Poly(L-lysine)-Graft-Chitosan Copolymers: Synthesis, Characterization, and Gene Transfection Effect

Haijun Yu,[†,‡] Xuesi Chen,[†] Tiancheng Lu,[†,‡] Jing Sun,[†,‡] Huayu Tian,[†] Jun Hu,[†,‡] Yu Wang,[†] Peibiao Zhang,[†] and Xiabin Jing*[,†]

State Key Laboratory of Polymer Physics and Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, People's Republic of China, and Graduate School of Chinese Academy of Sciences, Beijing 100039, People's Republic of China

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Polypeptide/polysaccharide graft copolymers poly(L-lysine)-graft-chitosan (PLL-g-Chi) were prepared by ring-opening polymerization (ROP) of ϵ-benzoxycarbonyl L-lysine *N*-carboxyanhydrides (Z-L-lysine NCA) in the presence of 6-*O*-triphenylmethyl chitosan. The PLL-g-Chi copolymers were thoroughly characterized by ^{1}H NMR, ^{13}C NMR, Fourier transform infrared (FT-IR), and gel permeation chromatography (GPC). The number-average degree of polymerization of PLL grafted onto the chitosan backbone could be adjusted by controlling the feed ratio of NCA to 6-*O*-triphenylmethyl chitosan. The particle size of the complexes formed from the copolymer and calf thymus DNA was measured by dynamic light scattering (DLS). It was found in the range of 120∼340 nm. The gel retardation electrophoresis showed that the PLL-g-Chi copolymers possessed better plasmid DNA-binding ability than chitosan. The gene transfection effect in HEK 293T cells of the copolymers was evaluated, and the results showed that the gene transfection ability of the copolymer was better than that of chitosan and was dependent on the PLL grafting ratio. The PLL-g-Chi copolymers could be used as effective gene delivery vectors.

Introduction

Gene therapy is promising for curing various inherited or acquired diseases. However, lack of safe and efficient DNA carriers is a main hurdle to the success of gene therapy. Great concerns about the safety of viral vectors make nonviral vectors more and more attractive.[1] Nonviral delivery systems for gene therapy have been increasingly proposed as safer alternatives to viral vectors since they have the potential to be administered repeatedly with minimal host immune response, and they are stable in storage and are easy to produce in large quantities.[2,3] Among numerous nonviral systems, cationic polymers have gained increasing attention because they can form polyelectrolyte complexes with plasmid DNA through charge interaction and self-assembling.[4] Several polycations have been reported to be used for gene transfection, including poly(L-lysine) (PLL),[5] polyethylenimine (PEI),[6] polyamidoamine dendrimers,[7] polyphosphoramidate,[8] poly(β-amino ester),[9] and chitosan.[10−14] These polymers mediate transfection via condensing DNA into nanoparticles, protecting DNA from enzymatic degradation, and facilitating the cellular uptake and endosomal escape of the complexes.[8]

PEI is generally accepted as one of the most efficient polymeric delivery vehicles.[15] Its high density of primary and secondary amines allows effective nucleic acid binding and compaction.[16,17] Higher molecular weight PEI (e.g., 25 kDa) displays effective intracellular gene delivery in almost all cell lines.[18] Unfortunately, it is in this efficacious molecular weight range that the PEI exhibits high cytotoxicity in most cases and its non-biodegradability may bring some circulatory cellular toxicities or organ damage to some extent. These shortcomings have inhibited its clinical development.[18−20]

In the past, PLL has been widely used in gene delivery because its structure facilitates various modifications of the polymer including conjugation with hydrophilic or amphiphilic polymers.[21,22] However, it is well-known that its transfection efficiency is low.[20,23,24]

Chitosan is also considered as a good candidate for the gene delivery system since it is already known as a biocompatible, biodegradable, and low toxic material with high cationic potential.[25] However, in spite of these excellent characteristics, chitosan always exhibits low transfection efficiency. Inefficient release of the DNA/chitosan complex from endocytic vesicles into the cytoplasm is one of the primary causes of its poor gene delivery.[13,26,27]

In the present study, to combine the strong cationic property of PLL and the biocompatibility and low toxicity of chitosan, great attempts were made to prepare biodegradable PLL-graft-chitosan copolymers. Kurita and co-workers have reported that poly(γ-methyl L-glutamate)-graft-chitin was prepared by ring-opening polymerization (ROP) of an α-amino acid *N*-carboxyanhydride (NCA) initiated by the free amino groups of water-soluble chitin.[28] However, the ROP of most kinds of NCA is not easy to control in a heterogeneous aqueous system. So, solubilization of the rigid chitosan in organic solvents is critical to obtain high degrees of substitution and to have control over its modification reactions under a homogeneous condition.[29,30] Kurita et al. have developed 6-*O*-triphenylmethyl chitosan as an intermediate for chitosan modification,[31] and recently this intermediate has been proved to be effective in *N*-acetylation because of its good solubility in several kinds of organic solvents.[30,32] So, we tried to synthesize the PLL-graft-chitosan copolymers through ROP of Z-L-lysine NCA initiated by 6-*O*-

* To whom correspondence should be addressed. Tel: +86-431-85262775; fax: +86-431-85685653.; E-mail address: xbjing@ciac.jl.cn. (Jing, X. B.).

† Changchun Institute of Applied Chemistry, Chinese Academy of Sciences.

‡ Graduate School of Chinese Academy of Sciences.

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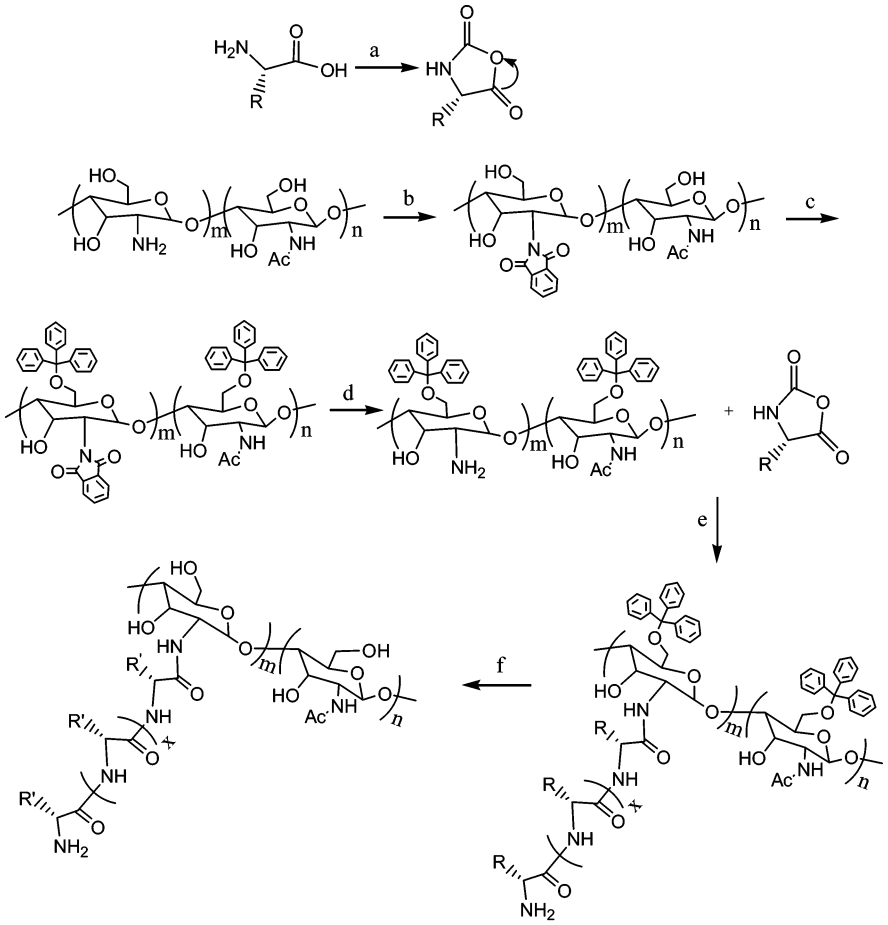
Scheme 1. Synthetic Route of the PLL-g-chitosan Copolymers



R=cbz protected ε-amine; R'= deprotected ε-amine.

a: $(CCl_3O)CO$, THF, 55 °C, 2 h; b: phthalic anhydride, DMF, 90 °C, 8 h; c: TrCl, pyridine, 90 °C, 24 h; d: $NH_2NH_2{\cdot}H_2O$, 100 °C, 15 h; e: DMAc, RT, 72 h; f: TFA, HBr/HAc, 0 °C, 30 min.

triphenylmethyl chitosan. The potential of the obtained copolymers to serve as a gene carrier was also explored.

