

Guanidinoamidized Linear Polyethyleneimine for Gene Delivery^{*}

Bo Zhang^{a, c, d†}, Xin-peng Ma^{d†}, Mei-hua Sui^b, Edward Van Kirk^e, William J. Murdoch^e,
Maciej Radosz^d, Neng-ming Lin^{a**} and You-qing Shen^{b**}

^a Institute for Individualized Medicine, Hangzhou First People's Hospital, Hangzhou 310006, China

^b Center for Bionanoengineering and the State Key Laboratory for Chemical Engineering, Department of Chemical and Biological Engineering, Zhejiang University, Hangzhou 310027, China

^c Laboratory of Clinical Pharmacy, Zhejiang Cancer Hospital, Hangzhou 310022, China

^d Department of Chemical and Petroleum Engineering, University of Wyoming, Laramie, Wyoming 82071, USA

^e Department of Animal Science, University of Wyoming, Laramie, Wyoming 82071, USA

Abstract Guanidine was introduced to low molecular weight linear polyethyleneimine (LPEI) *via* amide groups, to explore the effect of both guanidine degree and pendant chain length on its transfection behavior. The resulting guanidinoamidized LPEIs (GLPEIs) could dramatically reduce LPEI's toxicity, enhance its DNA-packaging capability, cellular uptake and therefore transfection efficiency. These polyplexes were taken up very efficiently *via* caveolae-mediated endocytosis and their transfection efficiencies in ovarian cancer cells were significantly improved compared to native LPEI_{10k} polyplexes. Among these GLPEIs, LPEI-C₃-G₁₀₀ showed higher DNA affinity even than LPEI_{25k} and the highest transfection efficiency, probably due to the optimization of polymer chain flexibility. Of notice, LPEI-C₃-G₁₀₀ polyplexes could more effectively accumulate into cytoplasm than LPEI_{25k}, although the transfection efficiency of LPEI-C₃-G₁₀₀ polyplexes was not superior to that of LPEI_{25k} polyplexes, which would be probably attributed to the more efficient release of LPEI_{25k} polyplexes than LPEI-C₃-G₁₀₀ polyplexes in the cytoplasm.

Keywords: Polyethyleneimine; Guanidine; Gene delivery; Cellular uptake.

INTRODUCTION

Non-viral gene vectors have attracted more and more attention as gene delivery carriers owing to their versatile functionality, low risk potentials and greatly reduced immunogenicity^[1–3]. Among various non-viral vectors, cationic polymers including polyethyleneimine (PEI)^[4, 5], polyamidoamine (PAMAM) dendrimer^[6], chitosan/dextran^[7] and cationic polypeptides such as poly(L-lysine)^[8] have been spotlighted. These polymers effectively condense pDNA into nanoparticles, facilitate cellular uptake *via* endocytosis, and have structural versatility in gene delivery^[9, 10]. However, their low transfection efficiencies relative to viral vectors limit their further applications.

^{*} This work was financially supported by the Qianjiang Talent Program of Zhejiang Province (2014.1–2015.12, Zhang Bo), the National Natural Science Fund for Distinguished Young Scholars (No. 50888001), the National Natural Science Foundation of China (No. 20974096), Zhejiang Provincial Program for the Cultivation of High-level Innovative Health Talents (2010-190-4), and the U.S. National Science Foundation (No. CBET 0753109, DMR-0705298) and Department of Defense (No. BC083821).

[†] These two authors are joint first authors.

^{**} Corresponding authors: You-qing Shen (申有青), E-mail: shenyq@zju.edu.cn

Neng-ming Lin (林能明), E-mail: lnm1013@163.com

Received October 22, 2014; Revised November 12, 2014; Accepted November 27, 2014

doi: 10.1007/s10118-015-1644-9

PEI has been considered as a gold standard of non-viral gene delivery carriers owing to its relatively high transfection efficiency^[11, 12]. Its gene transfer activity and cytotoxicity generally increase with increasing its molecular weight. Therefore, the cytotoxicity caused by PEI at the desirable doses for transfection is unavoidable^[13]. Low molecular weight PEI has very low transfection efficiency because of its poor DNA packaging capability. To take advantage of the low toxicity of low molecular weight PEI but to increase its gene transfection efficiency, low molecular weight PEI was linked to make high molecular weight PEI *via* cleavable poly(amino acid)^[14], disulfide groups^[15] and environmental sensitive bonds^[16]. Such degradable high molecular weight PEIs had low toxicity due to their ability to degrade into short PEI chains at physiological conditions, and simultaneously achieved similar transfection efficiency to high molecular weight PEI (e.g. PEI with molecular weight of 25 kDa).

Arginine-residue rich peptides such as transactivation (TAT) from HIV-1 virus are capable of translocating cell membranes freely through an energy-independent and non-receptor mechanism^[17, 18]. Homo-oligoarginines were found to greatly facilitate cellular uptake but oligopeptides of lysine, ornithine or histidine could not, suggesting the indispensable role of arginine residues in the great cell-penetrating ability of TAT^[19, 20]. Further reports showed that oligoarginines peptides had efficient cellular uptake^[21]. Oligoarginines with 7 to 9 arginine residues (R7-R9) were most efficient in entering the cell and nucleus, even at 4 °C or on the ice^[22]. Thus, oligoarginines or oligoguanidines were used to functionalize nanocarriers for enhanced endocytosis^[23]. Moreover, conjugation of arginine or guanidine groups to the periphery of dendrimers could also lead to high transfection efficiency^[24, 25]. These results suggested that, not only TAT or arginine conjugation, but simply chemical modification such as guanidinylation might be sufficient to transport polyplexes into cells^[26–28].

Meanwhile, the pendant chain length is also important to reach high transfection efficiency. For instance, previous report had gained the highest transfection efficiency and cellular uptake by using 4 carbon atoms in pendant primary amine of poly(amidoamine)s instead of 3 or 5 carbon atoms^[29]. Therefore, to study the combinational effect of both pendant chain length and guanidine degree in this gene vector, we proposed to introduce guanidine groups to LPEI *via* amide pendant chains, aiming to enhance the polymer's DNA packaging capability, the cellular uptake of the resulting polyplexes, and thereby its DNA transfection efficiency.

EXPERIMENTAL

Materials

2-Methyl-2-oxazoline (98%), methyl *p*-toluenesulfonate (98%), boc- β -alanine (Boc- β -Ala-OH, > 99.0%), 5-(boc-amino)valeric acid (Boc-5-Ava-OH, > 99.0%), sodium carbonate (> 99.0%), *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC, 99%), 4-(dimethylamino)pyridine (DMAP, $\geq$ 99.0%), 1H-pyrazole-1-carboxamide hydrochloride ($\geq$ 98.0%), kanamycin, chlorpromazine and Filipin III were purchased from Sigma-Aldrich Inc. (Milwaukee, WI). Linear polyethyleneimine (LPEI_{25k}, M_w 25000 Da), hydrochloric acid (37%), and sodium hydroxide (99.8%) were from Fisher Scientific (Pittsburgh, PA). pGL4 luciferase reporter vectors, *Escherichia coli*. competent cells and luciferase assay system were purchased from Promega CO. (Madison, WI). QIAGEN plasmid Midi Kit was purchased from QIAGEN Inc. (Valencia, CA). *LabelIT*[®] Nucleic Acid Labeling Kit, CyTM5 was purchased from Mirus Bio LLC (Madison, WI).

