

Effects of Chirality on Gene Delivery Efficiency of Polylysine^{*}

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Abstract Chirality is a key factor in the biological activity of many biomolecules. Poly(L-lysine) (PLL), a polypeptide synthesized from L-lysine, is one of the mostly used cationic polymers for gene delivery. The effect of chirality of polylysine (PL) on its gene delivery remains unknown. Herein, we prepared three polylysines (PLs) with the similar molecular weight but different backbone chiralities including poly(L-lysine) (PLL), poly(D-lysine) (PDL) and poly(DL-lysine) (PDLL). The side chains of each PL were modified with propylene oxide (PO) of different chiralities including (R)PO, (S)PO and (R,S)PO. These PL-POs with distinct chirality in main and side chains could condense pDNA into polyplexes. The polyplexes had approximately the same size, zeta potential and binding ability, but showed distinct gene transfection efficiency. We found that the PLs of L-configuration in the main chain had higher transfection efficiency than that of D or DL configuration due to their faster cellular uptake, while the side chain chirality had no effect on transfection efficiency.

Keywords: Gene delivery; Gene transfection; Chiral effects; Polylysine.

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INTRODUCTION

Over the past three decades gene delivery has become a potential approach to treat genetic disorders^[1, 2]. In order to deliver genes to the targeted cells efficiently, various viral and nonviral carriers have been developed^[3, 4]. As viral carriers are concerned with safety issues, the nonviral carriers as an alternative have attracted great attentions owing to their better safety and other unique strengths including biocompatibility, tumor/tissue targeting and the potential for large-scale production^[5–7]. Cationic polymers, which complex and condense negatively charged DNA into small nanoparticles for gene delivery, are one of the mostly studied nonviral vectors^[8–10]. Various polymers have been synthesized and the factors affecting polymer gene transfection efficiency including topographical structure^[11, 12], molecular weight^[13], α -helical structure^[14, 15], charge density^[16, 17] and the size of the complex^[18, 19] have been investigated.

Chirality is important in the organisms and biological processes, because there are many kinds of optically active molecules and macromolecules, such as proteins, polysaccharides in living systems^[20]. Recent years, tremendous kinds of chiral small molecules or polymers have been synthesized due to the significance of chirality^[21–23]. These exogenously synthetic chiral molecules, particular in chiral drugs, may show quite different biological activities due to their chiral or enantiomeric discrimination^[24, 25]. Therefore, we assume that the chiral discrimination may influence other biological processes, such as gene transfection. To our knowledge, the

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special study on polymers with different chiralities used as gene delivery carriers has never been reported.

Poly(L-lysine) (PLL), a polypeptide synthesized from L-lysine, is one of the mostly used cationic polymers for gene delivery. How the chirality of polylysine (PL) affects its gene delivery is an unanswered question. In this study, we prepared three kinds of polylysines including poly-L-lysine (PLL), poly-D-lysine (PDL) and poly-DL-lysine (PDLL) with different chiralities of main chain. PLL synthesized by L-isomer of amino acid has long been used as an effective gene vector^[1, 26, 27]. PDL synthesized by D-isomer of amino acid and PDLL of mesomer has rarely been used as gene carriers^[28]. Then three types of propylene epoxide (PO) including (R)PO, (S)PO and (R,S)PO were connected to the epsilon amino group of these three polylysines respectively. By introducing chirality to side chain, we obtained nine kinds of PL-POs with distinct chirality of main and side chains. We found that the chirality of the polymers' main chain but not the side chain could greatly affect their cellular uptake and gene transfection efficiency.

EXPERIMENTAL

Materials

All the solvents of reagent grade purity were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) and used without further purification, except for dimethylformamide (DMF), which was distilled under reduced pressure and dried with CaH₂. H-Lys(Z)-OH ($\geq 98.0\%$) and H-D-Lys(Z)-OH ($\geq 98.0\%$) were purchased from GL Biochem Co., Ltd. (Shanghai, China). HBr/CH₃COOH (33%) was purchased from Sigma-Aldrich (Milwaukee, WI). (R)-(+)- propylene oxide (99.0%), (S)-(–)- propylene oxide (99.0%) and (±)-propylene oxide ($\geq 99.7\%$) were purchased from Aladdin Chemistry Co. (Shanghai, China). All the other chemical reagents were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China). The dialysis membrane (MWCO = 3500) was purchased from Spectrum Laboratories Inc. (Rancho Dominguez, CA). The cell lines were obtained from the American Type Culture Collection. RPMI medium 1640 (RPMI-1640), Trypsin-EDTA (0.25%), fetal bovine serum (FBS), penicillin-streptomycin solution, phosphate-buffered saline (PBS) were purchased from Gibco (Shanghai, China). Cy5 was obtained from Mirus Bio(Madison, WI).