Materials and Methods

Materials. Chitosan (Jinke Biochemical Co. Ltd. Zhejiang, China) was used directly. Its number-average molecular weight (M_n), weight average molecular weight (M_w), and polydispersity (M_w/M_n), determined by gel permeation chromatography (GPC), were 64 400 Da, 238 000 Da, and 3.70, respectively. Its deacetylation degree determined by ^1H NMR was 90%. Pyridine and THF were distilled over freshly powdered calcium hydride (CaH_2) and were distilled under ambient pressure. DMSO, DMF, and DMAc were dried over CaH_2 and were distilled under a reduced pressure. Regenerated cellulose membranes with a cutoff of 7000 (Sigma) were used for dialysis. 3-(4,5-Dimethylthiozol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) supplied by Sigma was used witout further treatment. All other reagents were of analytical grade and were used directly without further treatment.

Methods. ^1H and ^{13}C NMR spectra were both recorded on a Bruker AVANCE DRX 400 spectrometer. The measurements were carried out at 303 K and the solvents of D_2O or DCl were used. Fourier transform infrared (FT-IR) spectra were recorded on KBr pellets with a Bruker Vertex 70 spectrometer. Elemental analyses were performed with a Vario EL elemental analyzer.

The molecular weights (M_w, M_n) and their distribution (M_w/M_n) of chitosan and the prepared graft copolymers were determined by a GPC system, which consists of a Waters 515 pump, an Ultrahydrogel TM 500 column, an Ultrahydrogel TM 250 column, and a Waters 2410 refractive index detector. A mobile phase of acetic acid (0.3 mol/L)/sodium acetate (0.2 mol/L) (pH 4.5) at a flow rate of 1 mL/min was used. Shodex standard P-82 Pullulan samples of known molecular weights were used as standards. All the calculated molecular weights were corrected using the refractive index increments of the sample and the standards.

Preparation of 6-*O*-Triphenylmethyl Chitosan (Tr-Chi). Tr-Chi was prepared by the same method as described in our previous study.[36]

Synthesis of ϵ-Benzoxycarbonyl L-Lysine (Z-L-Lysine) NCA. The Z-L-lysine NCA was synthesized according to the literature method.[20,33] Briefly, 10.0 g of Z-L-lysine (35.68 mmol) was dissolved in 100 mL of anhydrous THF. Following that, 6.0 g of triphosgene (20.24 mmol) was slowly added to the flask, and the reaction was carried out at 55 °C for 1 h. The solvent THF was evaporated in vacuum and the obtained solid was recrystallized three times by acetic ether and petroleum ether to obtain 6.5 g of dried product.

Synthesis of Poly(Z-L-lysine)-Graft-(6-*O*-Triphenylmethyl Chitosan) (PZLL-g-(Tr-Chi)) Copolymers. The PZLL-g-(Tr-Chi) copolymer was synthesized by (Tr-Chi)-initiating ROP of Z-L-Lysine NCA in DMAc. For example, 1.0 g of Tr-Chi (2.43 mmol) and 1.0 g of Z-L-lysine NCA (3.27 mmol) were dissolved in 30 mL of anhydrous DMAc. The reaction was continued for 72 h at room temperature. Then, the product was precipitated into diethyl ether and was dried to yield a white solid.

Preparation of Poly(L-lysine)-Graft-Chitosan (PLL-g-Chi) Copolymers. The obtained PZLL-g-(Tr-Chi) copolymers were deprotected

Table 1. Molecular Parameters of the PLL-g-Chi Copolymers[a]

	Molecular molecular weights by GPC			Grafting ratio of PLL determined by		DP_n
copolymers[a]	$M_w/10^3$	$M_n/10^3$	M_w/M_n	^{1}H NMR	elemental analysis[b]	^{1}H NMR
$PLL_{0.85}$-g-Chi	46.8	19.3	2.43	0.77	0.45	0.85
$PLL_{2.0}$-g-Chi	63.0	25.5	2.47	1.8	1.5	2.0
$PLL_{3.3}$-g-Chi	65.7	26.9	2.44	3.0	4.5	3.3
$PLL_{15.6}$-g-Chi	38.1	16.4	2.33	14.0	13.5	15.6

[a] The copolymers are coded by the DPn (number-average degree of polymerization) of PLL grafted onto chitosan backbone. [b] The measured C/N of chitosan is 5.29, and the theoretic C/N of PLL is 2.57. The C/N ratios of the graft copolymers are 4.69, 3.95, 3.27, and 2.85, respectively.

by HBr.[34] Typically, 1.0 g of the copolymer was dissolved in 15 mL of TFA, and 15 mL of HBr (33% in acetic acid) was added. After the reaction vessel was capped and stirred for 30 min in an ice bath, the reaction mixture was poured into diethyl ether to precipitate the PLL-g-Chi copolymer as the TFA salt. Then, the product was recovered by filtration and was dried in vacuum to yield a pale yellow or white solid. The solid was redissolved in distilled water and was dialyzed against phosphate buffer solution (PBS, pH 7.4) and distilled water each for 3 days to produce purified PLL-g-Chi copolymer.

Preparation of PLL Homopolymer. The PLL was prepared by ROP of Z-L-lysine NCA initiated by *n*-hexylamine (Aldrich, used as received) and by subsequent deprotection in the same procedure as described above. The number-average molecular weight (M_n 22 000 Da) of the obtained PLL was calculated from the ^{1}H NMR signals.

Buffering Capacity Determination of the Copolymers. The buffering capacity of the copolymer was determined by pH titration from high to low pH in a Hanna pH211 pH analyzer (Hanna Instruments Ltd, Italy).[35] The copolymer was dissolved in 0.15 mol/L NaCl to prepare solutions with a concentration of 0.5 mg/mL. Titrants of 1 mol/L HCl and 1 mol/L NaOH aqueous solutions were used. The buffering capacities of chitosan, PLL, and PEI were also determined for comparison. Because of the poor solubility of higher molecular weight chitosan in neutral or basic aqueous solution, a lower molecular weight chitosan (M_n 5400 Da, deacetylation degree 50%) was selected.[36] The PEI used was a commercial product from Alfa Aesar with M_w of 10 000 and linear structure.

Complex Size and Zeta-Potential Measurements. The commercially available calf thymus DNA (Sigma) was used to form complexes with the copolymers for particle size and zeta-potential measurement. The test copolymers and the DNA were dissolved in ultrapure water to make stock solutions with concentrations of 10 and 1.08 mg/mL, respectively. The solution of high molecular weight chitosan was prepared by dissolving the chitosan in an acetic acid aqueous solution (pH 4.5), and the pH of the chitosan solution was slowly adjusted to 6.0 with diluted sodium hydroxide solution. Both copolymers and DNA solutions were then diluted to the appropriate concentration depending on the required weight ratio of copolymer to DNA with a final complex concentration of 0.3 mg/mL.[37] The particle size and zeta-potential of the complexes were determined by a Zeta Potential Analyzer instrument (Brookhaven Instruments Corporation, Germany).

UV−Vis Absorption Spectra of the Copolymer/DNA Complexes. The UV absorption spectra of the complexes were recorded with a UV−vis spectroscopy instrument (PC-2401, Shimadzu), and the complexes were formed by calf thymus DNA with chitosan, PLL, and the copolymers in aqueous solution. The initial concentration of the DNA was fixed at 0.05 mg/mL. Three weight ratios of copolymer/DNA, that is, 30, 5, and 1, were selected for the measurements.

Atomic Force Microscopy (AFM) Measurement of the Polymers/DNA Complex Morphology. The AFM samples were prepared by coating a drop of the polymer/calf thymus DNA complex suspension onto a freshly treated single-crystal silicon wafer, followed by air-drying.

