

## Lipoic Acid Modified Low Molecular Weight Polyethylenimine Mediates Nontoxic and Highly Potent *in Vitro* Gene Transfection

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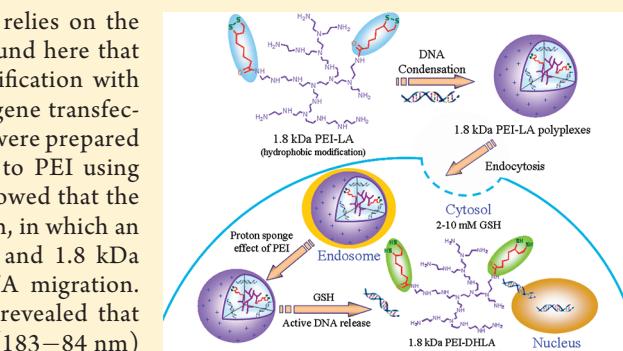
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**ABSTRACT:** The clinical success of gene therapy intimately relies on the development of safe and efficient gene carrier systems. We found here that 1.8 kDa polyethylenimine (PEI) following hydrophobic modification with lipoic acid (LA) mediated nontoxic and highly potent *in vitro* gene transfection in both HeLa and 293T cells. 1.8 kDa PEI-LA conjugates were prepared with controlled degree of substitution (DS) by coupling LA to PEI using carbodiimide chemistry. Gel electrophoresis measurements showed that the DNA binding ability of 1.8 kDa PEI was impaired by lipoylation, in which an N/P ratio of 2/1 and 4–6/1 was required for 1.8 kDa PEI and 1.8 kDa PEI-LA conjugates, respectively, to completely inhibit DNA migration. Interestingly, dynamic light scattering measurements (DLS) revealed that PEI-LA conjugates condensed DNA into much smaller sizes (183–84 nm) than unmodified 1.8 kDa PEI (444–139 nm) at N/P ratios ranging from 20/1 to 60/1. These polyplexes revealed similar surface charges of ca. +22 to +30 mV. 1.8 kDa PEI-LA<sub>2</sub> polyplexes formed at an N/P ratio of 10/1 were stable against exchange with 12-fold excess of negatively charged dextran sodium sulfate (DSS) relative to DNA phosphate groups while 1.8 kDa PEI controls dissociated at 6-fold excess of DSS, indicating that lipoylation of 1.8 kDa PEI resulted in stronger binding with DNA. Importantly, DNA was released from 1.8 kDa PEI-LA<sub>2</sub> polyplexes upon addition of 10 mM dithiothreitol (DTT). Reduction-triggered unpacking of 1.8 kDa PEI-LA<sub>2</sub> polyplexes was also confirmed by DLS. MTT assays demonstrated that all PEI-LA conjugates and polyplexes were essentially nontoxic to HeLa and 293T cells up to a tested concentration of 50  $\mu$ g/mL and an N/P ratio of 80/1, respectively. The *in vitro* gene transfection studies in HeLa and 293T cells showed that lipoylation of 1.8 kDa PEI markedly boosted its transfection activity. For example, 1.8 kDa PEI-LA<sub>2</sub> polyplexes displayed 400-fold and 500-fold higher levels of gene expression than unmodified 1.8 kDa PEI controls, which were ca. 2-fold and 3-fold higher than 25 kDa PEI controls, in serum-free and 10% serum media, respectively. The transfection efficiency decreased with increasing DS, following an order of 1.8 kDa PEI-LA<sub>2</sub> > 1.8 kDa PEI-LA<sub>4</sub> > 1.8 kDa PEI-LA<sub>6</sub> > 1.8 kDa PEI. Confocal laser scanning microscopy (CLSM) studies corroborated that 1.8 kDa PEI-LA<sub>2</sub> delivered and released DNA into the nuclei of HeLa cells more efficiently than 25 kDa PEI. These nontoxic 1.8 kDa PEI-LA conjugates form a superb basis for the development of targeting, biocompatible and highly efficient carriers of gene delivery.

**KEYWORDS:** polyethylenimine, hydrophobic modification, reduction-sensitive, plasmid DNA, polyplexes, gene delivery

### INTRODUCTION

The past decade has witnessed significant progress in the development of cationic polymer-based nonviral vectors for *in vitro* and *in vivo* gene transfer.<sup>1–4</sup> In contrast to viral vectors and other types of nonviral carriers including liposomes, polymeric vectors can be prepared reproducibly and inexpensively in large-scale and pharmaceutical grade, may be administered repeatedly due to low immune response, and may further be modified to achieve long circulation time and targeted delivery of therapeutic genes *in vivo*.<sup>5,6</sup> It should be noted, however, that despite remarkable development in vector design and understanding of transfection biology, therapeutic applications of polymeric gene delivery systems remain limited as a result of inadequate safety and transfection performance.<sup>7</sup>



Polyethylenimine (PEI) owing to its unique combination of high charge density and proton sponge effect is, undoubtedly, among the most efficient synthetic gene carriers.<sup>8–10</sup> Currently, 25 kDa branched PEI (denoted as 25 kDa PEI) and 22 kDa linear PEI are widely employed as golden standards for nonviral gene transfer.<sup>11,12</sup> Both PEI reagents, however, show varying levels of cytotoxicities *in vitro*, as well as acute and/or long-term toxicity *in vivo* due to their high molecular weight, excessive positive charge and lack of biodegradability. Low molecular weight PEIs (e.g., 1.8 kDa PEI) with favorable cytotoxicity profiles, on the

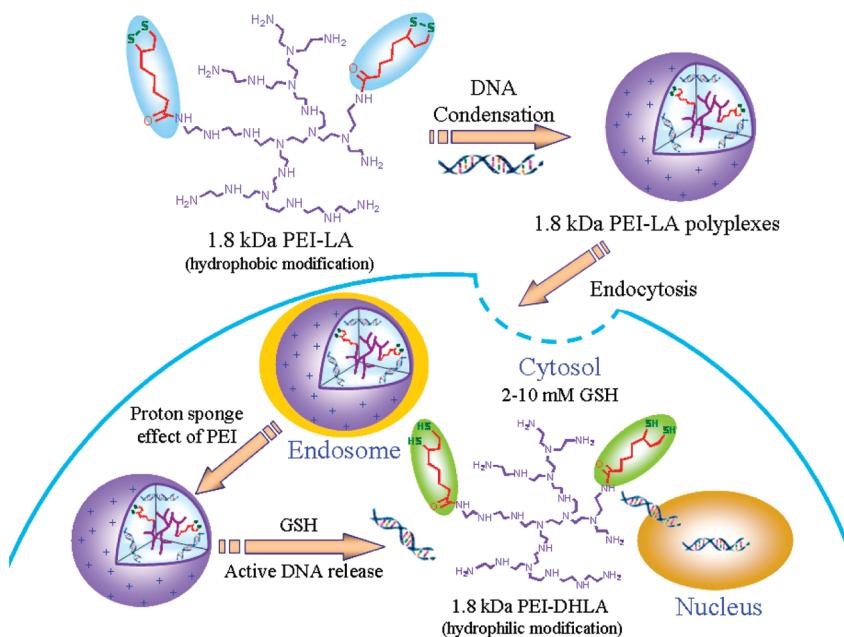
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**Scheme 1. Illustration of Reduction-Sensitive Hydrophobic Modification of 1.8 kDa PEI with Lipoic Acid (a Natural Biocompatible Compound) for Nontoxic and Efficient Intracellular Gene Transfection<sup>a</sup>**