Synthesis of PLL, PDL and PDLL with the Similar Molecular Weight (MW)

N- ϵ -Benzyloxycarbonyl-L-lysine-*N*- α -carboxyanhydride (Z-L-Lys-NCA), *N*- ϵ -benzyloxy carbonyl-D-lysine-*N*- α -carboxyanhydride (Z-D-Lys-NCA) and *N*- ϵ -benzyloxycarbonyl-DL-lysine-*N*- α -carboxyanhydride (Z-DL-Lys-NCA) were synthesized according to the literature^[29]. The usage of reagents, H-Lys(Z)-OH and H-D-Lys(Z)-OH, was half-and-half in the preparation of Z-DL-Lys-NCA. PLL, PDL and PDLL were synthesized by polymerization of Z-L-Lys-NCA, Z-D-Lys-NCA and Z-DL-Lys-NCA, respectively, initiated with *n*-butylamine in DMF and followed by deprotection in a mixture of HBr/CH₃COOH^[30]. The ratio of monomer and initiator was optimized to obtain polymers with similar molecular weight around 12 kDa. They all have similar ¹H-NMR spectra and the typical one is as follows: ¹H-NMR (400 MHz, D₂O, δ): 4.22 (t, 1H), 2.90 (t, 2H), 1.58–1.82 (m, 4H), 1.36 (m, 2H).

MW Measurement of the Z-PLL, Z-PDL and Z-PDLL

The molecular weights of Z-PLL, Z-PDL and Z-PDLL were determined by gel permeation chromatography (GPC) based on a PL-EMD 950 instrument. The three samples were measured in DMF at 25 °C.

Preparation of PL-POs —PLL-(R)PO, PLL-(S)PO, PLL-(R,S)PO, PDL-(R)PO, PDL-(S)PO, PDL-(R,S)PO, PDLL-(R)PO, PDLL-(S)PO, PDLL-(R,S)PO

A typical procedure to synthesize PO modified PLs is as follows: PLL (150 mg, 1.17 mmol NH₂) in a round-bottomed flask was dissolved in 4 mL deionized water and the pH was adjusted to 8.0–9.0 by sodium carbonate. (R)PO (10 molar excess, 11.7 mmol) was added to the solution and the mixture was stirred overnight at room temperature. The product (PLL-(R)PO) was purified by dialyzing against water (MWCO 3500) and then lyophilized to give product with a yield of 90%. Other PO modified PLs including PLL-(S)PO, PLL-(R,S)PO, PDL-(R)PO, PDL-(S)PO, PDL-(R,S)PO, PDLL-(R)PO, PDLL-(S)PO and PDLL-(R,S)PO were prepared

similarly. They all have similar ^1H -NMR spectra and the typical one is as follows: ^1H -NMR (400 MHz, D_2O , δ): 3.78–3.95 (m, 3H), 2.28–2.51 (m, 6H), 1.30–1.96 (m, 6H), 1.05 (m, 6H).

Optical Rotation Measurement of PLs and PL-POs

All the polymers were dissolved in HEPES (10 mmol/L pH 7.4) at a concentration of 1 g/dl and the optical rotation of these solutions was measured by using a PerkinElmer 341 instrument ($l = 1$ dm, $T = 20.0$ °C). Specific rotator powers $[\alpha]_{589}$ are expressed as $\text{deg dm}^{-1} \text{ g}^{-1} \text{ dl}$.

Circular Dichroism Measurement

CD spectra were measured with a J-600 spectropolarimeter (JASCO LTD, Tokyo, Japan) at room temperature. The instrument was equipped with a computerized data processing system and was calibrated with (+)-D-camphorsulfonic ammonium salt. 0.1 cm light path cell was used to determine the spectra. Each experiment was measured 3 times. Therefore, each curve gained from a number of independent experiments is a representative CD spectrum of the polymers.