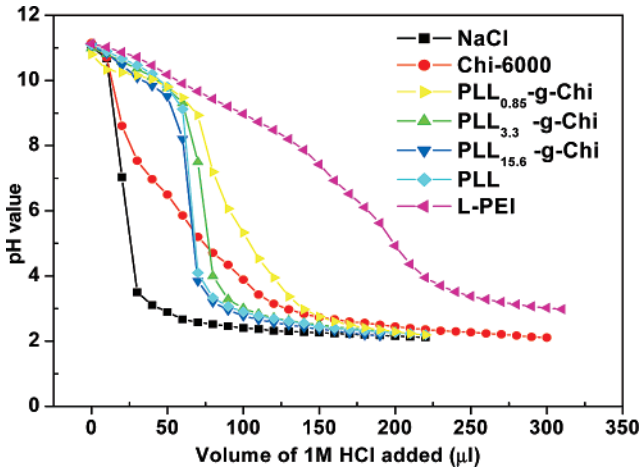


Figure 1. Buffering capacity of the PLL-g-chitosan copolymers.

Gel Electrophoresis Shift Assays. The plasmid DNA binding abilities of PLL-g-Chi copolymers and chitosan were examined by gel electrophoresis. Agarose gel (1.0%, w/v) containing ethidium bromide (0.06 μg/mL, Sigma) was prepared in TAE buffer (40 mmol/L Tris-acetate, 1 mmol/L EDTA). Ultrapure water was used as the solvent for all the copolymers and pDNA except for high molecular weight chitosan. The pEGFP-N_1 plasmid (Clontech Laboratories, Inc., United States) was extracted and purified with a Wizard purification system (Promega, United States). Three hundred nanograms of pDNA (3.0 μL, 100 ng/μL) was mixed with copolymer solutions to form complexes. The weight ratios of copolymer to pDNA were between 0 and 50, and the complexes were incubated for 30 min before the addition of loading buffer (1 μL, Blue Juice, Invitrogen, Carlsbad, CA). Then, the samples were subjected to gel electrophoresis at 60 V for 60 min.[21]

MTT Assay for Polymer Cytotoxicity. The common mouse fibroblast L929 cells were cultured onto a 96-well plate (1×10^4 cells/well) in complete DMEM (Dulbecco's modified eagle medium, Gibco) (with high glucose and 10% FBS (fetal bovine serum, from Gibco) supplemented) culture medium in a humidified atmosphere of 5% CO_2 at 37 °C for 24 h. The growth medium was replaced with 180 μL complete DMEM culture medium that contained the desired amount of the test polymer. The cells were incubated for another 24 h, and the cell viability was assayed by adding 20 μL of MTT (Sigma) PBS solution (5 mg/mL). After incubation at 37 °C for another 4 h, the formed crystals were dissolved in 150 μL of DMSO. The absorbance of each well was measured using a microplate reader (Stat Fax 2100, Awareness) at a test wavelength of 492 nm.[38]

In Vitro Gene Transfection. The cell line HEK 293T was selected for studying the gene transfection of the complexes. Before transfection, the test cells were seeded at a density of 6×10^3 cells/well onto 96-well tissue culture plates (Costar, Cambridge, United Kingdom) in complete DMEM culture medium, and the cells were incubated for 24 h in a humidified atmosphere of 5% CO_2 at 37 °C. The polymer solutions in ultrapure water and the pDNA solution in serum-free medium (Opti-MEM, pH 7.4, Gibco) were mixed at room temperature for 30 min to form the complexes, and the mixtures were then diluted to 45 μL with Opti-MEM. Each well of the cells was transfected with 15 μL of the complex solution after changing the fresh complete DMEM. The pDNA amount in each well was fixed at 300 ng. The weight ratios 5, 10, 15, and 20 of copolymer to pDNA were chosen. After 24 h incubation, the formulations were removed and 200 μL of complete DMEM culture medium was added. The experiments of studying gene transfection were continued to 72 h, and then the cells were analyzed for green fluorescence protein (GFP) expression with a fluorescence microscope (Nikon-2000U, Japan). After the cells were digested by trypsinase (Sigma, 0.05 wt % in PBS), the transfection

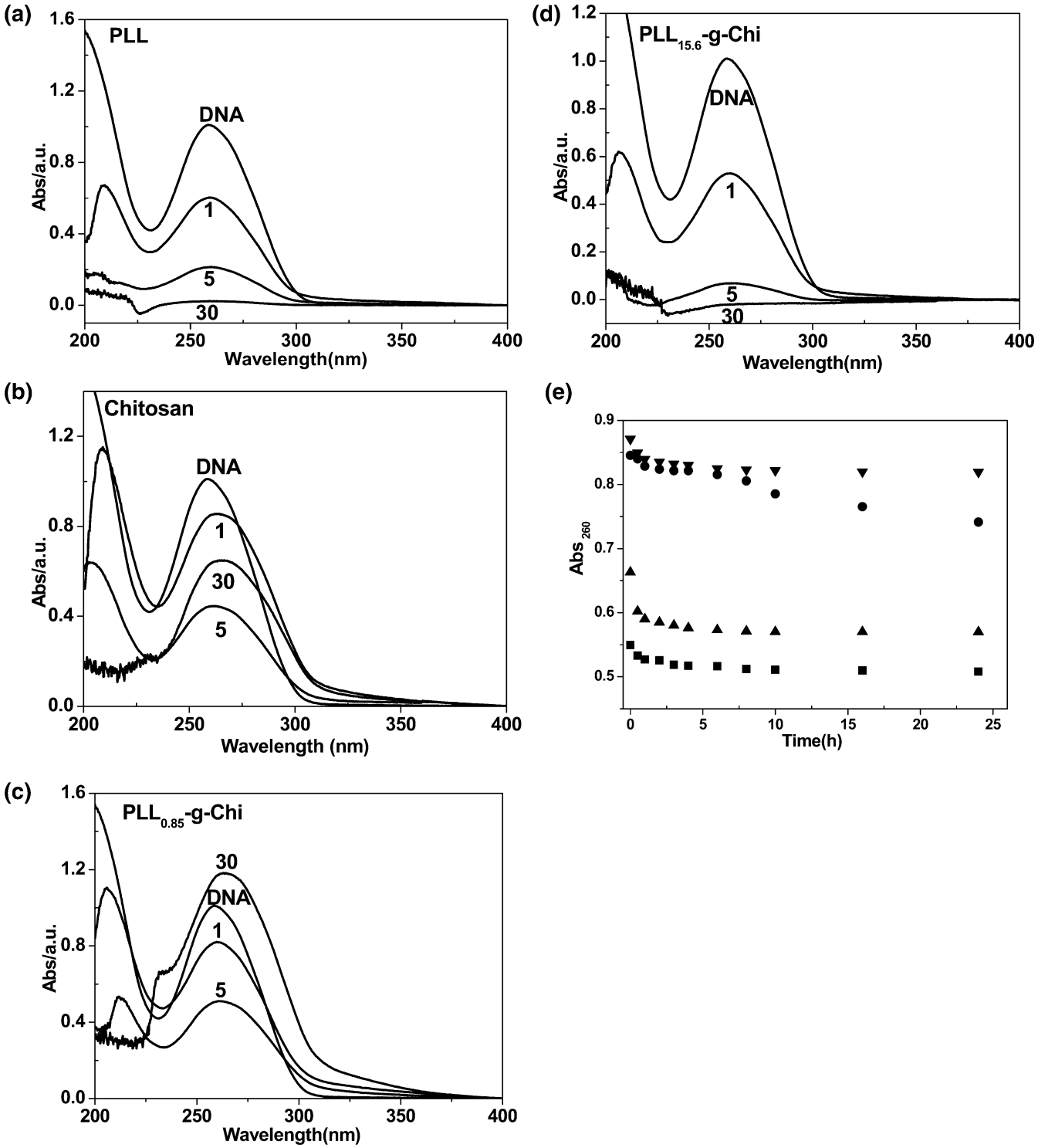


Figure 2. UV−vis absorption spectra of complexes in aqueous solution formed by polymers (chitosan, PLL, and copolymers) with calf thymus DNA at polymer/DNA weight ratios of 1, 5, and 30, respectively (a: PLL; b: chitosan; c: $PLL_{0.85}$-g-Chi; d: $PLL_{15.6}$-g-Chi). The spectra were recorded after the two solutions were mixed for 0.5 h. e: The spectra were recorded in a period of 0∼24 h after each polymer and DNA mixed at weight ratio of 1 (▼ chitosan/DNA; ● $PLL_{0.85}$-g-Chi/DNA; ▲ PLL/DNA; ■ $PLL_{15.6}$-g-Chi/DNA).