<sup>a</sup> The lipoylation of 1.8 kDa PEI gave rise to excellent DNA condensation ability and enhanced polyplex colloidal stability. However, the polyplexes of lipoylated 1.8 kDa PEI were readily unpacked, actively delivering and releasing DNA into the cell nuclei, once taken up by cells due to reduction-triggered reversal of hydrophobic modification.

other hand, display minimum transfection activities as a result of inadequate DNA complexation and protection.<sup>13</sup> In the past decade, with an aim to develop safe and efficient gene transfer agents, a number of novel gene vectors have been designed based on low molecular weight PEIs.<sup>14,15</sup> For example, several groups separately reported that hydrolytically or reductively degradable PEI polymers and networks derived from low molecular weight PEIs, while maintaining low cytotoxicities, mediate largely enhanced *in vitro* gene transfection as compared to the parent low molecular weight PEIs, with transfection efficiencies approaching or in few cases exceeding that of 25 kDa PEI control.<sup>16-24</sup> It is in general challenging, nevertheless, to obtain molecularly well-defined degradable PEI carriers because of complex coupling reactions (including branching and network-forming) between low molecular weight PEIs and coupling reagents. Feijen and Park reported multistep synthesis of structurally well-defined low molecular weight linear PEI-PEG-PEI triblock copolymers<sup>25</sup> and bioreducible linear PEI,<sup>26</sup> respectively, for low toxic and enhanced gene transfection comparable to 25 kDa PEI.

In the past years, hydrophobic modification has been established as an effective and straightforward approach to improve the transfection performances of many cationic polymers including chitosan, poly(amido amine) dendrimer, poly(L-lysine) and PEI.<sup>27,28</sup> PEI is amenable to versatile and selective chemical conjugations owing to the presence of many primary (25%) and secondary (50%) amino groups. For instance, Kim, Klibanov, Uludag, Lee, and Ramezani reported water-soluble lipopolymer through modification of 1.8 kDa PEI with cholesterol,<sup>29,30</sup> dodecylation of 2 kDa PEI,<sup>31</sup> substitution of 2 kDa PEI with aliphatic lipids including caprylic, myristic, palmitic, stearic, oleic and linoleic acids,<sup>32,33</sup> conjugation of 2 kDa PEI with dexamethasone,<sup>34,35</sup> and modification of 10 kDa PEI with

alkyl-oligoamine,<sup>36</sup> respectively. The transfection efficiencies were enhanced to different degrees over the unmodified low molecular weight PEIs, depending on types of ligands and degree of substitution. In a few cases, transfection efficiencies approximated or exceeded those of 25 kDa PEI control. The varying transfection performances are likely because hydrophobic modification yields delicate (mixed) effects on several key steps of nonviral gene transfer such as DNA condensation, cellular interaction and uptake, endosomal escape, and intracellular unpacking of polyplexes.<sup>27,28</sup> It should further be noted that hydrophobic modification mostly causes increased cytotoxicity over the parent low molecular weight PEIs (though still lower than 25 kDa PEI).<sup>32</sup>

In this paper, we report on novel reduction-sensitive hydrophobic modification of 1.8 kDa PEI with lipoic acid (LA) for nontoxic and superior nonviral gene transfection to 25 kDa PEI control (Scheme 1). LA is produced naturally in the human body and is commonly used for the treatment of varying diseases including Alzheimer's disease and diabetes.<sup>37,38</sup> We recently found that nanoparticles self-assembled from lipoylated dextran could efficiently deliver and release doxorubicin (an anticancer drug) into cancer cells owing to prompt reversal of disulfide bonds under the intracellular reductive environment.<sup>39</sup> Here, we hypothesized that lipoylation of 1.8 kDa PEI would facilitate DNA condensation and cellular uptake while rapid conversion of lipoyl group (hydrophobic) into dihydrolipoil group (hydrophilic) inside cells would result in "active" intracellular release of DNA (Scheme 1). The lack of "active" polyplex unpacking mechanism in cells is a bottleneck of many current nonviral gene vectors including 25 kDa PEI.<sup>40</sup> In recent years, reduction-responsive nanovehicles have emerged as a unique platform to achieve "active" intracellular drug release.<sup>41,42</sup>

Notably, LA modification has been shown to not only markedly enhance gene transfection but also reduce cytotoxicity of 1.8 kDa PEI. The synthesis and cytotoxicity of 1.8 kDa PEI–LA conjugates, DNA complexation, reduction-triggered unpacking of polyplexes, and *in vitro* gene transfection were investigated.

## MATERIALS AND METHODS

**Materials.** Lipoic acid (LA, 98%, Acros), *N,N'*-dicyclohexyl carbodiimide (DCC, 99%, Alfa Aesar), *N*-hydroxysuccinimide (NHS, 98%, Alfa Aesar), 1.8 kDa and 25 kDa branched polyethylenimine (1.8 kDa PEI and 25 kDa PEI, Sigma), 1,4-dithio-*D*, L-threitol (DTT, 99%, Merck), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Amresco), dextran sulfate sodium (DSS, Sigma), Cy-5 (Mirus), and Hoechst 33342 (Sigma) were used as received. Dichloromethane (DCM) was dried by refluxing over  $\text{CaH}_2$  and distilled before use. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin and trypsin were obtained from Invitrogen. Luciferase assay kits and Micro BCA protein assay kits were obtained from R-Protagen Co., Ltd. and Pierce, respectively.

**Synthesis of PEI–LA Conjugates.** PEI–LA conjugates were synthesized by coupling lipoic acid (LA) to 1.8 kDa PEI through carbodiimide chemistry. In a typical example, to a round-bottom flask containing a solution of LA (40 mg, 0.19 mmol) in DCM (5 mL) under nitrogen atmosphere were added NHS (67 mg, 0.58 mmol) and DCC (240 mg, 1.17 mmol). After 24 h activation, the reaction mixture was filtered. The filtrate was added dropwise to a DCM solution (30 mL) of 1.8 kDa PEI (420 mg, 9.5 mmol of nitrogen). The mixture was stirred for another 24 h under a nitrogen atmosphere at room temperature (rt). The product was isolated by precipitation in cold diethyl ether and dried in *vacuo*. Yield: 65%. DS: 1.9.  $^1\text{H}$  NMR (400 MHz,  $\text{CDOD}_3$ ):  $\delta$  1.40–1.76 (m,  $-\text{NHOCCH}_2\text{CH}_2\text{CH}_2\text{CH}_2-$ ), 1.89 (m,  $-\text{SSCH}_2\text{CH}_2\text{CH}-$ ), 2.21 (t,  $-\text{CH}_2\text{CONH}-$ ), 2.41 (m,  $-\text{SSCH}_2\text{CH}_2\text{CH}-$ ), 2.30–2.98 (m, PEI), 3.08–3.20 (m,  $-\text{SSCH}_2\text{CH}_2\text{CH}-$ ), 3.44 (t,  $-\text{COONHCH}_2-$ ), 3.59 (m,  $-\text{SSCH}_2\text{CH}_2\text{CH}-$ ). PEI–LA conjugates with DS of 3.8 and 5.8 were prepared similarly.