Synthesis of Guanidinoamidized LPEI (GLPEI)

LPEI with a molecular weight of 10000 Da (LPEI_{10k}, 230 repeating units) was synthesized as reported in the literature^[30]. The GLPEI was synthesized by a one-pot method (Scheme 1). Briefly, 1.2 equivalents of Boc- β -Ala-OH or Boc-5-Ava-OH, EDC relative to the NH group of LPEI and a catalytic amount of DMAP were dissolved in DMSO. After being stirred at room temperature for 1 h, the mixture was dropped into the solution of LPEI dissolved in deionized water. The mixed solution was maintained at pH 4.0 and stirred at room temperature for 24 h. Concentrated hydrochloride (37%) solution (3 mL) was added to the solution and the solution was stirred at room temperature for 24 h. Potassium carbonate was then gradually added to the solution until the solution pH reached 8.0. Three equivalents of 1H-pyrazole-1-carboxamide hydrochloride relative to the NH

groups in LPEI were added to the solution in portion. This mixture was allowed to stir at room temperature for 24 h. The resulting solution was then dialyzed using dialysis membrane (MWCO 3500 Da) for 12 h. The solution in the dialysis bag was concentrated to 2 mL and this solution was poured into 100 mL THF. The product was isolated by centrifugation and dried in vacuum. The structures of GLPEIs were confirmed by $^1\text{H-NMR}$.

LPEI-C₃-G₂₅ $^1\text{H-NMR}$ (400 MHz, D₂O, δ): 3.58–3.29 (b), 2.89–2.49 (b), 2.45 (t, $J = 7.2$ Hz). The spectra of LPEI-C₃-G₅₀ and LPEI-C₃-G₁₀₀ were similar except for the enhanced signals from the guanidine groups. LPEI-C₅-G₂₅ $^1\text{H-NMR}$ (400 MHz, D₂O, δ): 3.65–3.34 (b), 3.12 (m), 2.89–2.51 (b), 2.51 (t, $J = 7.2$ Hz), 2.09 (t, $J = 6.4$ Hz), 1.47 (b). The spectra of LPEI-C₅-G₅₀ and LPEI-C₅-G₁₀₀ were very similar except for the enhanced signals from the guanidine groups. LPEI_{25k}-C₃-G₃₀₀ $^1\text{H-NMR}$ (400 MHz, D₂O, δ): 3.59–3.22 (b), 2.92–2.59 (b), 2.43 (b), 1.58 (m).

FITC Labeling of LPEI (LPEI-FITC) and GLPEI (GLPEI-FITC)

FITC was added into the solution of GLPEI or LPEI in methanol and stirred at room temperature for 24 h in the dark. The product was precipitated in THF and dried in vacuum. The fluorescence absorption of the FITC-conjugation was verified by its fluorescence at an excitation wavelength of 488 nm. The averaged FITC number per GLPEI or LPEI chain was three.

LPEI_{10k}-FITC $^1\text{H-NMR}$ (400 MHz, D₂O, δ): 7.90 (m), 7.38–7.07 (m), 6.39 (m), 3.30–3.07 (b), 2.66 (b). LPEI_{25k}-FITC $^1\text{H-NMR}$ (400 MHz, D₂O, δ): 7.93 (s), 7.35–6.88 (m), 6.39 (m), 3.53–3.29 (b), 2.69 (b). LPEI-C₃-G₁₀₀-FITC $^1\text{H-NMR}$ (400 MHz, D₂O, δ): 7.96 (m), 7.36–6.94 (m), 6.44 (m), 3.58–3.29 (b), 2.97–2.54 (b), 2.36 (b).

Gel Electrophoresis

Polymer/pDNA complexes were formed by adding 10 μL of a pDNA solution (pGL4-luc, 40 $\mu\text{g/mL}$) to the same volume of polymer solutions at various concentrations to yield desired N/P ratios with 15 s vortexing. Polyplexes were electrophoresed on a 0.9% agarose gel at 100 V for 40 min. pDNA bands were visualized by staining with ethidium bromide and observed under UV light. In the heparin competitive assay, polyplexes were incubated with various concentrations of heparin for 30 min at RT before electrophoresis.

Ethidium Bromide Replacement Assay

Ethidium bromide (EB) 50 μL (0.1 mg/mL) and pDNA 50 μL (0.2 mg/mL) were mixed and incubated at 25 °C for 10 min. Polymer solution (50 μL) at a desired concentration to give an N/P of 5 was added to the pDNA/EB solution and the mixture was incubated for 30 min at RT. The fluorescence intensity of the mixture from 580 nm to 650 nm was measured by SpectraMax M2e at an excitation wavelength of 470 nm.

Size and Zeta-Potential Measurements

The sizes of polyplexes were determined using a nano-ZS zetasizer (Malvern Instrument Ltd., UK) with a laser light wavelength of 632.8 nm and scattering angle at 173°. The polyplexes were prepared as described above, except that the volume of polyplex solutions was increased to 1 mL. Each measurement was performed at 37 °C for 30 runs in triplicate, and the results were processed with DTS software version 3.32.

Cytotoxicity Assay

SKOV-3 or OVCAR-3 ovarian cancer cells were cultured in 96-well plates at an initial seeding density of 5000 cells/well in 200 μL McCoy's or RPMI-1640 medium supplemented with 10% FBS. Cells were grown for 24 h before treatment. LPEI_{10k} or GLPEI was added to the medium at polymer concentrations ranging from 2.5–40 $\mu\text{g/mL}$. Treated cells were incubated at 37 °C under a humidified atmosphere of 95% air and 5% CO₂ for 4 h, and the treatment was replaced with fresh medium. The cells were further cultured for 44 h. MTT reagent (10 μL) was added to each well and the cells were incubated for 5 h at 37 °C. Detergent reagent (100 μL) was added to each well and the plates were placed in incubator until all the crystals were dissolved. The absorbance at 570 nm of the solution in each well was recorded using a Model 680 microplate reader (Bio-Rad, Hercules, CA).

In vitro Transfection Efficiency

Cells were seeded in a 96-well plates at a density of 20000/well in 200 μ L complete medium 24 h prior to transfection. The medium in each well was removed and rinsed with PBS once. Polyplex solutions (50 μ L) prepared in FBS-free medium at a pDNA dose of 10 μ g/mL was added to each well and cultured for 4 h at either 4 °C or 37 °C. After 4 h incubation, the polyplex solutions were replaced with 200 μ L complete medium. The cells were incubated for an additional 44 h. Cell culture lysis reagent (CCLR, Promega Co., Madison, WI, 40 μ L) was added into each well to lyse the cells. Each of the cell lysis solution (40 μ L) was mixed with 100 μ L of luciferase assay reagent (Promega Co., Madison, WI) and the luminance was measured by SpectraMax M2e at 570 nm.

Transfection Mechanism Study

The endocytosis pathway of the polyplexes was probed using the inhibitor of clathrin- or caveolae-mediated endocytosis. Chlorpromazine (specifically inhibit clathrin-mediated endocytosis, 10 μ g/mL) or Filipin III (specifically inhibit caveolae-mediated endocytosis, 5 μ g/mL) was pre-incubated with the cells for 30 min before transfection in FBS-free medium. The solution was then replaced by polyplex solutions at a pDNA concentration of 10 μ g/mL and followed by the steps described above.

Intracellular uptake of the polyplexes was observed by confocal laser scanning fluorescence microscopy and flow cytometry. pDNA (pGL4-Luc) was labeled with Cy5 (Mirus, Madison, Wisconsin) according to the manufacturer's instruction. Briefly, pDNA was mixed with Cy5 *labelIT* reagent and incubated at 37 °C for 1 h. Sodium chloride and ethanol was applied to purify pDNA after labeling. The solution was centrifuged and the pDNA pellet was dissolved in TE buffer and the concentration of pDNA was measured at 260 nm by Nanodrop (ND-1000). Cell nuclei were stained with acridine orange (Sigma-aldrich, St. Louis). The polymers were labeled with FITC in methanol. Polyplexes were prepared as described above using Cy-5-pDNA and FITC-labeled polymer.