percents (positive cell percent) were calculated by dividing the number of fluorescent cells by the number of total cells in a certain area of a well.[39]

Fluorescence Assays Determining the Transfection Efficiency. The fluorescence assays were used to determine the GFP expression level. After the 293T cells were transfected by the complexes for 72 h, the cells were washed in PBS, and 150 μL of ultrapure water was added to each well. The GFP was extracted from the cells through three times of repeated freezing−thawing cycling treatment. Then, the solution was transferred into 96-well black microplates with clear plastic bottoms (Corning-Costar 3904, Cambridge, United Kingdom). The relative fluorescence intensity of the expressed GFP was determined by a FluoStar fluorescence plate reader (BMG Lab Technologies, Durham, United States), which was equipped with HQ500/20X (485 nm) excitation and HQ535/30M (520 nm) emission filters (Chroma, Rockingham, United States).[40]

Luciferase Assay Determining the Transfection Efficiency. The pGL3 plasmid (Promega, United States) was extracted and purified with a Wizard purification system. All the gene transfection procedures are the same as that mentioned previously. Seventy-two hours after

transfection, cell lysates were analyzed for luciferase activity with luciferase assay reagent (Promega, United States). For each sample, light units were integrated over 10 s with a luminometer (TD2020, Turner Designs, Sunnyvale, CA) and triplicate measurements were performed to get the average value.

Results and Discussion

Some reports claimed that chitosan of high molecular weight forms extremely stable complexes with pDNA compared to most of the other cationic polymers, which delays the release of pDNA from the pDNA/chitosan complexes, although chitosan itself and its derivatives have been used as gene carriers widely.[41−45] In the present study, PLL grafted chitosan copolymers were prepared through ROP of Z-L-lysine NCA, which was initiated by macroinitiator 6-*O*-triphenylmethyl chitosan under a homogeneous condition. To find a novel chitosan-based gene transfer agent with higher transfection efficiency, copolymers with different PLL grafting ratios and number-average degree of polymerization (DP_n) were designed.

Synthesis and Characterization of the PLL-Graft-Chitosan Copolymers. The synthesis route of the PLL-graft-chitosan copolymer is presented in Scheme 1. The amine groups of chitosan were first protected with phthalic anhydride. Then, the 6-CH_2OH groups on chitosan were reacted with triphenylchloromethane to get protected by triphenylmethyl. Finally, the amine groups were deprotected by hydrazine. The ROP reaction of Z-L-lysine NCA initiated by 6-*O*-triphenylmethyl chitosan was performed in DMAc at room temperature, followed by removal of the 6-*O*-triphenylmethyl and Z-groups with HBr (33 wt % in acetic acid solution). This method could be used for synthesizing other kinds of polypeptide-graft-chitosan copolymers.

FT-IR and NMR spectra were used to characterize the obtained copolymers. The peak at 1590 cm^{-1} in the FT-IR spectra of 6-*O*-triphenylmethyl chitosan (Tr-Chi) implied the presence of primary amine groups in Tr-Chi. After the ROP of Z-L-lysine NCA, the disappearance of the peak at 1590 cm^{-1} and the appearance of the peak at 1650 cm^{-1} belonging to the amide I band both indicated the formation of PZLL-g-(Tr-Chi). In the deprotection product, the peaks at 3100−3000 (C−H) and 703 cm^{-1} (arom) belonging to the triphenylmethyl disappeared completely, and the peak at 1735 cm^{-1} of ester bond in the Z-group also disappeared, suggesting complete removal of the chitosan and PLL protections. The obtained PLL-g-Chi copolymer showed the characteristic peak of CH_2 vibrations of PLL chains at 2944 cm^{-1}, the amide stretching vibration of PLL chains at 1650 cm^{-1}, and the typical peak of Chi at 1075 cm^{-1}, which proved that the PLL-g-Chi copolymer was synthesized successfully.

The grafting ratios of PLL in the final products, defined as molar ratio of the grafted lysine units to the total saccharide units in the chitosan, were determined by comparing the ^1H NMR signal integrals from ϵ-CH_2 protons of PLL with integrals of the polysaccharide backbone proton signals.[30] By assuming that all of the free amine groups present in 6-*O*-triphenylmethyl chitosan could initiate the ROP of Z-L-lysine NCA, the number-average degree of polymerization (DP_n) of each PLL graft can be calculated as

$$DP_n = \text{grafting ratio}/0.9$$

where factor 0.9 corresponded to the degree of deacylation in Chi, as determined by ^1H NMR. The results were collected in Table 1, and the DP_n values were used to distinguish different PLL-g-Chi's prepared.

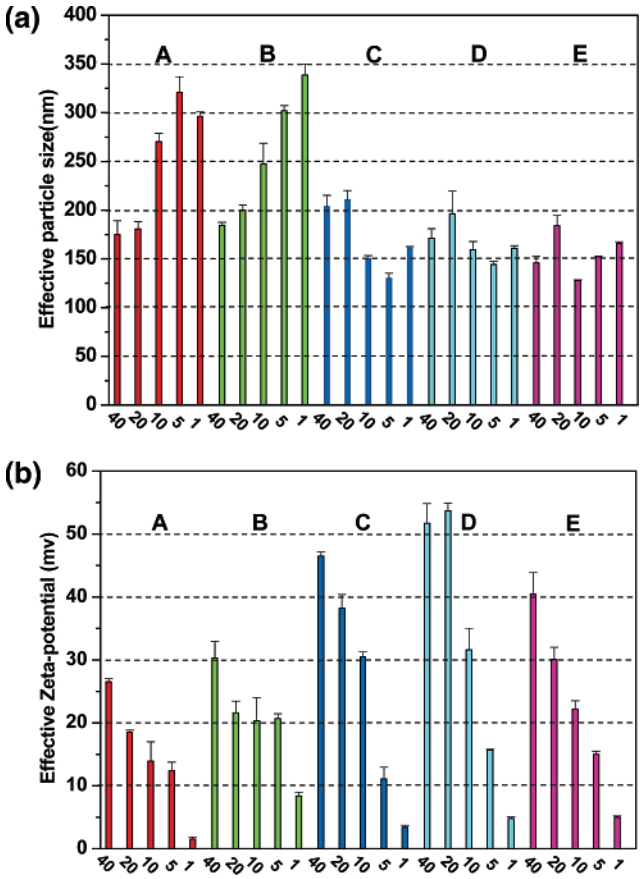


Figure 3. Effective particle size (a) and Zeta-potential (b) of the complexes formed by polymers with calf thymus DNA. A: chitosan; B: $PLL_{0.85}$-g-Chi; C: $PLL_{3.3}$-g-Chi; D: $PLL_{15.6}$-g-Chi; E: PLL (weight ratios of polymer to calf thymus DNA were 40, 20, 10, 5, and 1, respectively).