**Acid–Base Titration.** The buffer capability of PEI–LA conjugates was determined by acid–base titration assays over a pH range from 2.0 to 11.0. Briefly, the polymer (0.1 mmol of nitrogen atoms) was dissolved in 5 mL of 150 mM NaCl solution. The solution was brought to a starting pH of 2.0 with 0.1 M HCl, and then was titrated with 0.1 M NaOH using a pH meter (DELTA 320). For comparison, 1.8 kDa and 25 kDa PEI were also titrated in the same way. The buffer capacity, defined as the percentage of amine groups becoming protonated from pH 5.1 to 7.4, was calculated according to the following equation:

$$\text{buffer capacity (\%)} = 100 \times (\Delta V_{\text{NaOH}} \times 0.1 \text{ M}) / N \text{ mol}$$

wherein  $\Delta V_{\text{NaOH}}$  is the volume of NaOH solution (0.1 M) required to bring the pH value of the polymer solution from 5.1 to 7.4, and N mol is the total moles of protonable amine groups in the tested polymer (0.1 mmol).

**Particle Size and  $\zeta$ -Potential Measurements.** The polyplexes were prepared at varying N/P ratios from 10/1 to 60/1 by adding a HEPES buffer solution (20 mM, pH 7.4) of polymer (600  $\mu\text{L}$ , varying concentrations) to a HEPES buffer solution (20 mM, pH 7.4) of plasmid DNA (150  $\mu\text{L}$ , 37.5  $\mu\text{g/mL}$ ), followed by vortexing for 5 s and incubating at rt for 30 min. The

surface charge and size of polyplexes were measured at 25 °C with a Zetasizer Nano ZS instrument (Malvern) equipped with a standard capillary electrophoresis cell and dynamic light scattering (DLS, 10 mW He–Ne laser, 633 nm wavelength), respectively. The measurements were performed in three independent replicates. The data are presented as mean value  $\pm$  standard deviation.

**Gel Retardation Assays.** The DNA binding ability of 1.8 kDa PEI–LA conjugates was studied by agarose gel electrophoresis. The polymer/DNA complexes prepared as above at varying N/P ratios from 1/1 to 6/1 were electrophoresed through a 0.8% agarose gel containing ethidium bromide at 100 V in TAE solution (40 mM Tris–HCl, 1 v/v % acetic acid, and 1 mM EDTA).

**Competitive Binding Assays and Reduction-Triggered Unpacking of Polyplexes.** The competitive binding assays were performed using negatively charged DSS. 1.8 kDa PEI–LA<sub>2</sub> polyplexes were prepared at an N/P ratio of 10/1 as described above. 5  $\mu\text{L}$  of DSS solution in HEPES was added into polyplex dispersions, to give varying sodium sulfate/DNA phosphate mole ratios from 6/1 to 8/1 and 12/1. Following incubation for 30 min, the polyplexes were electrophoresed through a 0.8% agarose gel containing ethidium bromide at 100 V in TAE solution (40 mM Tris–HCl, 1 v/v % acetic acid, and 1 mM EDTA). 1.8 kDa PEI was used as a control.

Reduction-triggered unpacking of polyplexes was investigated by gel retardation assays and DLS. For gel retardation assays, 5  $\mu\text{L}$  of DTT solution in HEPES was added to 40  $\mu\text{L}$  of polyplex dispersions in HEPES to reach a final DTT concentration of 10 mM. After 1 h, 5  $\mu\text{L}$  of DSS solution in HEPES was added into polyplex dispersions, to give varying sodium sulfate/DNA phosphate mole ratios from 6/1 to 8/1 and 12/1. After 30 min incubation, the polyplexes were electrophoresed through a 0.8% agarose gel containing ethidium bromide at 100 V in TAE solution (40 mM Tris–HCl, 1 v/v % acetic acid, and 1 mM EDTA).

For DLS measurements, a predetermined amount of DTT was added under a nitrogen flow into a cuvette containing 0.5 mL of 1.8 kDa PEI–LA<sub>2</sub>/DNA complexes, to yield a final DTT concentration of 10 mM. The cuvette was sealed with a septum, and hydrodynamic sizes of polyplexes were monitored in time.

**Cytotoxicity Assays.** The cytotoxicity of 1.8 kDa PEI–LA conjugates was evaluated in 293T and HeLa cells by MTT assays. 1.8 kDa and 25 kDa PEI were used as controls. In brief, 293T and HeLa cells were seeded in a 96-well plate ( $6 \times 10^3$  cells/well) in 100  $\mu\text{L}$  of DMEM medium containing 10% FBS for 1 day. The culture medium was removed and replenished with 100  $\mu\text{L}$  of fresh medium containing varying amounts (from 0.25 to 5  $\mu\text{g}$ ) of PEI–LA conjugates, 1.8 kDa PEI or 25 kDa PEI, which corresponded to final polymer concentrations ranging from 2.5 to 50  $\mu\text{g/mL}$ . The cells were incubated with polymers for 1 day. The medium was replaced with 100  $\mu\text{L}$  of fresh medium containing 100  $\mu\text{g}$  of MTT, and cells were further cultured for 4 h at 37 °C. The medium was aspirated, the MTT-formazan generated by live cells was dissolved in 150  $\mu\text{L}$  of DMSO, and the absorbance at a wavelength of 570 nm of each well was measured using a microplate reader. The relative cell viability (%) was determined by comparing the absorbance at 570 nm with that of control wells containing only cell culture medium. Data are presented as average  $\pm$  SD ( $n = 4$ ).

The cytotoxicity of 1.8 kDa PEI–LA polyplexes prepared at N/P ratios of 10/1, 20/1, 40/1 and 80/1 was also evaluated in