Agarose Gel Electrophoresis

30 μL different concentrations of PL-POs dissolved in HEPES (10 mmol/L pH 7.4) were added to plasmid DNA (pDNA, 30 μL , 40 $\mu\text{g/mL}$) to yield the desired PL-POs/pDNA and the atomic nitrogen-to-phosphorus (N/P) ratios. The mixture was vortexed for 10 s and incubated at room temperature for 30 min. Polyplexes were electrophoresed on a 0.9% (W/V) agarose gel for 30 min at 100 V. The gel was stained with ethidium bromide (EB) (0.5 $\mu\text{g/mL}$) and pDNA bands were visualized on a UV illuminator to show the location of the pDNA.

Size and Zeta Potential Measurements

The sizes of PL-POs/pDNA were measured by using a Nano-ZS zetasizer (Malvern Instrument, UK) with a scattering angle of 173° and a laser light wavelength of 632.8 nm. The polyplexes were obtained according to the method described above. Each measurement was carried out at 25 °C in triplicate, and the results were processed with DTS software version 6.20. The zeta potentials were measured by using zetasizer. The measurements were carried out in disposable zeta capillary cells at 25 °C in triplicate, and the results were processed with DTS software version 6.20.

Ethidium Bromide Exclusion Assay

50 μL different concentrations of PL-POs were added to pDNA (50 μL , 40 $\mu\text{g/mL}$) to obtain polyplexes with different N/P ratios (*e.g.*, 0.528 mg/mL of PL-POs gave an N/P ratio of 16) followed by 10 s of vortexing. Then the mixture was incubated at room temperature for 30 min. After adding 5 μL (4 $\mu\text{g/mL}$) EB, the mixture was incubated at room temperature for another 30 min. The fluorescence intensity was monitored by SpectraMax M2e. The excitation wavelength is 510 nm and emission wavelength is 608 nm.

In vitro Cytotoxicity Assay

The cytotoxicity of polymers (PLs and PL-POs) was determined in Hela cells using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Hela cells were seeded at a density of 4000–5000 cells/well in 100 μL complete medium onto 96-well plates and allowed to attach for 24 h prior to the assay. The culture medium of the cells was replaced with 200 μL of fresh medium containing polymers with different concentrations equivalent with the concentration used for transfection study. After 48 h of incubation, the original culture medium was replaced with 100 μL of fresh medium containing 10% MTT reagent and incubated for another 4 h at 37 °C. Finally, 100 μL DMSO was added to each well. The absorbance of each well was measured by using a microplate spectrophotometer (Molecular Devices, SpectraMax M2e, USA) at the wavelength of 562 nm. The cells without any treatment were used as control.

In vitro Transfection Assay

The *in vitro* transfection assay of the polyplexes was performed as follows: Hela cells were cultured in RPMI-1640 containing 10% FBS, 1% penicillin and streptomycin at 37 °C with 5% CO_2 . Hela cells were seeded into 48-well plates in 400 μL complete medium at a density of 40000 cells/well and incubated for 24 h before

transfection. The complete medium was then replaced with serum-free medium. 50 μL (pDNA dose of 20 $\mu\text{g}/\text{mL}$) polyplexes was added into each well and the cells were cultured for 4 h. The serum-free medium was then replaced with the complete medium and the cells were cultured for another 4 h. The medium of all wells was removed and each well was added with 100 μL lysis reagent (Promega) to lyse the cells. Protein content of the lysed cells was measured by Bradford protein assay. The luciferase activity was measured with a luminometer equipped with a photomultiplier tube (Guangdong, China). The luciferase expression was expressed as relative light units per mg protein (RLU/mg protein).

Cellular Endocytosis

Hela cells were seeded into glass bottom cell culture dishes (NEST, China) at a density of 150000 cells per dish in 1.5 mL complete medium and cultured for 24 h. The complete medium was replaced with the serum-free medium. 50 μL polyplexes (pDNA dose of 20 $\mu\text{g}/\text{mL}$) solutions were then added to each well and cultured for 2 h or 6 h. After 2 h or 6 h of incubation, the medium was removed and the cells were washed three times with cold PBS before observation by confocal microscopy (CLSM, Nikon-A1 system, Japan).

Statistical Analysis

Data were presented as means $\pm$ standard errors (SE). Student's t-test was used to determine the significant difference of the data between individual groups.