We also tried to figure out the composition of the copolymers by elemental analysis. The weight ratio of C to N in the copolymers decreased with the increase of PLL content because of the higher N content in PLL than that in chitosan, and this change was utilized to calculate the PLL grafting ratio (see Table 1). A consistency between the results calculated from ^1H NMR spectra and elemental analysis data was obtained. The molecular weights and polydispersity indexes of the starting material chitosan and the obtained copolymers were analyzed by GPC, and the results are shown in Table 1. A significant decrease in molecular weight of chitosan during the synthetic procedure was found. For example, the M_n of chitosan used was 64.4×10^3, and it decreased to 19.3×10^3 for $PLL_{0.85}$-g-Chi. This was mainly caused by the two reaction steps in aqueous environment, that is, *N*-phthalimido deprotection in a basic and 6-*O*-trityl and Z-deprotection in an acidic environment (see Scheme 1). Similar results have been reported that a significant decrease in the molecular weight of chitosan would occur with such a protection and deprotection strategy.[30] The M_n of the copolymers determined by GPC increased from 25.5×10^3 to 26.9×10^3 when the DP_n of PLL increased from 2.0 to 3.3. This is reasonable. However, the M_n of sample $PLL_{15.6}$-g-Chi became 16.4×10^3. Because all samples were prepared under the same conditions except the feed ratio of Z-L-lysine NCA to chitosan, the extents of degradation of the chitosan backbones in different samples should be similar, and higher DP_n should correspond to higher M_n. This unexpected low M_n of $PLL_{15.6}$-g-Chi was explained as follows: compared with the first three PLL-g-Chi's, $PLL_{15.6}$-

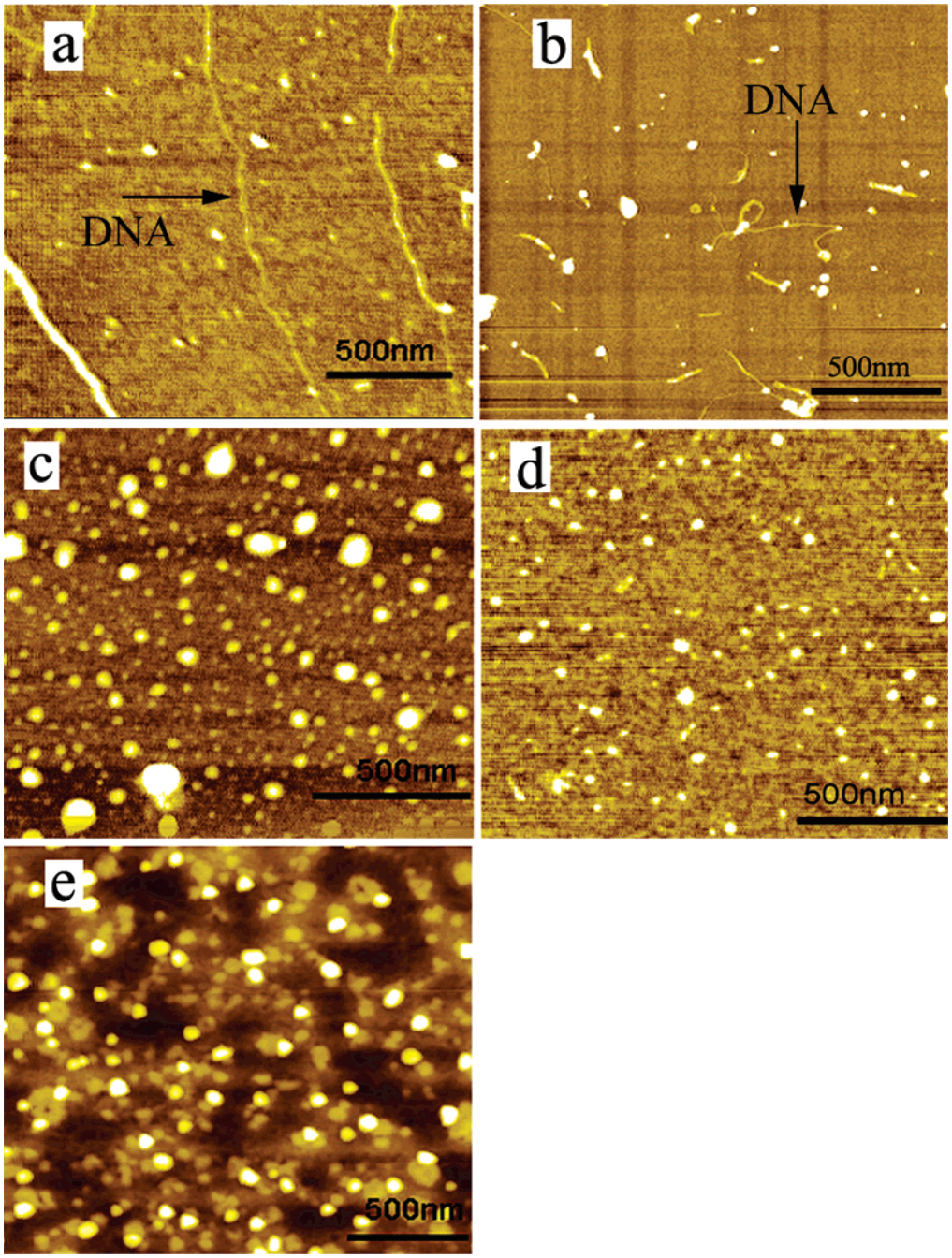


Figure 4. AFM images of polymer/DNA complexes at certain weight ratio of polymer to calf thymus DNA. a: calf thymus DNA; b: chitosan/DNA(10); c: $PLL_{3.3}$-g-Chi/DNA(10); d: $PLL_{15.6}$-g-Chi/DNA(5); e: PLL/DNA(5).

g-Chi was special in that its PLL grafts took more than 90% of the total molecular weight. Therefore, its interaction with the GPC column was different from that of the other three, causing longer retention time and lower M_n.

Buffering Capacity of the PLL-Graft-Chitosan Copolymers. An appropriate buffering capacity is important to a novel cationic polymer designed for gene delivery, because the buffering capacity of polycationic vectors affected the stability in cell culture medium, endolysis, and extents released from the endosomes of the polymer/pDNA complexes.[42] The buffering capacities of the obtained copolymers, linear PEI, water-soluble chitosan, and PLL were determined by an acid−base titration method, and a 0.15 mol/L NaCl aqueous solution was used as control. The polymers of high buffering ability would undergo a small change in pH when the same amount of HCl was added into the polymer solutions during titration.[46] As shown by the steepness of the curve slopes in Figure 1, PEI displays a high buffering ability over pH range of 11∼5, whereas chitosan displays a buffering range between pH 7 and 3.5, corresponding to the protonation of the primary and secondary amine groups on PEI and the primary amine groups (pK_a = 6.5) on chitosan, respectively. The PLL shows identical titration curve to PEI over pH 11∼9 but gives a steep slope below pH 9. It seems that the protonation of the ϵ-amine groups on PLL begins at pH 11 and completes at pH 9.[23] Among the four samples containing chitosan backbones, pure chitosan shows the lowest slope, $PLL_{15.6}$-g-Chi shows the steepest slope over pH 7∼3 (the curve overlaps with that of PLL), and $PLL_{0.85}$-g-Chi and $PLL_{3.3}$-g-Chi are in-between. This indicates that both chitosan backbones and PLL grafts make contributions to the pH buffering. Chitosan is responsible for the pH range of 7∼3 and PLL is responsible for the pH range of 11∼9.

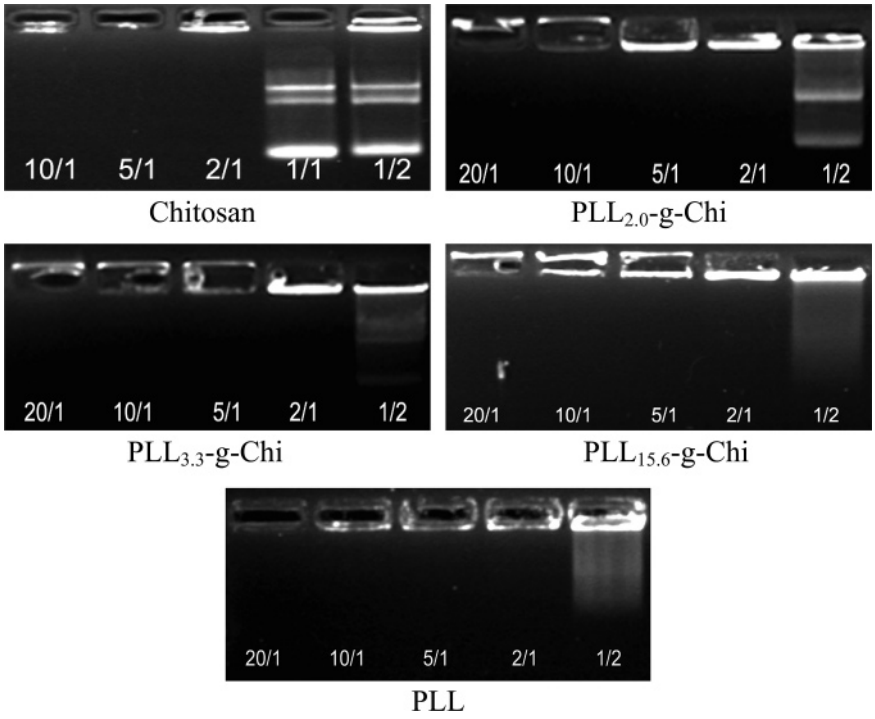


Figure 5. Gel electrophoresis shift assays for the pDNA binding ability of the copolymers at various copolymer/pDNA weight ratios.