Scheme 2. Synthesis of 1.8 kDa PEI–LA Conjugates

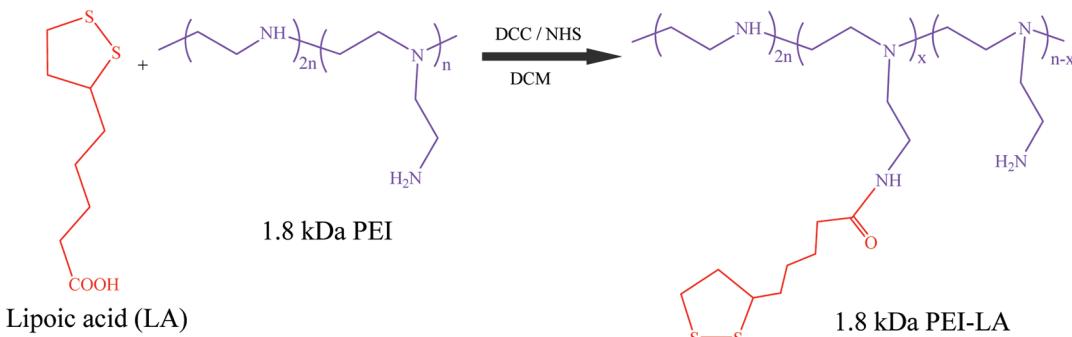


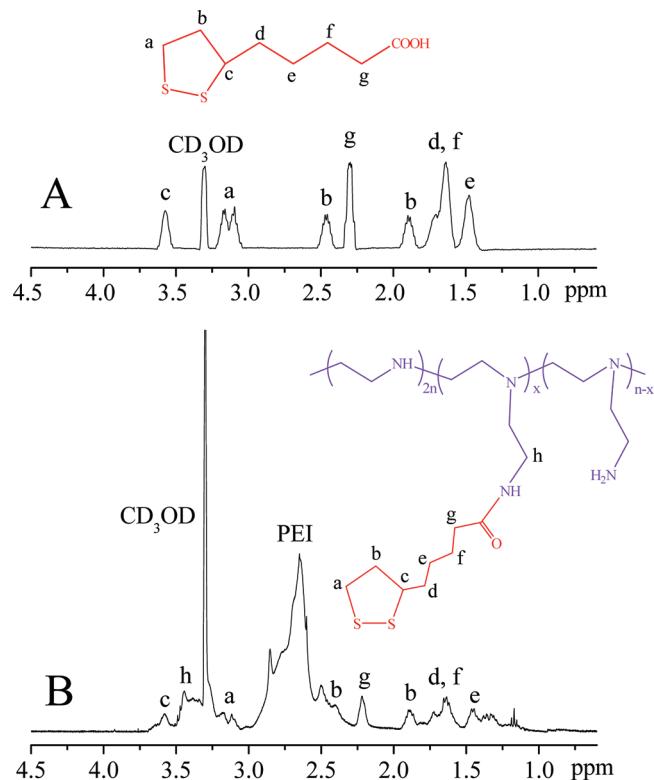
Table 1. Characteristics of 1.8 kDa PEI–LA Conjugates

entry	polymer <sup>a</sup>	DS (theory)	DS <sup>b</sup> ( <sup>1</sup> H NMR)	buffer capacity <sup>c</sup> (%)
1	1.8 kDa PEI–LA <sub>2</sub>	2	1.9	13.2
2	1.8 kDa PEI–LA <sub>4</sub>	4	3.8	13.5
3	1.8 kDa PEI–LA <sub>6</sub>	6	5.8	13.4
4	1.8 kDa PEI		14.3	
5	25 kDa PEI		14.8	

<sup>a</sup> 1.8 kDa PEI–LA<sub>x</sub> wherein *x* represents degree of substitution (DS, defined as number of substituents per 100 nitrogen of PEI). <sup>b</sup> Determined by <sup>1</sup>H NMR. <sup>c</sup> Determined by acid–base titration.

HeLa and 293T cells by MTT assays. Polyplexes of 1.8 kDa and 25 kDa PEIs were used as controls. To facilitate comparison, the experiment was performed using the same protocol as transfection studies. In brief, HeLa and 293T cells were seeded in a 96-well tissue culture plate at 6000 cells/well in 100  $\mu$ L DMEM medium containing 10% FBS for 1 day. 1.8 kDa PEI–LA polyplex dispersions were added, and the cells were cultured for 4 h at 37 °C. Next, the polyplexes were removed, 200  $\mu$ L of fresh culture medium containing 10% serum was added, and the cells were cultured for 2 days. The medium was replaced with 100  $\mu$ L of fresh medium containing 100  $\mu$ g of MTT, and cells were further incubated for 4 h at 37 °C. The relative cell viability (%) was determined as described above.

**In Vitro Gene Transfection.** Transfection experiments were performed in HeLa and 293T cells using the plasmid pGL3 as a reporter gene. Transfections were conducted using polyplexes formed at N/P ratios ranging from 30/1 to 60/1. The cells were plated in 24-well plates ( $6 \times 10^4$  cells/well) and maintained in DMEM supplemented with 10% FBS at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub> until 70% confluence. In a standard transfection experiment, the cells were rinsed with PBS and incubated with 100 mL of polyplex dispersions (1  $\mu$ g of plasmid DNA per well) and 400 mL of culture medium with or without 10% serum for 4 h at 37 °C. Next, the polyplexes were removed, 500  $\mu$ L of fresh culture medium containing 10% serum was added, and the cells were cultured for 2 days. Luciferase quantification was done using a commercial luciferase assay kit (R-Protagen Co., Ltd.) and a multifunctional microplate reader (Multiskan Flash, Thermo). The total protein was measured according to a BCA protein assay kit (Pierce). Transfection efficiency was expressed as relative light units (RLU) per mg of protein. 25 kDa PEI/DNA formulation prepared at an optimal

Figure 1. <sup>1</sup>H NMR spectra (400 MHz, CD<sub>3</sub>OD) of (A) lipoic acid and (B) 1.8 kDa PEI–LA<sub>6</sub> (Table 1, entry 3).

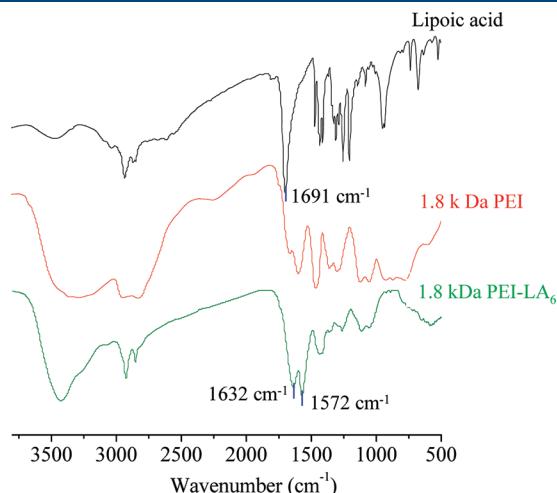
N/P ratio of 10/1 was used as a reference. All the experiments were carried out in triplicate.

**Confocal Microscopy.** The cellular uptake and intracellular DNA release behaviors of 1.8 kDa PEI–LA<sub>2</sub> polyplexes at an N/P ratio of 40/1 were studied in HeLa cells with CLSM using Cy5-labeled DNA. 25 kDa PEI polyplexes at an N/P ratio of 10/1 were used as a control. In brief, HeLa cells were plated on coverslips in 24-well plates (cell density  $6 \times 10^4$  cells/well) and maintained in DMEM supplemented with 10% FBS at 37 °C for 1 day. 100  $\mu$ L of polyplex dispersions (1  $\mu$ g of Cy-5 labeled plasmid DNA per well) was added. The cells were cultured for 4 or 8 h at 37 °C. The polyplexes were removed, and the cells were washed with PBS three times and fixed with 4% paraformaldehyde for 15 min. The nuclei were stained with 200  $\mu$ L of Hoechst 33342 (20  $\mu$ g/mL)

for 15 min at rt. The cells following three times rinsing with PBS were observed with a confocal laser scanning microscope (TCS SP5 Leica).