OVCAR-3 cells were plated into glass-bottom petri dishes (MatTek, Ashland, MA) at 200000 cells per dish in 2 mL RPMI-1640 medium supplemented with 10% FBS and incubated at 37 °C for 24 h before use. The medium was replaced with the polyplex solutions at a pDNA dose of 10 μ g/mL and then incubated for 2–6 h. The cells were then rinsed with PBS twice before observation with a confocal microscope (TCS SP2 Laser Scanning Spectral Confocal Microscope, Leica, IL). Cy-5 was excited at 643 nm and its fluorescence emission was observed using the 670–710 nm bandpass filter. FITC and acridine orange were both excited at 488 nm and the emission was detected in the range of 500–530 nm for FITC and 560–600 nm for acridine orange. Transmission images were obtained in the same scan.

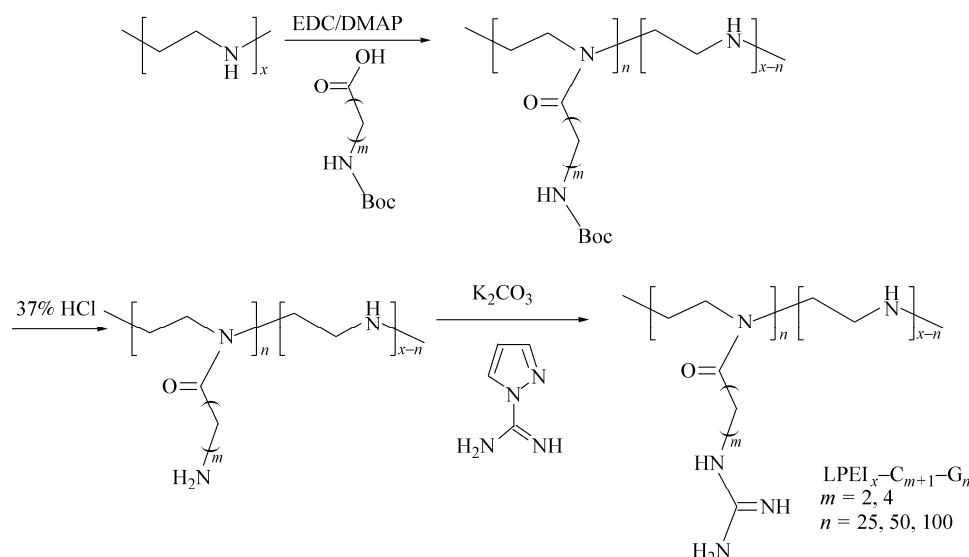
Cells in other dishes treated with transfection reagents were rinsed with PBS, detached by Trypsin and suspended in PBS. 5000 cells were then analyzed using flow cytometry (easyCyte 8HT, Millipore, MA) and data analysis was performed by guavaSoft software (Millipore, MA).

RESULTS AND DISCUSSION

Synthesis of GLPEIs

Recent research suggested that polymers with similar structure but only differed in chain length could behave differently in transfection efficiency. Ou *et al.* found that one more methylene unit in the side chain and main chain of polymers resulted in enhanced binding affinity of polymers with DNA^[31]. These results indicated that different spacer on the side chain might yield different transfection behavior, but the optimal spacer to achieve the highest efficient transfection required further exploitation. On the other hand, as demonstrated by previous reports that conjugation of arginine/guanidine to original polymers could greatly improve their cellular uptake and even gene transfection efficiency^[32–34]. In this study we simultaneously evaluated the effect of pendant chain length and guanidinoamidization degree in low molecular weight LPEI-based gene carrier, by introducing guanidine to LPEI *via* amide groups with different chain lengths (Scheme 1). The resulting GLPEIs (expressed in a general formula: LPEI_x-C_{m+1}-G_n; where $x = 230$ standing for 230 repeating units) had two tunable factors;

one was the pendant chain length ($m + 1 = 3$ or 5 carbon atoms interval between polymer backbone and the guanidine group), and the other was the number of the guanidine groups per LEPI chain ($n = 25, 50$ or 100 as determined by $^1\text{H-NMR}$). Guanidinoamidized LPEI_{25k} (LPEI_{25k}-C₃-G₃₀₀) was also synthesized as a standard to compare the DNA packaging ability and transfection efficacy among different GLPEIs.



Scheme 1 Synthesis of GLPEIs

Characterization of Polyplexes

Agarose gel electrophoresis was used to determine the ability of GLPEIs to form polyplexes with pDNA. As shown in Fig. 1, all of the GLPEIs were able to completely retard pDNA at an N/P ratio of 2.5, but native LPEI_{10k} (Fig. 1g) started to retard pDNA at an N/P of 5. It was in consistent with previous reports that, guanidine modification of PEI dramatically increased the pDNA binding affinity of parent PEI^[35, 36]. As determined by nano-ZS zetasizer, the hydrodynamic sizes of GLPEI polyplexes (Figs. 2a and 2b) were very suitable to be used as efficient gene carriers. Interestingly, GLPEI-C₅-G_x had zeta-potentials of about 10 mV higher than those from LPEI-C₃-G_n (Figs. 2c and 2d), which could be attributed to that the flexibility of longer pendant chains made the guanidine groups easier to extend onto the particles surface and caused higher positive charged density^[37]. The positively charged surface of the polyplexes was also beneficial to their interactions with negatively charged plasma membrane for subsequent cellular uptake^[38].

Stability of Polyplexes

The stability of the polyplexes was examined using heparin replacement assay and EB exclusion assay. The presence of 100 $\mu\text{g/mL}$ heparin in the polyplex solutions did not influence the structure of the LPEI-C₃-G₁₀₀/pDNA polyplexes (Fig. 3a). On the contrary, 25 $\mu\text{g/mL}$ of heparin already released some pDNA from the LPEI_{25k}/pDNA polyplexes. In the EB exclusion assay (Fig. 3b), the tested polymers were added to the EB/pDNA complexes at a dose of an N/P ratio of 5 (0.2 mg/mL pDNA). EB/pDNA has a characteristic fluorescence peaked at 610 nm. The presence of the cationic polymers competes with EB to complex with pDNA and thus reduces the fluorescence intensity (RFU) of EB/pDNA. Therefore, the more decrease in fluorescence intensity suggests stronger binding affinity of the polymers to pDNA. As shown in Fig. 3(b), LPEI_{10k} with 25 guanidine groups (LPEI-C₃-G₂₅) already had a comparable pDNA-binding affinity to LPEI_{25k}, and LPEI-C₃-G₁₀₀ had an even stronger affinity, close to that of LPEI_{25k} conjugated with 300 pendant guanidine groups (LPEI_{25k}-C₃-G₃₀₀).