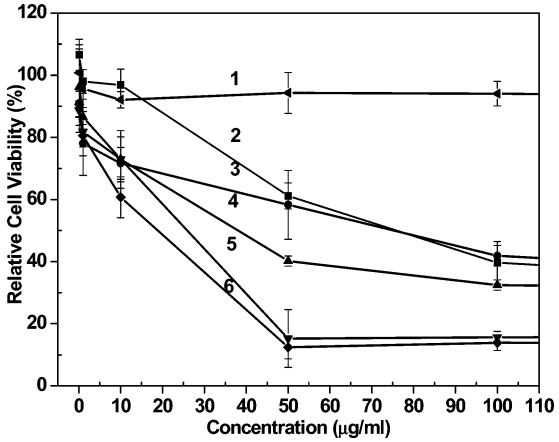


Figure 6. MTT assay for the cytotoxicity of chitosan (1), $PLL_{2.0}$-g-Chi (2), $PLL_{3.3}$-g-Chi (3), $PLL_{15.6}$-g-Chi (4), PLL (5), and B-PEI (25 k Da) (6) in L929 cells.

Interaction between the PLL-Graft-Chitosan Copolymers and DNA. In the present study, UV−vis spectra were used to investigate the interaction between the copolymer and DNA. The absorption spectra of copolymer/DNA complexes in solution were examined as a function of polymer/DNA weight ratio for polymers PLL and chitosan and PLL-g-Chi copolymers, and the results are shown in Figure 2. A broad absorption band centered at 260 nm can be found for free DNA. Its intensity is influenced by the complexation of DNA with cationic polymers. For example, when DNA is mixed with PLL at PLL/DNA weight ratio of 1, this peak is weakened. When this ratio becomes 5, it is weakened further. Also, the peak almost disappears when this ratio is continuously added to 30 (Figure 2a). For chitosan/DNA complexation, the peak intensity changes in a more complicated way. It decreases with increasing chitosan/DNA weight ratio from 0 through 1 to 5 but changes reversely when the ratio goes to 30 (Figure 2b). It is noticed that sample $PLL_{15.6}$-g-Chi behaves like pure PLL while $PLL_{0.85}$-g-Chi behaves like pure chitosan (Figure 2c, d). In the case of $PLL_{0.85}$-g-Chi, the peak intensity for weight ratio copolymer/DNA of 30 is even greater than that for pure DNA. It is well-known that when a DNA molecule is complexed with cationic polymer chains, its molecular chain gets compact. At the extreme case, solid small particles can be formed and can even separate from the solution because of the complexation of the negative DNAs and the cationic polymers. Obviously, because the same DNA concentration was used for all UV−vis measurements, the intensity change of the 260-nm peaks is a reflection of the molecular status of the DNA. Its lowering corresponds to the complexation and compaction of the DNA molecules. When the weight ratio of polymer/DNA goes higher from zero, more and more negative charges on DNA are neutralized by positive charges of the cationic polymers. Correspondingly, more and more cationic polymer chains come in contact with the DNA chains, and its molecular chains become more and more compact. Therefore, absorption of the DNA molecules decreases. If the electric charges are entirely neutralized, the most compactness of the DNA molecular chains can be achieved; solid particles can probably be formed and get suspended or separated from the solution. That is why the absorption of DNA is reduced or even diminishes. When too many cationic polymers are added to the system, the repulsion caused by the net positive charges leads to enlargement of the molecular size. Correspondingly, the 260-nm peak is enhanced because of scattering of the aggregates.[47] This phenomenon is observed for pure chitosan and $PLL_{0.85}$-g-Chi when the polymer/DNA weight ratio is 30 but not for pure PLL or $PLL_{15.6}$-g-Chi. The possibility is that at the ratio of 30, DNA chains are compressed by PLL or $PLL_{15.6}$-g-Chi more seriously than that operated by pure chitosan and $PLL_{0.85}$-g-Chi, and a more compact structure is formed. The stability of the complexes is weakened accordingly, so aggregation of the complexes occurs instantaneously after the mixing of DNA and copolymers, which is followed by depositing of the aggregates.

To examine the stability of the copolymer/DNA complexes formed, their UV−vis spectra were recorded at different time intervals in the time range from 0 to 24 h after the complex formation. The typical results are shown in Figure 2e for the weight ratio of 1. In fact, for the weight ratio of 5 and 30, the

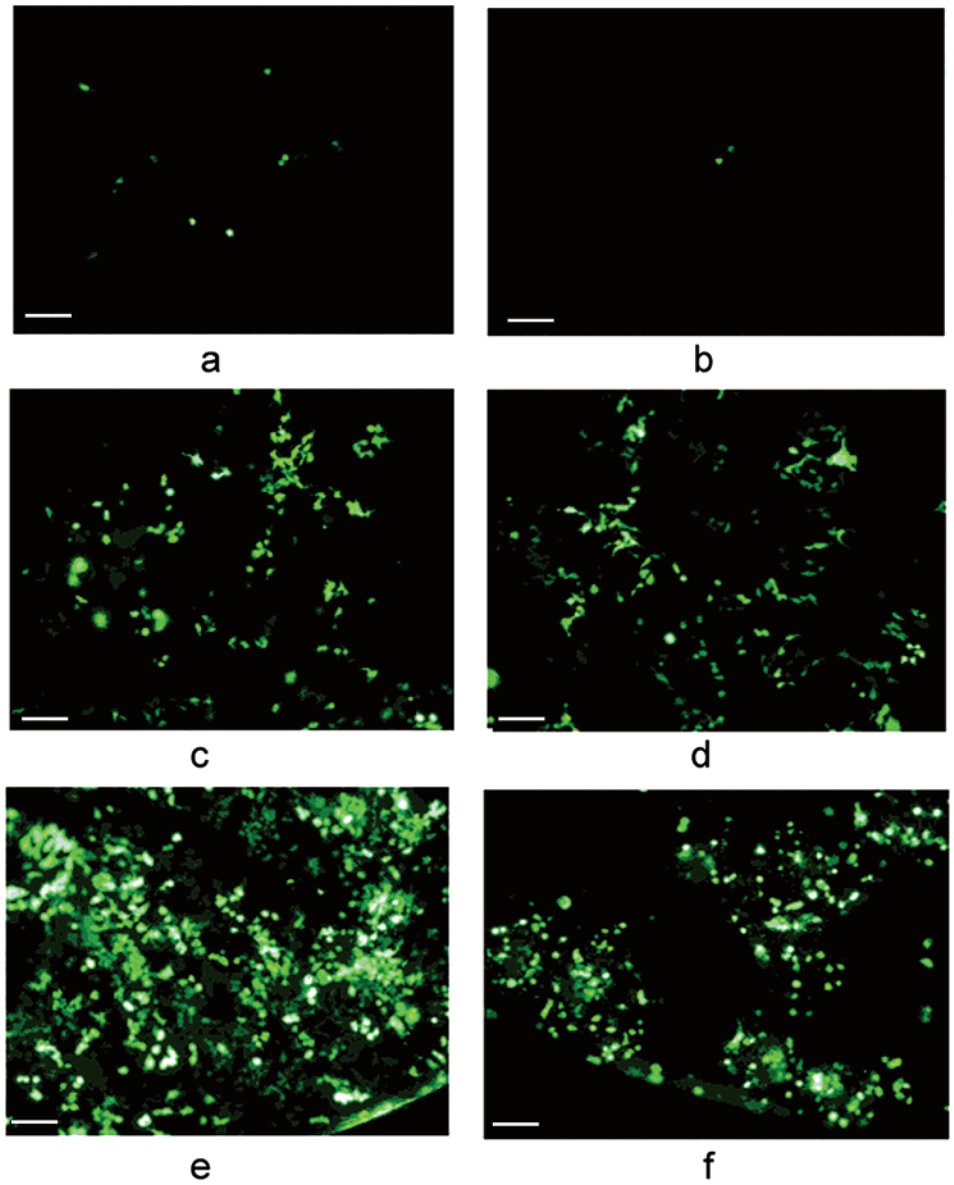


Figure 7. Typical fluorescence images of the 293T cells transfected by PLL-g-Chi copolymer/pDNA complexes at optimized polymer/pDNA weight ratio (scale bar: 40 μm). a: pDNA; b: chitosan (10); c: $PLL_{0.85}$-g-Chi (10); d: $PLL_{3.3}$-g-Chi (10); e: $PLL_{15.6}$-g-Chi (10); f: PLL (20).

same variation trend was observed: the peak intensity reduces slowly with increasing standing time. It means that the complexes formed are quite stable under the experimental conditions on one hand, and the complexation is improved to some extent during the storage periods on the other hand.