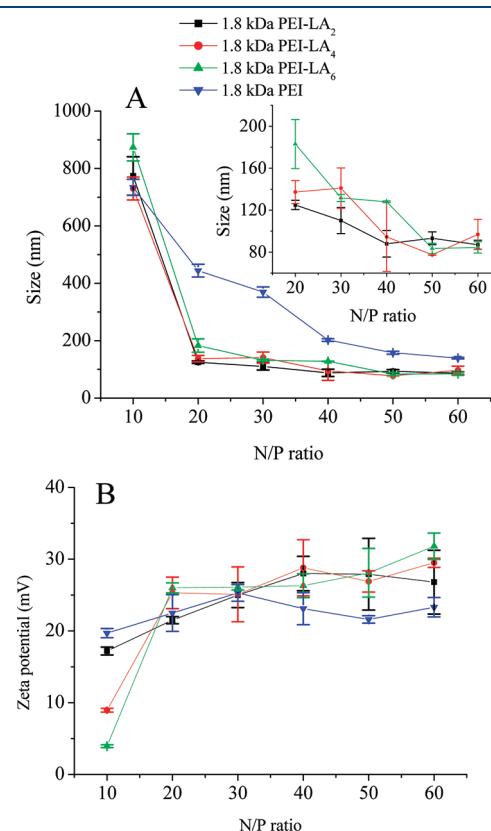
## ■ RESULTS AND DISCUSSION

**Synthesis of 1.8 kDa PEI–LA Conjugates.** Lipoic acid (LA) was conjugated to 1.8 kDa PEI via amide bonds using carbodiimide chemistry (Scheme 2). The resulting PEI–LA conjugates were isolated by precipitation in cold diethyl ether. The results of synthesis are summarized in Table 1. <sup>1</sup>H NMR of PEI–LA conjugates in CD<sub>3</sub>OD displayed, besides a broad peak at  $\delta$  2.30–2.98 assignable to the methylene protons of PEI, signals at  $\delta$  3.59 and 2.21/3.44 attributable to the lipoyl methine proton and methylene protons next to the carbonyl group, respectively (Figure 1). The degree of substitution (DS), defined as number of lipoyl groups per 100 nitrogen atoms of PEI, could be estimated by comparing the integrals of signals at  $\delta$  2.21 and  $\delta$  2.30–2.98. The results revealed that 1.8 kDa PEI–LA conjugates with varying DS values of 1.9, 3.8 and 5.8, close to the designed DS of 2, 4 and 6, respectively, were obtained, indicating

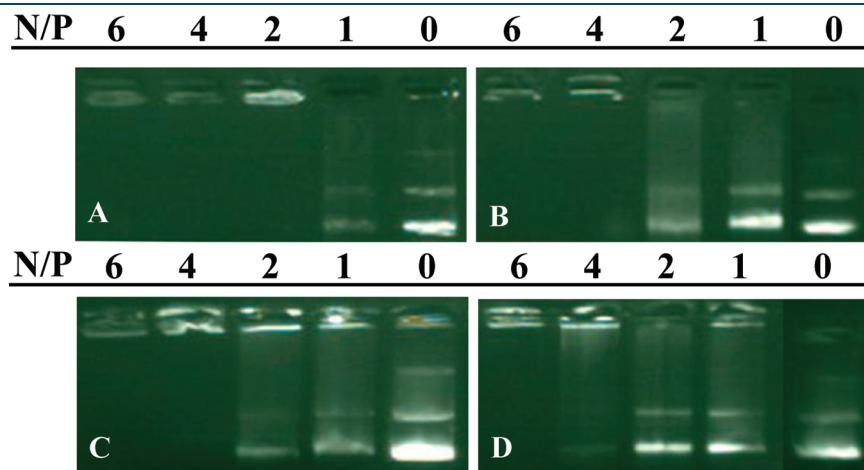


**Figure 2.** FT-IR spectra of lipoic acid, 1.8 kDa PEI and 1.8 kDa PEI–LA<sub>6</sub> (Table 1, entry 3).

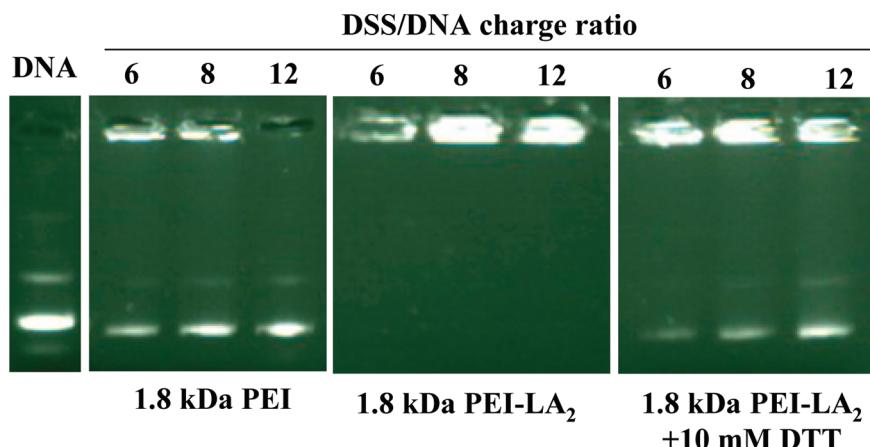
controlled lipoylation of PEI. The successful coupling was further confirmed by FT-IR analyses in which absorptions characteristic of amide groups were observed at 1572 and 1632 cm<sup>-1</sup> for 1.8 kDa PEI–LA conjugates (Figure 2). Notably, acid–base titrations showed that lipoylated PEIs had buffer capacities ranging from 13.2% to 13.5%, which were not much lower than 1.8 kDa and 25 kDa PEIs (14.3% and 14.8%) (Table 1), indicating that PEI–LA conjugates inherited good proton sponge effect of PEI.



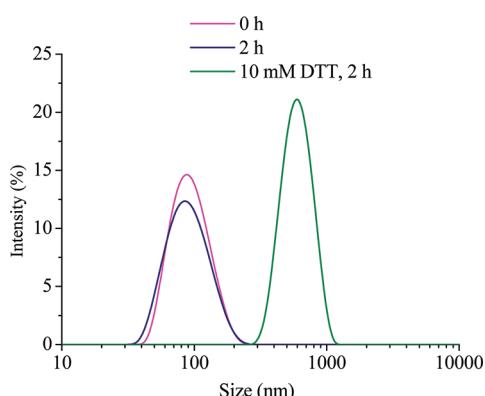
**Figure 4.** Average particle sizes (A) and  $\zeta$ -potentials (B) of 1.8 kDa PEI–LA polyplexes formed at N/P ratios ranging from 10/1 to 60/1. 1.8 kDa PEI was used as a control. Data are shown as mean  $\pm$  SD ( $n = 3$ ).



**Figure 3.** Gel retardation assays of 1.8 kDa PEI and PEI–LA polyplexes formed at N/P ratios ranging from 0/1 to 6/1: (A) 1.8 kDa PEI; (B) 1.8 kDa PEI–LA<sub>2</sub>; (C) 1.8 kDa PEI–LA<sub>4</sub>; (D) 1.8 kDa PEI–LA<sub>6</sub>.



**Figure 5.** Agarose gel electrophoresis of 1.8 kDa PEI polyplexes formed at an N/P ratio of 10/1 following incubation with negatively charged dextran sodium sulfate (DSS) at varying DSS/DNA charge ratios of 6, 8, and 12 in the absence or presence of 10 mM DTT. 1.8 kDa PEI was used as a control.