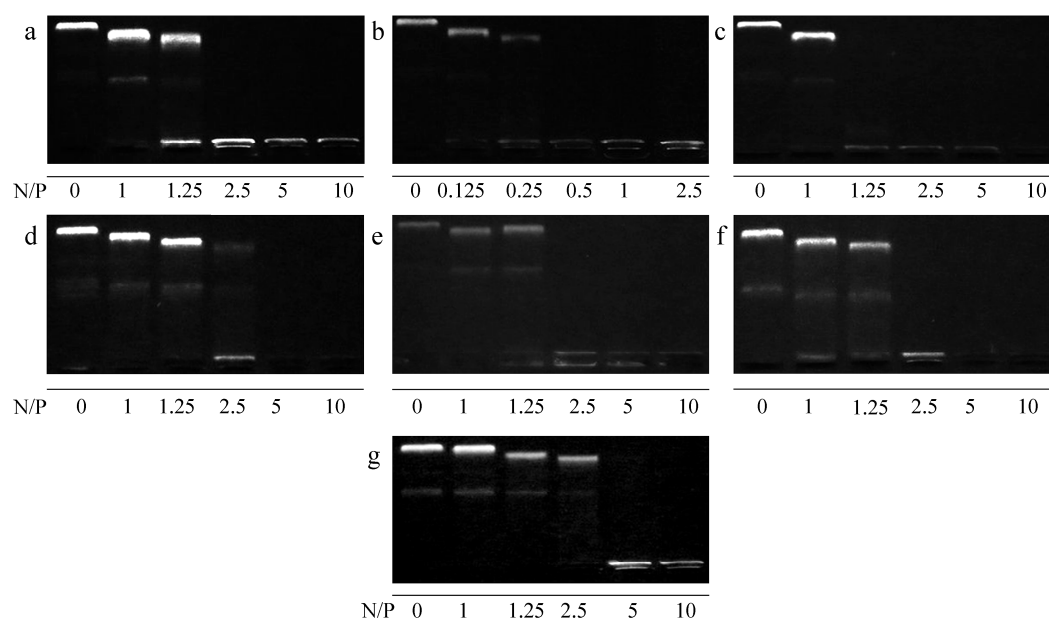


Fig. 1 Agarose gel electrophoresis results of LPEI-C₃-G₂₅ (a), LPEI-C₃-G₅₀ (b), LPEI-C₃-G₁₀₀ (c), LPEI-C₅-G₂₅ (d), LPEI-C₅-G₅₀ (e), LPEI-C₅-G₁₀₀ (f) and LPEI_{10k} (g) at different N/P ratios (Polyplexes were run on the 0.9% agarose gel at 100 mV for 40 min before observation.)

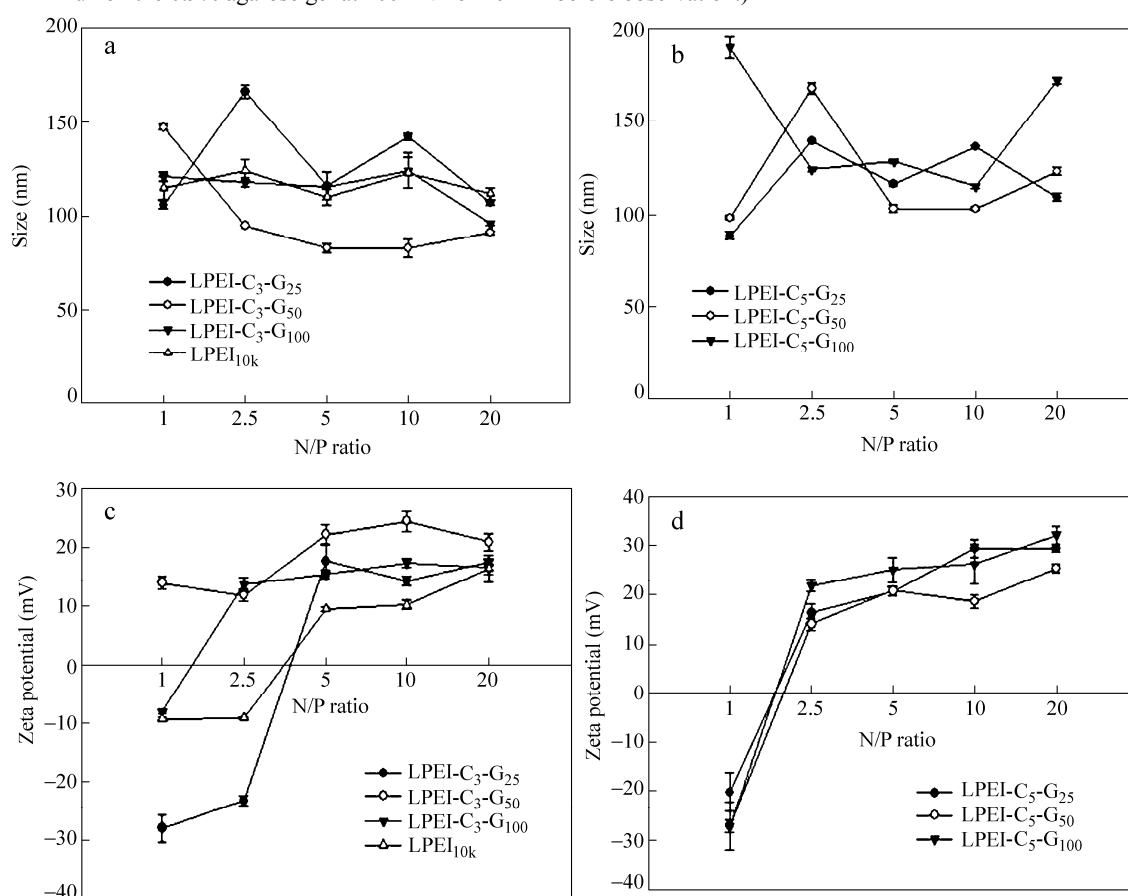


Fig. 2 The size (a and b) and zeta potential (c and d) of polyplexes as a function of N/P ratio

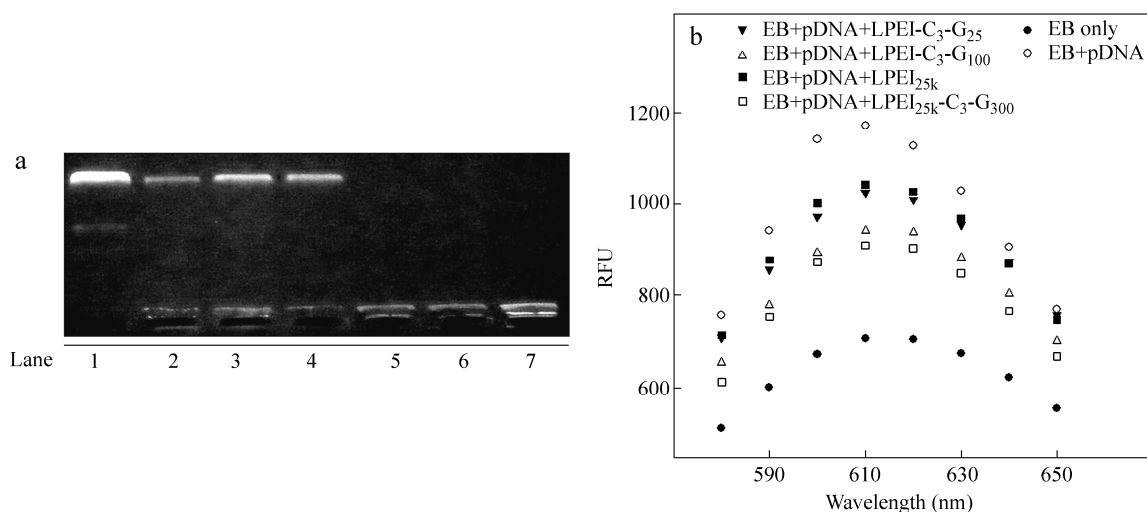


Fig. 3 (a) Agarose gel electrophoresis results of LPEI-C₃-G₁₀₀ (N/P = 20) and LPEI_{25k} (N/P = 10) in the presence of heparin: Lane 1, naked pDNA; Lanes 2–4, polyplexes of LPEI_{25k} with 25, 50 and 100 µg/mL heparin respectively; Lanes 5–7, polyplexes of LPEI-C₃-G₁₀₀ with 25, 50 and 100 µg/mL heparin respectively; (b) The fluorescence emission spectra of EB, EB/pDNA (weight ratio=1/2) and EB/pDNA in the presence of the polymers (The concentrations of polymers were set at an N/P ratio of 5 (0.2 mg/mL pDNA). And fluorescence was excited at the wavelength of 470 nm.)

