method and expressed as the percentages of cell viability by setting the untransfected cells as 100% (mean ± SD; n = three independent transfection experiments).

The direct conjugation of folate onto PEI, however, hardly promoted cell viability. Additional folate on the conjugated dextran was also found to have little effect on further improving the viability.

Effects of Relative Buffering Capacity on Cytotoxicity and Transfection Efficiency. Several recent studies have demonstrated that buffering capacity is an important characteristic of PEI for its high efficiency of transgene expression.[10,11,13,37] Conjugation onto the amines of PEI diminished the extent of protonation and so abolished buffering capacity as revealed by other reports[30,38] as well as shown in this study. Such decreases in buffering capacity suggested that using the fluorophore-labeled PEI to monitor the intracellular amount of polymer might be unable to reflect the actual sponge effect of either unmodified PEI or the PEI conjugates. Therefore, we estimated the intracellular amounts of the PEI conjugates by assuming that the polymer entering the cells was quantitatively proportional to the amount of EMA-labeled plasmid in the DNA−polymer complexes. The fluorescence intensity of the intracellular EMA-labeled plasmid was used to represent the intracellular amount of the corresponding conjugate. When the conjugate got pro-

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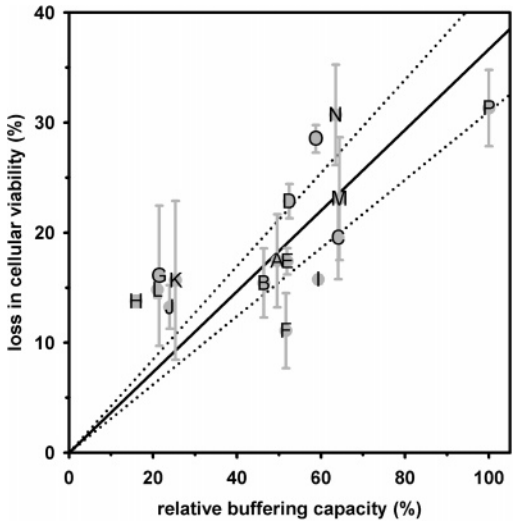

Figure 6. Dependence of cytotoxicity and the relative buffering capacity. Loss in cellular viability was used to indicate the cytotoxicity by setting the untransfected cells as 0%. The relative buffering capacity was defined as the multiplication of the mean fluorescence intensity of intracellular EMA-plasmid (Figure 3, panel B) and the buffering capacity of the corresponding conjugate (Figure 4, panel B) by setting the unmodified PEI as 100%. The solid line shows a linear regression between the loss in cellular viability and the relative buffering capacity, and the dotted line shows the linear regression with 99% confidence. The letters indicate each conjugate as listed in Table 1.

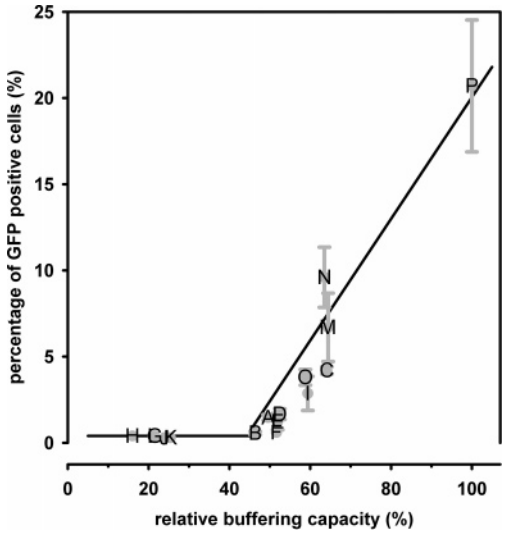

Figure 7. Dependence of transfection efficiency and the relative buffering capacity. The relative buffering capacity was defined as the multiplication of the mean fluorescence intensity of intracellular EMA-plasmid (Figure 3, panel B) and the buffering capacity of the corresponding conjugate (Figure 4, panel B) by setting the unmodified PEI as 100%. The relationship shows that a minimal value of the relative buffering capacity was required for a detectable level of GFP positive cells.

tonated within endosomes, the required amount of proton for a pH change from 8 to 5.5, which was determined by in vitro titration, was used as a quantitative indicator referring to the level of buffering capacity of the conjugate. The relative buffering capacity was then defined as the multiplication of the required amount of proton (Figure 3, panel B) and the intracellular EMA fluorescence intensity (Figure 4, panel B), and was further expressed in terms of percentages by setting the value of unmodified PEI as 100%. Figure 6 revealed an approximately linear dependence between the loss in cellular viability and the relative buffering capacity when the relationship of these two parameters was examined. The high relative buffering capacity might contribute to the sponge effect of PEI for disruption of the endosomes, and also induced high cellular cytotoxicity presumably due to the unbalances of intracellular protons and the disruption of intracellular organelles. On the other hand, the decreases in the relative buffering capacity might be unable to induce a sufficient influx of chloride ion into the endosomes, and therefore maintained high cell viability.

When the relative buffering capacity was used as a parameter to examine its relationship with the transfection efficiency, a threshold of relative buffering capacity, about 50% of the unmodified PEI capacity, was found for the minimal detection of GFP positive cells by flow cytometry (Figure 7). Beyond the threshold, the percentage of GFP positive cells followed a trend increasing with the relative buffering capacity roughly in a linear way. It could be realized that the low relative buffering capacity was insufficient to disrupt the endosomes for the release of plasmid. Therefore, dextran−PEI and folate−dextran−PEI conjugates produced very minimal levels of transgene expression mainly due to the severe decreases in the relative buffering capacity whereas the folate−PEI conjugates still maintained the moderate percentages of GFP expressing cells due to the smaller decreases in the relative buffering capacity.

Conclusions

In this study, the cellular cytotoxicity and the efficiency of transgene expression were found to quantitatively depend upon the relative buffering capacity of the PEI conjugates. The conjugation onto PEI through primary amine resulted in decreases in relative buffering capacity presumably because the conjugated molecule hindered the protonation of the conjugates. The low level of relative buffering capacity might be attributed to the accompanying decreases in transfection efficiency and cell cytotoxicity. A high-degree conjugation of a large-size molecule, such as dex-10000, could stabilize the DNA−polymer complexes, and improve cell viability. Nevertheless such conjugation failed to produce detectable transgene expression presumably because of the significant decreases in the relative buffering capacity. The quantitative descriptions of the dependence between the relative buffering capacity and transfection efficiency as well as cellular cytotoxicity could be expected to provide a basis for the design of efficient polycationic vectors.

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