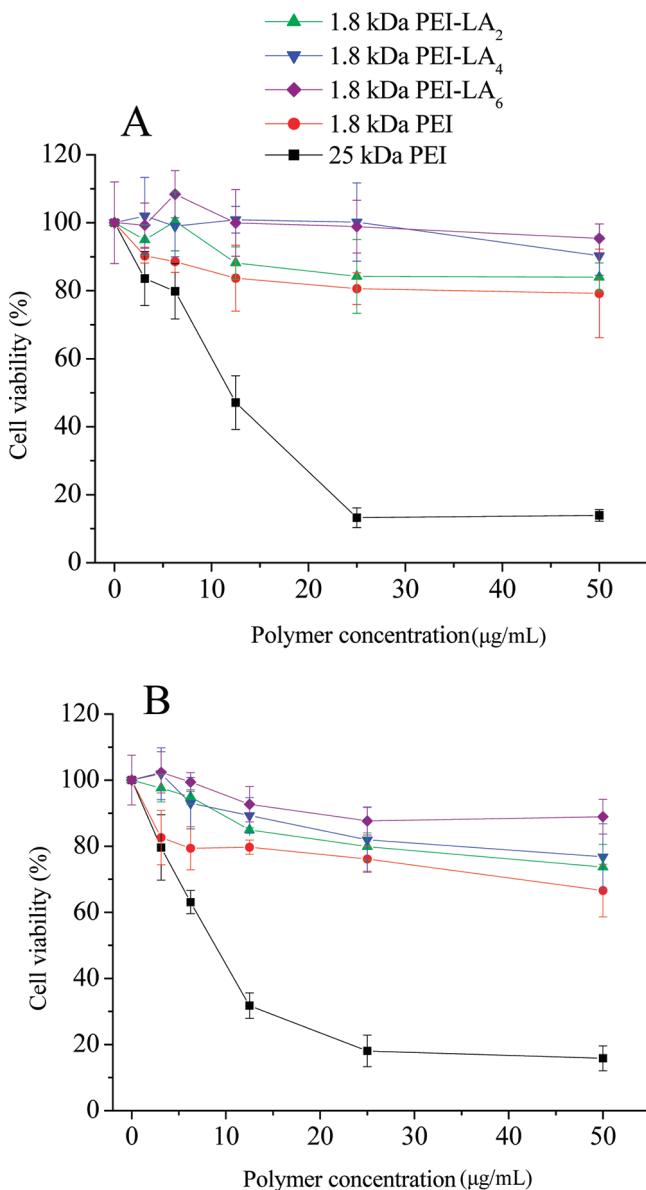
**Figure 6.** Size change of 1.8 kDa PEI-LA<sub>2</sub> polyplexes formed at an N/P ratio of 20/1 in response to 10 mM DTT.

**Biophysical Characterization of 1.8 kDa PEI-LA Polyplexes.** We assumed that lipoylation of 1.8 kDa PEI would result in better DNA condensation due to the presence of additional hydrophobic interactions between PEI-PEI as well as PEI-DNA. To confirm this, DNA complexation behaviors of PEI-LA conjugates were studied using gel electrophoresis, dynamic light scattering (DLS) and zeta potential measurements. Gel electrophoresis results revealed that an N/P ratio of 4/1, 4/1, 6/1 and 2/1 was required to completely inhibit DNA migration for 1.8 kDa PEI-LA<sub>2</sub>, 1.8 kDa PEI-LA<sub>4</sub>, 1.8 kDa PEI-LA<sub>6</sub> and unmodified 1.8 kDa PEI, respectively (Figure 3). The impaired DNA retardation of 1.8 kDa PEI by lipoylation could be due to decreased cationic charge density and increased steric hindrance pertinent to the bulky and rigid lipoyl groups. Pack et al. reported that acetylation of 25 kDa PEI weakened PEI-DNA interactions.<sup>43</sup>

Interestingly, DLS measurements showed that all three PEI-LA conjugates were able to condense DNA into nanosized particles (84–183 nm) at N/P ratios ranging from 20/1 to 60/1 (Figure 4). In comparison, unmodified 1.8 kDa PEI formed much larger DNA complexes at the same N/P ratios, in which small-sized polyplexes (139–202 nm) were obtained only at high N/P ratios above 40/1 (Figure 4A). It is also interesting to note that 1.8 kDa PEI-LA<sub>2</sub> gave the smallest polyplexes at N/P ratios of 20/1 to 40/1, hinting that both primary amine and

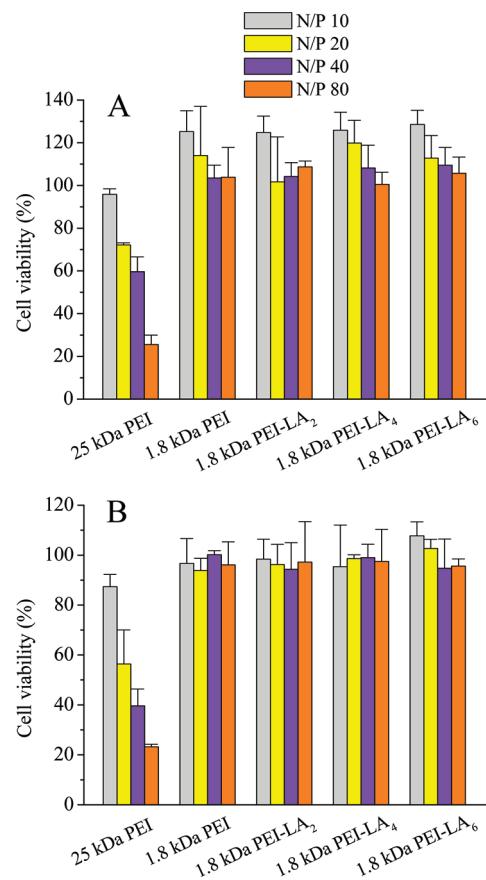
hydrophobic ligands play an important role in DNA condensation. Zeta potential measurements showed that, at a low N/P ratio of 10/1, surface charges decreased with increasing degrees of LA substitution (Figure 4B), which agrees well with decreasing cationic charge density of 1.8 kDa PEI by lipoylation as shown by above gel electrophoresis studies (Figure 3). However, at higher N/P ratios ranging from 20/1 to 60/1, PEI-LA polyplexes displayed elevated positive surface charges (+21.5 to +31.8 mV) comparable to or higher than 1.8 kDa PEI counterparts (+21.6 to +25.3). Increased surface charges were also observed for polyplexes of lipid modified low molecular weight PEIs.<sup>33</sup> The better DNA condensation ability of PEI-LA conjugates over unmodified 1.8 kDa PEI at N/P ratios above 20/1 is most probably due to their largely increased surface charge density resulting from micellization. It appeared, therefore, that conjugation of LA to low molecular weight PEI impairs its DNA binding ability at low N/P ratios due to reduction of net cationic charge density, while it enhances DNA condensation at high N/P ratios due to the presence of hydrophobic interactions.

**Reduction-Triggered Active Unpacking of Polyplexes.** The ideal gene carriers should be able to effectively condense and protect DNA under extracellular conditions while efficiently delivering and releasing DNA inside cells. We hypothesized that the hydrophobic lipoyl group would be transformed into the hydrophilic dihydrolipoyl group under intracellular reductive environment, promoting active intracellular release of DNA. Gel retardation assays showed that DNA was released at 6-fold excess of negatively charged dextran sodium sulfate (DSS) relative to DNA phosphate groups from 1.8 kDa PEI polyplexes formed at an N/P ratio of 10/1 (Figure 5). In contrast, 1.8 kDa PEI-LA<sub>2</sub> polyplexes formed at an N/P ratio of 10/1 were stable against 12-fold excess of DSS (Figure 5), confirming that lipoylation of 1.8 kDa PEI results in stronger binding with DNA. Interestingly, release of DNA was clearly observed for 1.8 kDa PEI-LA<sub>2</sub> polyplexes following incubation with 10 mM DTT under otherwise the same conditions (Figure 5), supporting our hypothesis that intracellular level of reductive condition might trigger unpacking of PEI-LA polyplexes. DLS measurements showed that 1.8 kDa PEI-LA<sub>2</sub> polyplexes formed at an N/P ratio of 20/1 maintained essentially the same particle sizes in 2 h (Figure 6), indicating excellent polyplex colloidal stability. The polyplexes were, nevertheless, rapidly expanded to ca. 635 nm



**Figure 7.** Cytotoxicity of 1.8 kDa PEI-LA conjugates at polymer concentrations ranging from 2.5 to 50  $\mu\text{g}/\text{mL}$ . 1.8 kDa and 25 kDa PEIs were used as controls. Cell viabilities are shown as mean  $\pm$  SD ( $n = 4$ ). (A) HeLa cells, (B) 293T cells.