Cytotoxicity

As reported in other systems^[39], modification with guanidine groups would greatly decrease the cytotoxicity of original polymers. In this study, MTT assay was used to test the cytotoxicity of the polymers to two ovarian cancer cell lines, SKOV-3 (Figs. 4a and 4c) and OVCAR-3 (Figs. 4b and 4d). Except for LPEI-C₃-G₁₀₀ that showed some toxicity at high doses, other GPEIs exhibited almost no cytotoxicity to SKOV-3 cells even at the dose that LPEI_{10k} killed about 70% of cells.

In vitro Transfection

The *in vitro* transfection of GLPEIs was tested using pGL4-luc as the reporter gene. LPEI_{25k} polyplexes (N/P = 10) were used as a standard control, and the N/P ratios of the GLPEI and native LPEI_{10k} polyplexes ranged from 5 to 20. As shown in Fig. 5(a), at an N/P of 5, the luciferase transfection of the polyplexes in OVCAR-3 cells increased sharply as LPEI_{10k} was guanidinoamidized. Introducing 50 guanidine groups to the LPEI chain already increased the gene transfection efficiency by two or three orders of magnitude compared to that of the LPEI_{10k} polyplexes. At higher N/P ratios (*e.g.* 10 or 20), low degree of guanidinoamidization was sufficient to reach similar gene transfection efficiency as high guanidinoamidization degree. Similarly, LPEI-C₅-G_n had substantially increased transfection efficiency (10 to 100 times) compared to LPEI_{10k} at N/P ratios of 10 and 20 (Fig. 5b). At low N/P ratio (*e.g.* 5), LPEI-C₃-G_n was more efficiently in gene transfection than LPEI-C₅-G_n. Noteworthy, LPEI_{25k}-C₃-G₃₀₀ had only one-tenth transfection efficiency of original LPEI_{25k} (Fig. 5a), possibly due to the insufficient release of pDNA from LPEI_{25k}-C₃-G₃₀₀ polyplexes. It had been reported that, ten-fold increase in transfection efficiency of chitosan was observed after complete substitution of its primary amines with guanidine^[40, 41]. In a biodegradable di-block polymer a high guanidine-containing one (over 65%) gave better transfection efficiency than the low guanidine-containing one. Therefore, it seemed that in those systems fully or almost fully guanidine substitution was needed for high transfection. In this study, conjugation with 100 guanidinoamidized side chains out of 230 ethyleneimine repeating units, or 42% guanidine degree, gave the most efficacious transfection on OVCAR-3 cells (Fig. 5a).

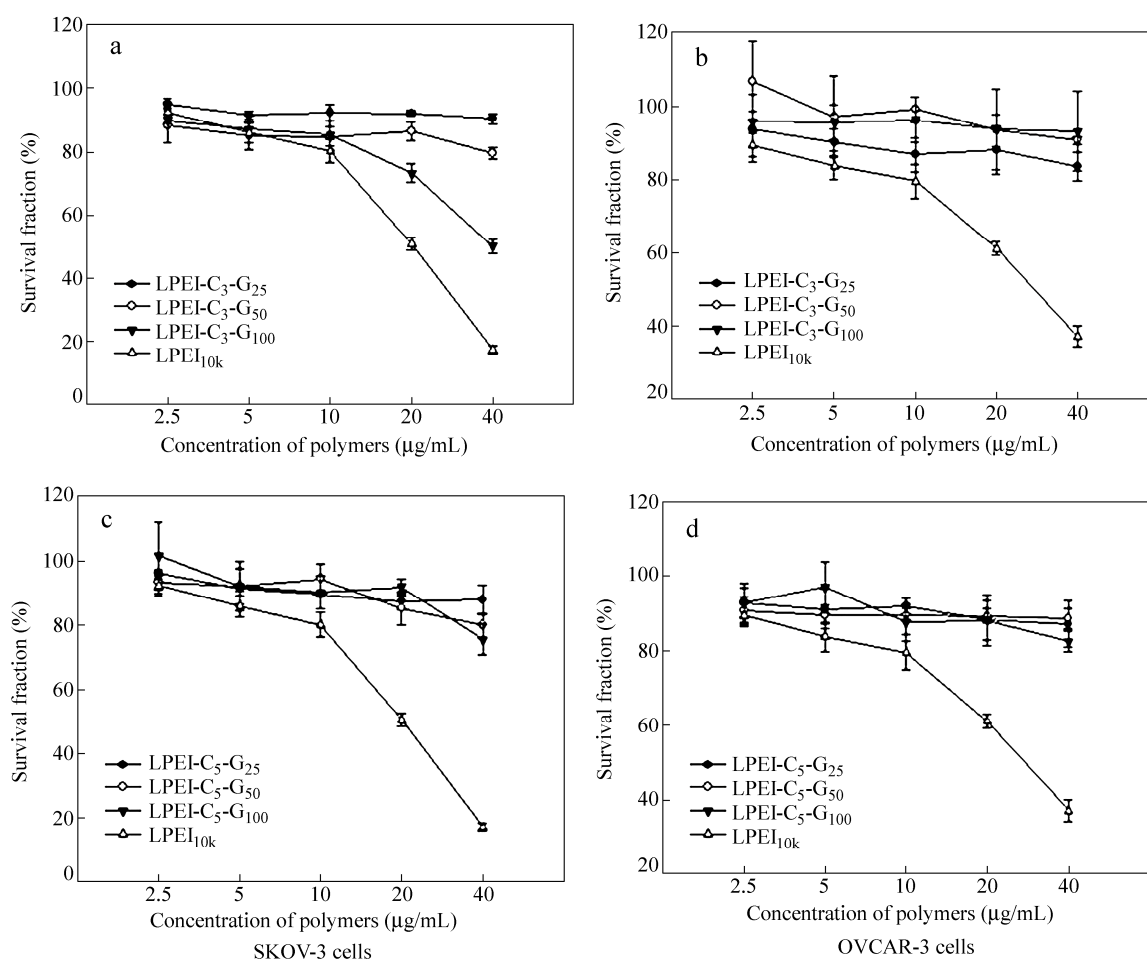


Fig. 4 The cytotoxicity of GLPEI and LPEI_{10k} to SKOV-3 cells (a and c) and OVCAR-3 cells (b and d) at various polymer concentrations. Cells were treated with polymers for 4 h, and incubated with complete medium for another 44 h before MTT assay.