As far as UV−vis spectra are concerned, PLL-g-Chi copolymers combine the characteristics of both PLL and chitosan. Once again, $PLL_{15.6}$-g-Chi tends to have more characteristics of PLL, while $PLL_{0.85}$-g-Chi has more characteristics of chitosan. When the peak intensity changes are compared between $PLL_{15.6}$-g-Chi and $PLL_{0.85}$-g-Chi, it is found that at the same weight ratios of 1 and 5, the peak height reduced to 0.53 and 0.07 for $PLL_{15.6}$-g-Chi/DNA but only to 0.81 and 0.50 for $PLL_{0.85}$-g-Chi/DNA, respectively. It implies that the grafting ratio of PLL is one of the determining factors for the complexation and compaction of DNA in addition to the weight ratio of polymer/DNA. By adjusting both the grafting ratio and the polymer/DNA weight ratio, the desired complexation and molecular chain compaction can be achieved.

Particle Size and Zeta-Potential of the Complexes. The efficient compaction of pDNA into nanoparticles is an essential process that mediates the endocytosis of the polymer/DNA complexes, because cells typically uptake particles ranging from about 50 to several hundred nanometers.[15] Dynamic light scattering (DLS) measurements were performed to examine the size of the complexes formed by chitosan, PLL, and PLL-g-Chi copolymers with calf thymus DNA. Figure 3a shows that all polymers tested are able to form particles of 120∼340 nm in diameter to be endocytosed. Typically, chitosan forms complexes of 175∼320 nm. PLL complexes are around 175 nm (145∼200 nm), and the three PLL-g-Chi copolymers tested give the complexes of 130∼340 nm. Among the three PLL-g-Chi samples, $PLL_{0.85}$-g-Chi usually gives the largest particles, which are similar to chitosan. However, $PLL_{15.6}$-g-Chi gives the smallest particle size at a certain weight ratio of copolymer/DNA. The results indicate that the particle size of the complexes is obviously affected by the grafting ratios of PLL.

It has been proved by UV−vis spectra that the DNA complexation and compaction ability of chitosan is comparable to that of $PLL_{0.85}$-g-Chi. This can be further confirmed by the DLS measurements. As shown in Figure 3a, a similarity can be found between the hydrodynamic diameters of the complexes formed by chitosan and $PLL_{0.85}$-g-Chi with DNA. The DNA binding and condensing ability of the copolymers is enhanced

by increasing the PLL grafting ratios. Accordingly, the complexes generally become smaller. This can explain why the copolymers $PLL_{3.3}$-g-Chi and $PLL_{15.6}$-g-Chi can form smaller complexes than that of $PLL_{0.85}$-g-Chi at certain weight ratios. The size of the chitosan ($PLL_{0.85}$-g-Chi) complexes also depends on the weight ratio of chitosan to DNA. When it is increased, the complexes generally become smaller. For example, when the weight ratio of chitosan to DNA was increased from 5 to 40, the particle size of the complexes decreased from 320 to 150 nm. The situation is the same as that of $PLL_{0.85}$-g-Chi: the complexes change from 300 to 184 nm with an increased weight ratio from 5 to 40. However, the weight ratios do not significantly influence the particle size of the complexes once the DP_n of PLL is higher than 3.3.

The positive charge of the complexes facilitates nonspecific interactions with negatively charged cell surface proteins that trigger cellular uptake. Zeta-potential measurements were conducted to determine the net charges on the complex surface. The results are collected in Figure 3b. It is seen that all complexes are positively charged and the positive charges increase with weight ratio of copolymer to DNA for a given copolymer/DNA pair. $PLL_{0.85}$-g-Chi/DNA and $PLL_{3.3}$-g-Chi/DNA have zeta-potentials comparable to chitosan/DNA or PLL/DNA for a given polymer/DNA weight ratio, while $PLL_{15.6}$-g-Chi/DNA gives higher zeta-potential than both chitosan/DNA and PLL/DNA. These results illuminate that the positive charge on the surface of the copolymer/DNA complexes can be improved by increasing the grafting ratio of PLL at certain weight ratio of copolymer to DNA.

The AFM Morphology of the Complexes. The morphology of the complexes formed from calf thymus DNA and PLL, chitosan, and PLL-g-Chi copolymers were characterized by AFM. As shown in Figure 4a, free calf thymus DNA has linear structure and average chain lengnth of about 1.5 μm. When it is mixed with chitosan, heterogeneous morphology of rodlike, toroidal, and spherical shapes appears. This result agrees with the literature reports.[48,13] Part of the free DNA strucuture can still remain, which indicates that chitosan itself cannot completely condense DNA into nanoparticles at a weight ratio of 10 (Figure 4b). PLL can condense DNA into spherical nanoparticles with relatively homogeneous particle size even at a lower PLL/DNA weight ratio of 5 (see Figure 4e). After grafted with PLL, the positive charge of chitosan is improved, and the DNA binding and condensing ability of chitosan is also enhanced. This can be confirmed by the AFM investigation shown in Figure 4 (c, d). Both the tested copolymers possess the ability to compress calf thymus DNA into spherical nanoparticles, and the complexes formed with both copolymers reveal similar morphologies as PLL. However, $PLL_{15.6}$-g-Chi generally forms smaller particles than that of $PLL_{3.3}$-g-Chi. This observation is consistent with the DLS results, and it also proved the conclusion that the PLL grafting ratio affects the DNA binding and compaction ability of the PLL-g-Chi copolymers.

The pDNA-Binding Ability of the Copolymers. As an essential characteristic of a gene delivery carrier, pDNA-binding ability of the copolymers was determined by an agarose gel electrophoresis (AE) method. The results are displayed in Figure 5. Complete retardation of pDNA was achieved at a copolymer/DNA weight ratio of 2 for three copolymers, chitosan, and PLL. At the weight ratio of 0.5, chitosan could not completely neutralize the pDNA, so part of the pDNA in the corresponding channel was released. On the other hand, an improved pDNA retardation effect comparable to PLL is observed for the PLL-g-Chi copolymers. It becomes obvious that higher PLL grafting ratio would be helpful for the condensation and retardation of pDNA.

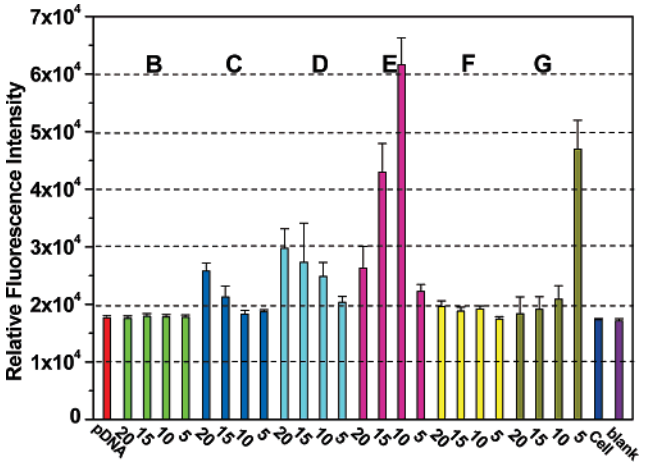


Figure 8. Fluorescence assays for the gene transfection efficiency of the PLL-g-Chi copolymers/pDNA complexes in 293T cells. A: pDNA; B: chitosan; C: $PLL_{0.85}$-g-Chi; D: $PLL_{3.3}$-g-Chi; E: $PLL_{15.6}$-g-Chi; F: PLL; G: PEI (weight ratios of polymer to pDNA were 20, 15, 10, and 5, respectively).