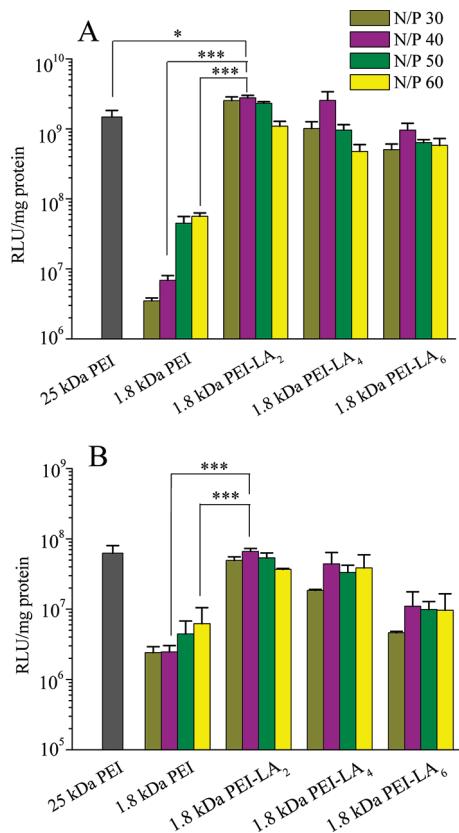
following 2 h incubation with 10 mM DTT (Figure 6), supporting unpacking of polyplexes. In a control experiment, 1.8 kDa PEI-LA conjugates were treated with 100 mM DTT, yielding dihydrolipoylated 1.8 kDa PEIs (1.8 kDa PEI-DHLA conjugates) following ultrafiltration (MWCO 1000). In contrast to 1.8 kDa PEI-LA, 1.8 kDa PEI-DHLA was not able to condense DNA into nanosized particles at N/P ratios ranging from 10/1 to 60/1 under otherwise the same conditions, corroborating that dihydrolipoylated 1.8 kDa PEI has much inferior DNA condensation ability to unmodified 1.8 kDa PEI. These results show that lipoylation of 1.8 kDa PEI might elegantly resolve the DNA condensation and release dilemma of nonviral gene carriers, i.e. on one hand improving DNA condensation ability and polyplex colloidal stability and on the other hand actively releasing DNA under intracellular-mimicking reductive environments.



**Figure 8.** Cytotoxicity of 1.8 kDa PEI-LA polyplexes at N/P ratios of 10/1, 20/1, 40/1 and 80/1. The polyplexes of 1.8 kDa and 25 kDa PEIs were used as controls. Cell viabilities are shown as mean  $\pm$  SD ( $n = 4$ ). (A) HeLa cells, (B) 293T cells.

**Cytotoxicity and *in Vitro* Gene Transfection.** The cytotoxicity of PEI-LA conjugates and polyplexes was investigated in 293T and HeLa cells by MTT assays. Notably, the results showed that all PEI-LA derivatives were practically nontoxic to 293T and HeLa cells (cell viabilities  $>80\%$ ) up to a tested polymer concentration of 50  $\mu\text{g}/\text{mL}$  (corresponding to N/P ratios of 172/1, 156/1 and 140/1 for 1.8 kDa PEI-LA<sub>2</sub>, 1.8 kDa PEI-LA<sub>4</sub>, and 1.8 kDa PEI-LA<sub>6</sub>, respectively in the following transfection experiments) (Figure 7). As previously reported,<sup>11,14</sup> 1.8 kDa PEI revealed significantly lower cytotoxicity as compared to 25 kDa PEI. The lipoylation of 1.8 kDa PEI was shown to further minimize its cytotoxicity, in which increasing cell viabilities were observed at increasing degrees of lipoylation (Figure 7). This is in sharp contrast to lipid modification of low molecular weight PEIs, which gave rise to increased cytotoxicities as compared to unmodified PEIs.<sup>32,33</sup> Moreover, polyplex cytotoxicity assays displayed that 1.8 kDa PEI-LA polyplexes formed at N/P ratios of 10/1, 20/1, 40/1 and 80/1 were essentially nontoxic to HeLa and 293T cells (cell viabilities  $>95\%$ ) (Figure 8). The excellent safety profile of 1.8 kDa PEI-LA conjugates is likely associated with decreased number of primary amine groups as well as excellent biocompatibility of lipoic acid.

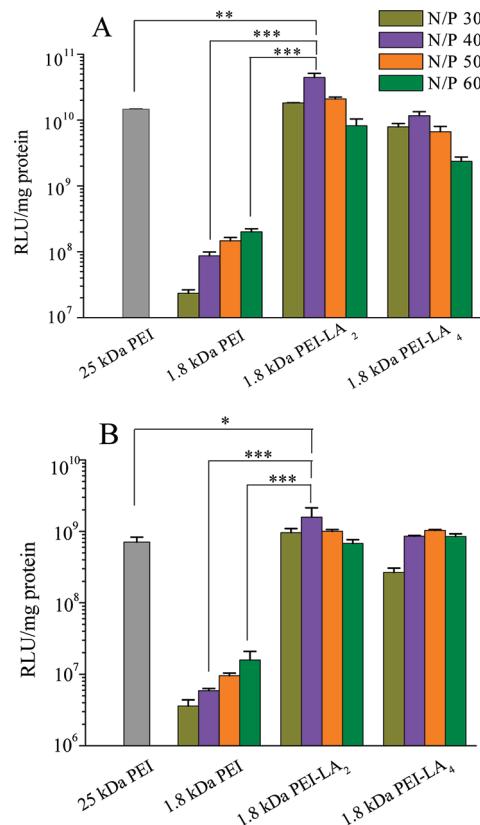
The *in vitro* transfection activity of PEI-LA conjugates was evaluated in HeLa and 293T cells using pGL3 as a reporter gene at N/P ratios ranging from 30/1 to 60/1. Interestingly, all three 1.8 kDa PEI-LA conjugates were shown to mediate markedly



**Figure 9.** Transfection efficiencies of 1.8 kDa PEI-LA polyplexes in 293T (A) and HeLa (B) cells at N/P ratios of 30/1, 40/1, 50/1 and 60/1 in serum-free conditions. 25 kDa PEI formulation at its optimal N/P ratio of 10/1 and 1.8 kDa PEI formulations at N/P ratios of 30/1, 40/1, 50/1 and 60/1 were used as controls. Data are shown as mean  $\pm$  SD ( $n = 3$ ) (Student's *t* test, \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001).

enhanced transfection as compared to the parent 1.8 kDa PEI under serum-free conditions (Figure 9). For example, 1.8 kDa PEI-LA<sub>2</sub> polyplexes formed at an N/P ratio of 40/1 displayed 400-fold augmentation of transfection efficiency in 293T cells as compared to 1.8 kDa PEI controls under otherwise the same conditions, reaching a high gene expression level of about 2 times that of 25 kDa PEI control (Figure 9A). 1.8 kDa PEI-LA<sub>2</sub> polyplexes revealed also an elevated transfection efficiency comparable to 25 kDa PEI control in HeLa cells (Figure 9B). It appeared that transfection efficiency of 1.8 kDa PEI-LA conjugates decreased with increasing DS (Figure 9), signifying the importance of balancing vector cationic charge density and hydrophobicity.