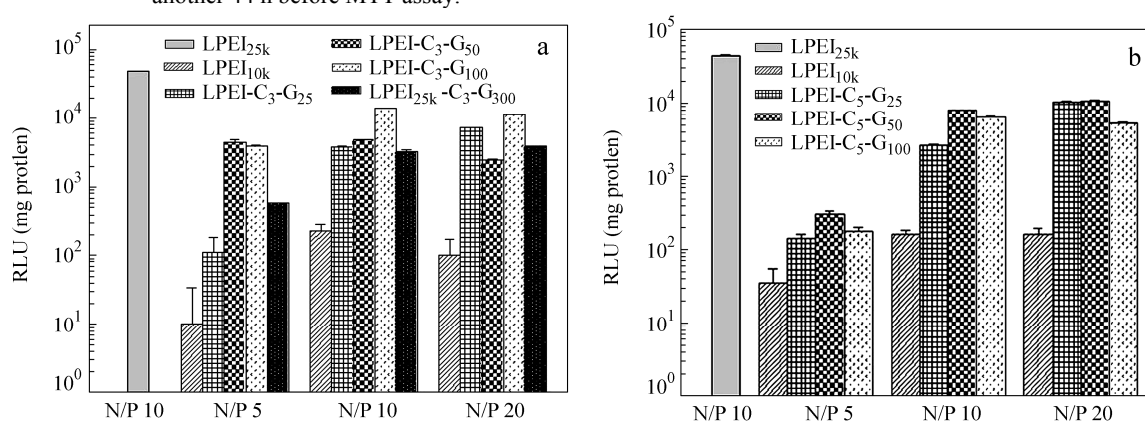


Fig. 5 The transfection efficiency of LPEI-C₃-G_n (a), LPEI-C₅-G_n (b), LPEI_{10k} and LPEI_{25k} polyplexes on OVCAR-3 cells.

Polyplexes solution (50 μL) prepared in FBS-free medium at a pDNA dose of 10 μg/mL was added to each well and cultured for 4 h. Cells were incubated for another 44 h before luciferase analysis and protein concentration measurement.

Cellular Uptake and Mechanism Exploration

The cellular uptake mechanisms of GLPEI polyplexes were studied by selectively blocking the known endocytosis pathways. The transfection efficiencies of LPEI-C₃-G₂₅, LPEI-C₃-G₁₀₀ and LPEI_{25k} polyplexes decreased to 25% or less at 4 °C of those at 37 °C, indicating an energy dependent endocytosis mechanism (Fig. 6a). Furthermore, the endocytosis pathways were probed using two types of inhibitors (Fig. 6b). Pre-treatment of cells with chlorpromazine did not decrease, but slightly increased the transfection efficiency of LPEI-C₃-G₂₅ and LPEI_{25k} polyplexes. Meanwhile, pre-treatment of cells with Filipin III reduced the transfection efficiency to 10% or less of the control. The confocal laser scanning microscopy (CLSM) results also confirmed the effect of Filipin III on OVCAR-3 cells. Compared with the cells without inhibitors (Fig. 6c), visualized red dots (Cy5-pDNA) dramatically decreased in the cells pre-treated with Filipin III (Fig. 6d). These results confirmed that the cellular uptake of LPEI-C₃-G_x and LPEI_{25k} polyplexes was mainly through caveolae-dependent pathway, which was agreeable to other reports^[42]. Therefore, the guanidinoamidization of LPEI did not change its polyplexes' endocytosis pathway. More importantly, caveolae-dependent pathway was preferred for gene delivery, since the cargos might not be fused to lysosomes and thus avoided lysosomal degradation^[43]. This explained the increase in gene transfection after clathrin-dependent pathway was blocked because of the reinforced caveolae-mediated endocytosis pathways^[44]. Therefore, it was potentially advantageous for GLPEIs to achieve high transfection efficiency by escaping enzymatic degradation in lysosomes.

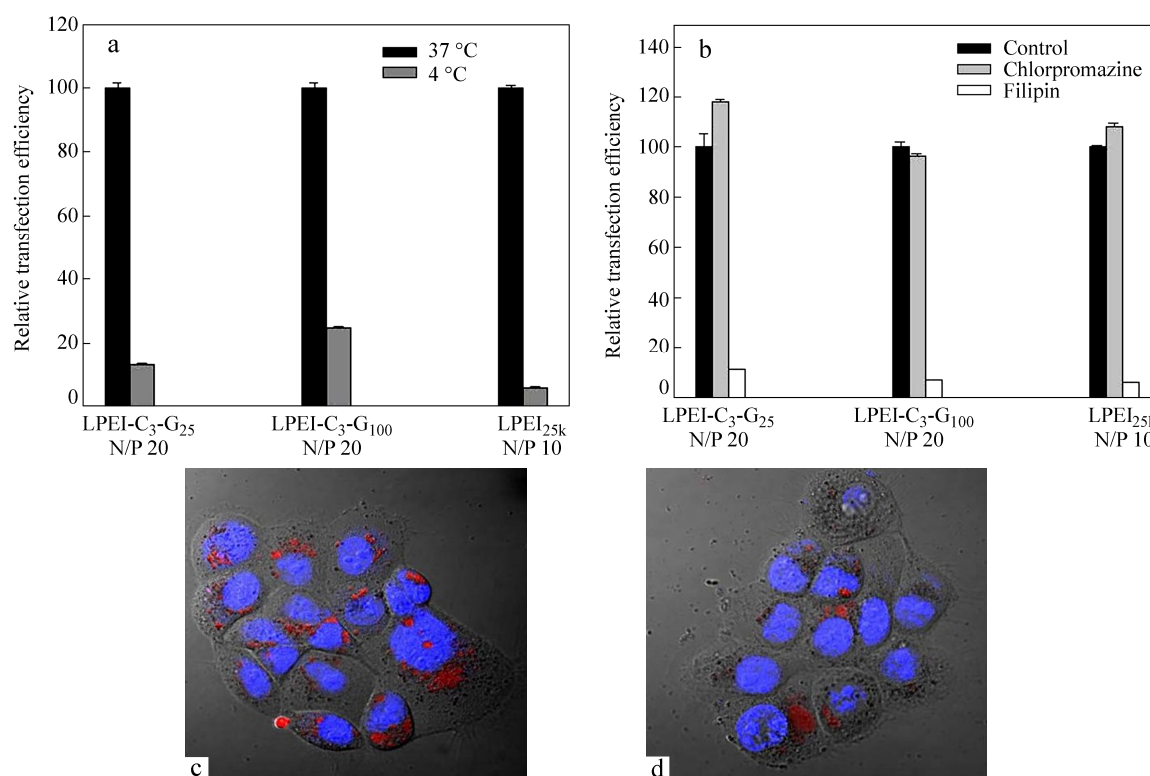


Fig. 6 (a) The influence of temperature on transfection efficiency of GLPEI and LPEI_{25k} polyplexes; (b) The transfection efficiency of the polyplexes of LPEI-C₃-G₂₅ (N/P = 20), LPEI-C₃-G₁₀₀ (N/P = 20) and LPEI_{25k} (N/P = 10) in the presence of chlorpromazine (10 µg/mL) and Filipin III (5 µg/mL) respectively; Intracellular uptake of LPEI-C₃-G₁₀₀ polyplexes (Cy5-pDNA, Red) with (d) or without (c) Filipin III (The concentration of pDNA was 10 µg/mL. Cell nucleus were stained by acridine orange (blue).)