The Cytotoxicity of the Copolymers. A standard MTT test was introduced to evaluate the cytotoxicity of the PLL-g-chitosan copolymers. PLL (M_n 22 000 Da) was used as a control considering our polymers were derived partially from L-lysine and were of comparable molecular weight. Branched PEI (B-PEI, M_n 25 000 Da) was also used as a control because this kind of PEI was considered as the most effective synthetic cationic polymer for gene transfection but with high cytotoxicity. Comparisons between the graft copolymers and the controls were made at equivalent weight concentrations. The results (see Figure 6) show that PLL-g-Chi copolymers have higher cytotoxicity to L929 cells in a concentration range of 1∼100 μg/mL than chitosan but have lower cytotoxicity than B-PEI or PLL homopolymer. Among the three PLL-g-Chi copolymers, $PLL_{15.6}$-g-Chi displays the highest cytotoxicity. It is well-known that the cytotoxicity of PLL arises from its ϵ-primary amine groups. Although chitosan also has primary amine groups present on its monosaccharide rings, its cytotoxicity is much lower. In our case, because the PLL chains are grafted onto the chitosan backbone, and they are relatively short, their cytotoxicity is significantly lowered because of the presence of chitosan backbone. This is in agreement with the results reported by Metzke et al.,[49] where reduced cytotoxicity of hybrid polymer was achieved by breaking the cationic polypeptide into short segments with monosaccharide spacers.

Gene Transfection. The gene transfection efficiency of the PLL-g-Chi copolymers was evaluated by in vitro gene transfection experiments. The HEK 293T cells were transfected with pEGFP-N_1 pDNA. Figure 7 contains the typical fluorescence images of the transfected 293T cells. It can be seen that PLL-g-Chi copolymers possess reasonable gene transfection ability compared with PLL and chitosan. A dependence of the transfection efficiency on the polymer/pDNA weight ratio is found for a given copolymer, and the $PLL_{15.6}$-g-Chi always exhibits the strongest GFP expression among the copolymers at an optimized weight ratio of 10. These observations are confirmed by the GFP fluorescence intensity data (see Figure 8). The GFP expressed level of $PLL_{15.6}$-g-Chi/pDNA complexes in 293T cells is much higher than that of the complexes formed from pDNA with other copolymers, chitosan, and PLL and is

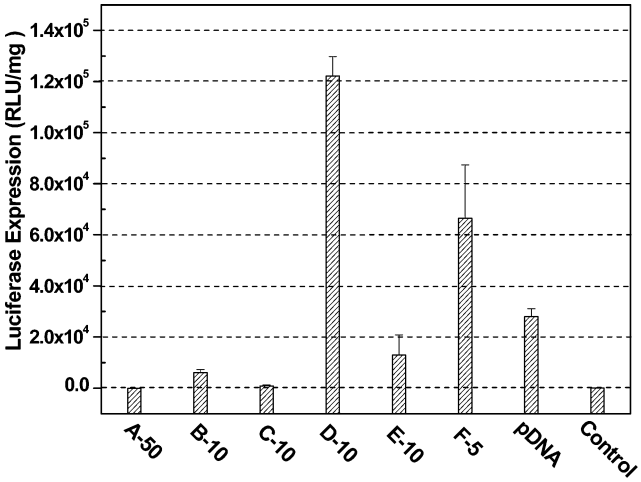


Figure 9. The luciferase gene expression in 293T cells observed with the complexes formed by pGL3 pDNA with (A) chitosan, (B) $PLL_{2.0}$-g-Chi, (C) $PLL_{3.3}$-g-Chi, (D) $PLL_{15.6}$-g-Chi, (E) PLL, and (F) B-PEI at optimized weight ratios. Untransfected cells were used as control. The gene expression values are shown as relative light units per milligram of protein (RLU/mg protein).

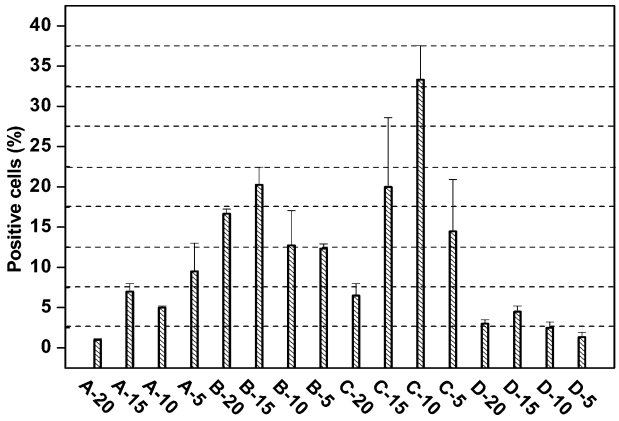


Figure 10. Gene transfection percent in 293T cells mediated by copolymer/pDNA complexes. A: $PLL_{0.85}$-g-Chi; B: $PLL_{3.3}$-g-Chi; C: $PLL_{15.6}$-g-Chi; D: PLL (Weight weight ratios of polymer to pDNA were 20, 15, 10, and 5, respectively).

even higher than that of B-PEI positive control, although B-PEI (25 kDa) has been considered to be the most effective synthetic cationic gene carrier. We also make effort to investigate the gene transfection efficiency of the copolymers by luciferase assay (Figure 9), which is a much more sensitive method than fluorescence intensity determination. The optimized weight ratio is selected according to the fluorescence intensity determination results. The highest luciferase expression level of 1.2×10^5 RLU/mg protein is achieved by $PLL_{15.6}$-g-Chi at weight ratio of 10 (nearly 2-folds higher than that of B-PEI). The complexes' mediated gene transfection percents in 293T cells are shown in Figure 10. For $PLL_{15.6}$-g-Chi/pDNA complexes at weight ratio of 10, the transfected cell percent is 33%, however, PLL (4.5%) and chitosan (lower than 1.0%) just give very low or negligible transfection yield. Lower PLL grafting ratio generally results in gradual decrease in transfection yield. It is decreased to 20% for $PLL_{3.3}$-g-Chi and only 9.5% for $PLL_{0.85}$-g-Chi. We had tried to simply refer these exciting results achieved by $PLL_{15.6}$-g-Chi to higher PLL grafting ratio. However, it seems indefinite. Because the pK_a ($\approx$ 10.5) of PLL ϵ-NH_2 is above physiological pH whereas the endosomal environment is acidic,[20,50] PLL cannot facilitate endosomal escape of the polymer/pDNA complexes. The PLL control exhibits very low GFP and luciferase expression levels in 293T cells. A primary assumption is that the enhanced gene transfection efficiency may be caused by enhanced osmolarity, subsequent endosomal rupture, and escape into the cytoplasm of the copolymer/pDNA complexes. An easy release of pDNA from the complexes after entering cells may be another main cause. The real mechanism remains to be investigated.

Conclusion

In summary, a novel method for synthesizing PLL-g-Chi copolymers is developed, that is, ROP of a Z-L-lysine NCA initiated by the amine groups present on 6-*O*-triphenylmethyl chitosan, and subsequent removal of the protective groups on chitosan and PLL. The solubility of the prepared copolymers in neutral water is much better than that of chitosan with comparable molecular weight. This improved synthetic route can also be used for preparing other kinds of polypeptide-graft-chitosan copolymers. The interaction between PLL-g-Chi copolymers and DNA was investgated by UV−vis spectra. The gene transfection effect of the graft copolymers in 293T cell line was tested. It was found that the copolymers possess better DNA binding and condensing ability than chitosan. The gene transfection efficiency of the obtained copolymers is higher than that of chitosan and PLL. A primary conclusion can be drawn that both the PLL grafting ratio and the weight ratio of copolymer to pDNA have influence on the gene transfection efficiency. The effect of PLL chain length on the gene transfection of the copolymers is being investigated in detail.

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Supporting Information Available. FT-IR spectra, ^1H NMR spectra, and ^{13}C and GPC curves of the PLL-g-chitosan copolymers. This material is available free of charge via the Internet at http://pubs.acs.org.

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