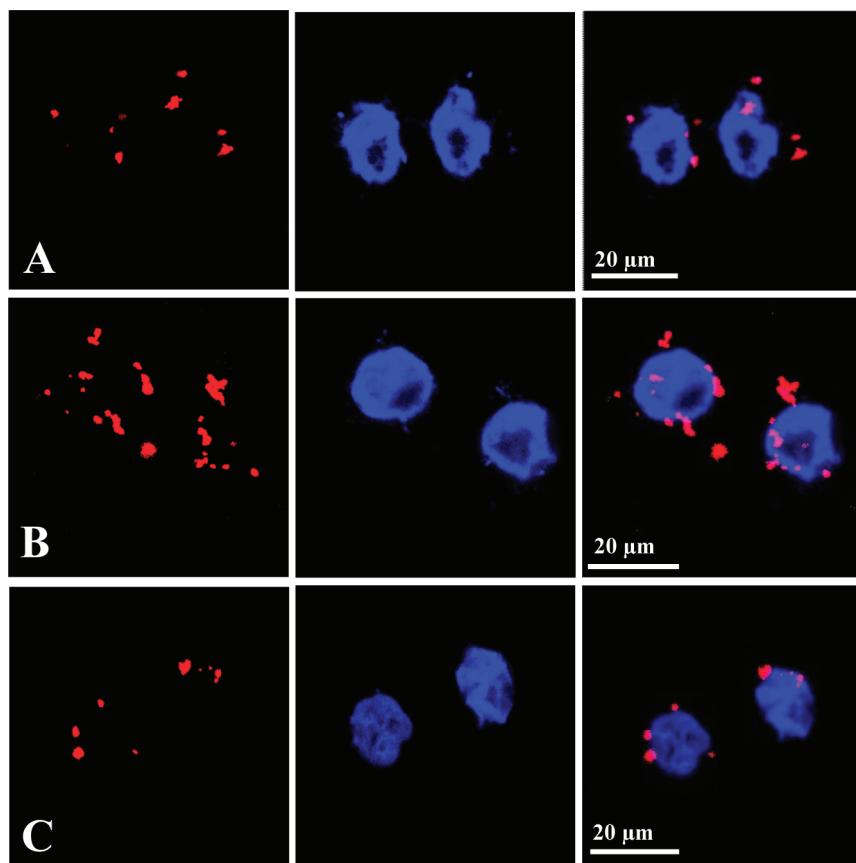
The *in vitro* transfection activity of 1.8 kDa PEI-LA<sub>2</sub> and 1.8 kDa PEI-LA<sub>4</sub> conjugates was also investigated in the presence of 10% serum. The results showed that 1.8 kDa PEI-LA<sub>2</sub> polyplexes formed at an N/P ratio of 40/1 yielded the greatest transfection in both 293T and HeLa cells, which was approximately 500-fold and 180-fold higher than 1.8 kDa PEI control, and 3-fold and 2-fold higher than 25 kDa PEI control, respectively (Figure 10). The superior transfection activity of 1.8 kDa PEI-LA polyplexes to 25 kDa PEI controls in the presence of serum is likely related to their elegant combination of enhanced colloidal stability under extracellular conditions and rapid release of DNA inside cells.



**Figure 10.** Transfection efficiencies of 1.8 kDa PEI-LA polyplexes in 293T (A) and HeLa (B) cells at N/P ratios of 30/1, 40/1, 50/1 and 60/1 in 10% serum condition. 25 kDa PEI formulation at its optimal N/P ratio of 10/1 and 1.8 kDa PEI formulations at N/P ratios of 30/1, 40/1, 50/1 and 60/1 were used as controls. Data are shown as mean  $\pm$  SD ( $n = 3$ ) (Student's *t* test, \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001).

**Intracellular DNA Trafficking.** To obtain insight into the intracellular fate of DNA complexed with 1.8 kDa PEI-LA conjugates, transfection experiments were performed using Cy-5 labeled DNA in HeLa cells. The cell nuclei were stained with Hoechst 33242 (blue). Interestingly, confocal laser scanning microscope (CLSM) showed that 1.8 kDa PEI-LA<sub>2</sub> polyplexes formed at an N/P ratio of 40/1 effectively delivered DNA (red) into HeLa cells as well as cell nuclei following 4 h transfection (Figure 11A). The amount of DNA delivered and released into the cell nuclei increased when transfection time was prolonged to 8 h (Figure 11B). In comparison, cells transfected with 25 kDa PEI control under otherwise the same conditions displayed obviously less DNA fluorescence in the cell nuclei (Figure 11C). These results confirm that 1.8 kDa PEI-LA<sub>2</sub> is much more efficient for intracellular DNA delivery than 25 kDa PEI. Uludag et al. also observed that linoleic acid modification of 2 kDa PEI resulted in enhanced association with the nucleus.<sup>44</sup> This is likely because hydrophobically modified PEI has better interactions with the nuclear membrane.

Hence, reduction-sensitive hydrophobic modification of low molecular weight PEIs has appeared to be a novel and unique approach for the development of structurally defined, biocompatible and highly efficient gene transfer agents. The superior transfection activity of 1.8 kDa PEI-LA conjugates is likely associated with their better DNA condensation and protection, enhanced polyplex colloidal stability under extracellular conditions,



**Figure 11.** CLSM images of HeLa cells transfected with 1.8 kDa PEI–LA<sub>2</sub>/Cy5-labeled pDNA complexes at an N/P ratio of 40/1 in the presence of 10% serum (1  $\mu$ g of DNA/well). 25 kDa PEI/Cy5-labeled pDNA complexes at an N/P ratio of 10/1 was used as a control. Cells were incubated with polyplexes for 4 or 8 h. For each panel, images from left to right show Cy5 labeled pDNA (red), cell nuclei stained by Hoechst 33342 (blue), and overlays of two images. The bar represents 20  $\mu$ m. (A) 1.8 kDa PEI–LA<sub>2</sub> polyplexes, 4 h; (B) 1.8 kDa PEI–LA<sub>2</sub> polyplexes, 8 h; (C) 25 kDa PEI polyplexes, 8 h.

efficient intracellular polyplex unpacking and nuclear delivery, and minimal cytotoxicity.

## CONCLUSIONS

We have demonstrated that hydrophobic modification of 1.8 kDa PEI with lipoic acid, a natural reduction-sensitive biocompatible compound, affords nontoxic and superior nonviral gene transfection to 25 kDa PEI control in serum-free as well as 10% serum media. These novel reduction-sensitive PEI-based vectors have several unique features: (i) they are readily prepared with well-defined structures and compositions; (ii) they on one hand exhibit excellent DNA condensation ability and yield enhanced polyplex colloidal stability, and on the other hand actively deliver and release DNA into the cell nuclei following cellular uptake due to reduction-triggered reversal of hydrophobic modification, elegantly resolving DNA condensation and release dilemma; (iii) they are practically nontoxic to cells even at a high polymer concentration of 50  $\mu$ g/mL; and (iv) they show superior transfection activity to 25 kDa PEI control. These nontoxic 1.8 kDa PEI–LA conjugates form a superb basis for the development of targeting, biocompatible and highly efficient carriers for delivery of therapeutic genes.

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