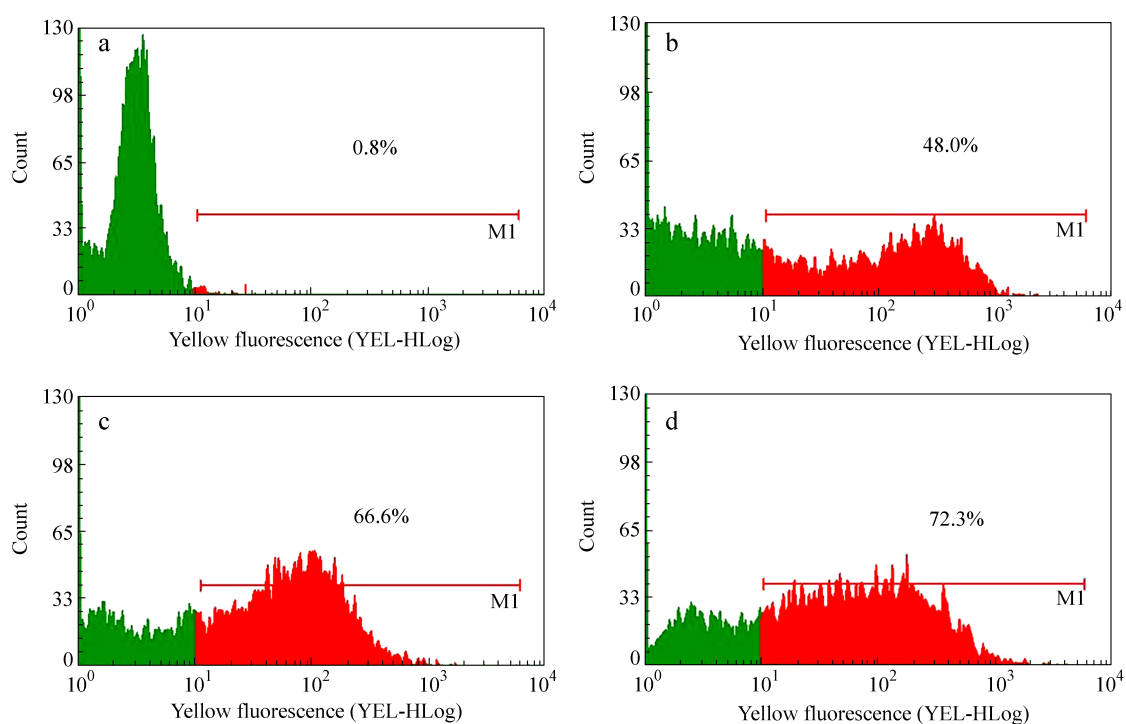


Fig. 7 Quantification of cellular uptake by flow cytometry: (a) OVCAR-3 cells as control and the cells treated with polyplexes of (b) FITC-LPEI_{10k} (N/P = 20), (c) FITC-LPEI_{25k} (N/P = 10) and (d) FITC-LPEI-C₃-G₁₀₀ (N/P = 20) at a pDNA dose of 10 μ g/mL and analyzed after 4 h incubation

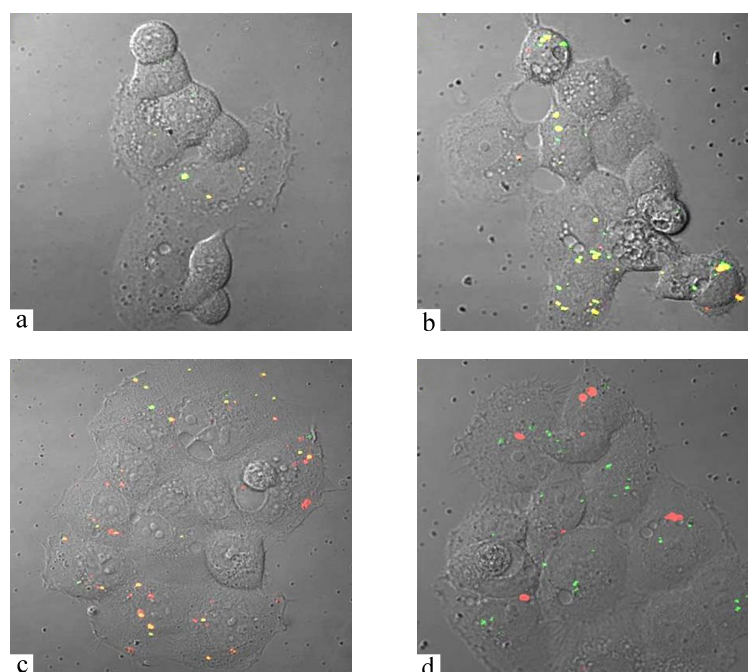


Fig. 8 Observation of intracellular localization of FITC-polymer (green) and Cy5-pDNA (red) polyplexes: The FITC-LPEI-C₃-G₁₀₀ (N/P = 20, 10 μ g/mL pDNA) polyplexes were incubated with OVCAR-3 cells for 2 h (a), 4 h (b) and 6 h (c) respectively; The FITC-LPEI_{25k} polyplex (N/P = 10, 10 μ g/mL pDNA) incubated with OVCAR-3 cells for 4 h before observation (d)

We also found that guanidinoamidization greatly improved the cellular uptake of LPEI-C₃-G₁₀₀ polyplexes compared to the original LPEI_{10k} polyplexes. Moreover, LPEI-C₃-G₁₀₀ polyplexes were taken up by OVCAR-3 cells even more rapidly than LPEI_{25k} polyplexes as quantitatively determined by flow cytometry (Fig. 7). Interestingly, although the cellular uptake of LPEI-C₃-G₁₀₀ polyplexes was faster than that of the LPEI_{25k} polyplexes, its transfection efficiency was slightly lower than that of LPEI_{25k} polyplexes. To explore the underlying reason, we observed the intracellular localization of both Cy5-pDNA (red) and FITC-polymer (green) in cells at different treatment times (Fig. 8). Yellow dots suggested the overlap of pDNA with the polymers, representing the intact structure of the polyplexes. The LPEI-C₃-G₁₀₀ polyplexes inside the cells were mostly intact within 4 h, and the polyplexes partially disassembled after 6 h incubation (Fig. 8c). Whereas, LPEI_{25k} and pDNA completely disassociated at 4 h incubation (Fig. 8d), which was consistent with the previous report that PEI polyplexes could be fully unpacked after 2 h incubation^[45]. Although the binding of GLPEI and pDNA seemed too strong to disassociate at the desired position and hinder higher *in vitro* transfection efficiency, it could be potentially promising in the *in vivo* experiments as the strong pDNA binding ability of GLPEIs may avoid the undesired dissociation of the polyplexes in the blood stream^[46].

CONCLUSIONS

In this study, we conjugated low molecular weight LPEI with guanidine groups to increase its transfection efficiency and meanwhile minimize the toxicity. The resulting GLPEIs had much lesser toxicity than native LPEI_{10k} at the doses of transfection. The formed GLPEI polyplexes were about ~100 nm with positive surface charges. These GLPEI polyplexes had preferable caveolae-dependent endocytosis, which could possibly bypass lysosomal degradation because of the neutral pH in caveosomes. Conjugation with guanidine groups enhanced LPEI's binding affinity to pDNA, as demonstrated by heparin competitive assay and EB exclusion assay. Moreover, these GLPEI polyplexes had greatly improved cellular uptake and increased gene transfection efficiency compared to native LPEI_{10k}, and their transfection efficiencies were even comparable to LPEI_{25k}. Among these GLPEIs, LPEI-C₃-G₁₀₀ polyplexes could more effectively accumulate into cytoplasm than LPEI_{25k} polyplexes although the transfection efficiency of LPEI-C₃-G₁₀₀ polyplexes was not superior to that of LPEI_{25k} polyplexes, which would be probably attributed to the more efficient release of LPEI_{25k} polyplexes than LPEI-C₃-G₁₀₀ polyplexes in the cytoplasm.

REFERENCES

- 1 Vercauteren, D., Rejman, J., Martens, T.F., Demeester, J., De Smedt, S.C. and Braeckmans, K.J., *Control. Release*, 2012, 161: 566
- 2 McCarthy, H.O., McCaffrey, J., McCrudden, C.M., Zholobenko, A., Ali, A.A., McBride, J.W., Massey, A.S., Pentlavalli, S., Chen, K.H., Cole, G., Loughran, S.P., Dunne, N.J., Donnelly, R.F., Kett, V.L. and Robson, T., *J. Control. Release*, 2014, 189: 141
- 3 Xu, C.H., Sui, M.H., Tang, J.B. and Shen, Y.Q., *Chinese J. Polym. Sci.*, 2011, 29(3): 274
- 4 Jang, Y.L., Ku, S.H., Lee, S.J., Park, J.H., Kim, W.J., Kwon, I.C., Kim, S.H. and Jeong, J.H., *J. Nanosci. Nanotechnol.*, 2014, 14: 7388
- 5 Jiang, X.L., Chu, Y.F., Liu, J., Zhang, G.Y. and Zhuo, R.X., *Chinese J. Polym. Sci.*, 2011, 29(4): 421
- 6 Wang, F., Wang, Y., Wang, H., Shao, N., Chen, Y. and Cheng, Y., *Biomaterials*, 2014, 35: 9187
- 7 Bao, X., Wang, W., Wang, C., Wang, Y., Zhou, J., Ding, Y., Wang, X. and Jin, Y., *Biomaterials*, 2014, 35: 8450
- 8 Du, J., Shi, Q.S., Sun, Y., Liu, P.F., Zhu, M.J., Du, L.F. and Duan, Y.R., *J. Gene Med.*, 2011, 13: 312
- 9 Canine, B.F. and Hatefi, A., *Adv. Drug Deliver. Rev.*, 2010, 62: 1524
- 10 Levine, R.M., Scott, C.M. and Kokkoli, E., *Soft Matter*, 2013, 9: 985
- 11 Duncan, R. and Richardson, S.C.W., *Mol. Pharmaceut.*, 2012, 9: 2380

- 12 Yamano, S., Dai, J., Hanatani, S., Haku, K., Yamanaka, T., Ishioka, M., Takayama, T., Yuvienco, C., Khapli, S., Moursi, A.M. and Montclare, J.K., *Biomaterials*, 2014, 35: 1705
- 13 Wang, M., Tucker, J.D., Lu, P., Wu, B., Cloer, C. and Lu, Q., *Bioconjugate Chem.*, 2012, 23: 837
- 14 Duan, X., Xiao, J., Yin, Q., Zhang, Z., Mao, S. and Li, Y., *Int. J. Nanomed.*, 2012, 7: 4961
- 15 Aravindan, L., Bicknell, K.A., Brooks, G., Khutoryanskiy, V.V. and Williams, A.C., *Macromol. Biosci.*, 2013, 13: 1163
- 16 Muthiah, M., Park, I.K. and Cho, C.S., *Expert Opin. Drug Del.*, 2013, 10: 1259
- 17 Todorova, N., Chiappini, C., Mager, M., Simona, B., Patel, I.I., Stevens, M.M. and Yarovsky, I., *Nano Lett.*, 2014, 14: 5229
- 18 Sakuma, S., Suita, M., Yamamoto, T., Masaoka, Y., Kataoka, M., Yamashita, S., Nakajima, N., Shinkai, N., Yamauchi, H., Hiwatari, K.I., Hashizume, A., Tachikawa, H., Kimura, R., Ishimaru, Y., Kasai, A. and Maeda, S., *Eur. J. Pharm. Biopharm.*, 2012, 81: 64
- 19 Tung, C.H. and Weissleder, R., *Adv. Drug Del. Rev.*, 2003, 55: 281
- 20 Mann, A., Shukla, V., Khanduri, R., Dabral, S., Singh, H. and Ganguli, M., *Mol. Pharmaceut.*, 2014, 11: 683
- 21 Martin, I., Teixido, M. and Giralt, E., *Curr. Pharm. Design*, 2013, 19: 2924
- 22 Futaki, S., Hirose, H. and Nakase, I., *Curr. Pharm. Design*, 2013, 19: 2863
- 23 Oh, D., Shirazi, A.N., Northup, K., Sullivan, B., Tiwari, R.K., Bisoffi, M. and Parang, K., *Mol. Pharmaceut.*, 2014, 11: 2845
- 24 Theodossiou, T.A., Pantos, A., Tsogas, I. and Paleos, C.M., *ChemMedChem*, 2008, 3: 1635
- 25 Son, S.J., Yu, G.S., Choe, Y.H., Kim, Y.J., Lee, E., Park, J.S. and Choi, J.S., *B. Korean Chem. Soc.*, 2013, 34: 579
- 26 Nimesh, S. and Chandra, R., *Eur. J. Pharm. Biopharm.*, 2008, 68: 647
- 27 Cheng, Q., Huang, Y., Zheng, H., Wei, T., Zheng, S., Huo, S., Wang, X., Du, Q., Zhang, X., Zhang, H.Y., Liang, X.J., Wang, C., Tang, R. and Liang, Z., *Biomaterials*, 2013, 34: 3120
- 28 Zhai, X., Sun, P., Luo, Y., Ma, C., Xu, J. and Liu, W., *J. Appl. Polym. Sci.*, 2011, 121: 3569
- 29 Peng, L., Liu, M., Xue, Y.N., Huang, S.W. and Zhuo, R.X., *Biomaterials*, 2009, 30: 5825
- 30 Brissault, B., Kichler, A., Leborgne, C., Danos, O., Cheradame, H., Gau, J., Auvray, L., and Guis, C., *Biomacromolecules*, 2006, 7: 2863
- 31 Ou, M., Xu, R., Kim, S.H., Bull, D.A. and Kim, S.W., *Biomaterials*, 2009, 30: 5804
- 32 Zern, B.J., Chu, H., Osunkoya, A.O., Gao, J. and Wang, Y., *Adv. Funct. Mater.*, 2011, 21: 434
- 33 Yu, G.S., Bae, Y.M., Kim, J.Y., Han, J., Ko, K.S. and Choi, J.S., *Macromol. Res.*, 2012, 20: 1156
- 34 Carlson, P.M., Schellinger, J.G., Pahang, J.A., Johnson, R.N. and Pun, S.H., *Biomater. Sci.-UK*, 2013, 1: 736
- 35 Kim, T.I., Rothmund, T., Kissel, T. and Kim, S.W., *J. Control. Release*, 2011, 152: 110
- 36 Liu, B.R., Lin, M.D., Chiang, H.J. and Lee, H.J., *Gene*, 2012, 505: 37
- 37 Pack, D.W., Hoffman, A.S., Pun, S. and Stayton, P.S., *Nat. Rev. Drug Discov.*, 2005, 4: 581
- 38 Shen, J., Kim, H.C., Su, H., Wang, F., Wolfram, J., Kirui, D., Mai, J., Mu, C., Ji, L.N., Mao, Z.W. and Shen, H., *Theranostics*, 2014, 4: 487
- 39 Morris, V.B. and Sharma, C.P., *Biomaterials*, 2010, 31: 8759
- 40 Ryu, K., Kim, K. and Kim, T.I., *Macromol. Res.*, 2014, 22: 264
- 41 Beloor, J., Choi, C.S., Nam, H.Y., Park, M., Kim, S.H., Jackson, A., Lee, K.Y., Kim, S.W., Kumar, P. and Lee, S.K., *Biomaterials*, 2012, 33: 1640
- 42 El-Sayed, A. and Harashima, H., *Mol. Ther.*, 2013, 21: 1118
- 43 Nassoy, P. and Lamaze, C., *Trends. Cell Biol.*, 2012, 22: 381
- 44 Yao, J., Fan, Y., Li, Y. and Huang, L., *J. Drug Target.*, 2013, 21: 926
- 45 Lee, H., Kim, I.K. and Park, T.G., *Bioconjugate Chem.*, 2010, 21: 289
- 46 Yin, H., Kanasty, R.L., Eltoukhy, A.A., Vegas, A.J., Dorkin, J.R. and Anderson, D.G., *Nat. Rev. Genet.*, 2014, 15: 541