RESULTS AND DISCUSSION

Characterization of PLs

The structure and optical activity of the three PLs (Scheme 1) with different configurations were characterized by ^1H -NMR and optical rotation, respectively. These PLs have similar ^1H -NMR spectra (Supporting Fig. S1) but different optical activity. The specific rotator powers $[\alpha]_{589}$ of the three PLs are shown in Table 1. It was found that the PLL and PDL had opposite optical activity and PDLL had no optical activity. To study chiral effects on their applications in gene delivery, these PLs were synthesized with approximately identical molecular weights (Supporting Fig. S2). The molecular weights of all PLs were about 12 kDa and the PDI were ranging from 1.11 to 1.18 as measured by GPC (Table 1).

Table 1. Molecular weights and optical rotation of three PLs with different chirality

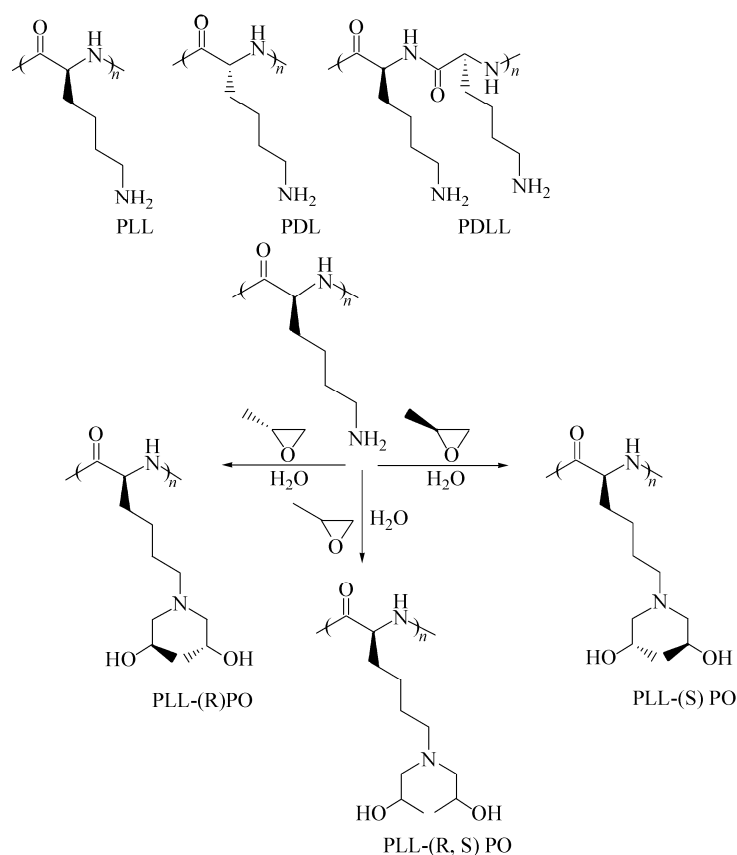
Polylysine	M_n	PDI	Optical rotation
PLL	1.14×10^4	1.18	-67.8°
PDL	1.26×10^4	1.11	67.0°
PDLL	1.17×10^4	1.15	0.6°

Characterization of PL-POs

PL-POs were synthesized by the ring opening of PO with the primary amine of the PLs in alkaline condition (Scheme 1). Excessive PO was used to guarantee each NH_2 group reacted with two PO molecules. The products were dialyzed against water for two days to remove the excess of PO. The structure of PL-POs was confirmed by ^1H -NMR spectroscopy (Supporting Fig. S3). Table 2 shows the optical rotation of all the PL-POs. The nine different PL-POs exhibited different rotators powers which indicated their differences in chirality and the successful synthesis of chiral PL-POs.

Table 2. Optical rotation of all the PL-POs

Main chain	Side chain		
	(R)PO	(S)PO	(R, S)PO
PLL	-84.0°	38.7°	-14.3°
PDL	-54.3°	83.1°	16.3°
PDLL	-62.8°	44.7°	-0.4°



Scheme 1 Synthetic route of PL-POs

Chiral Characterization of the PLs and PL-POs

The chiral properties of PL-POs and PLs were investigated by circular dichroism (CD) spectra, and the results are shown in Fig. 1. It was found from the CD signal that PLL have large negative ellipticities while PDL have large positive ellipticities. Moreover, the spectra of PLL and PDL are symmetrical, which is in agreement with their L and D configuration. The ellipticity of meso polymer PDLL was close to zero, showing the molecule had no chiral or optical activity. CD spectra of PLL-(R)PO, PLL-(S)PO and PLL-(R,S)PO were all similar to PLL, indicating that PO and its chirality had no effect on their CD spectra. Same results were found from PO modified PDL and PDLL.

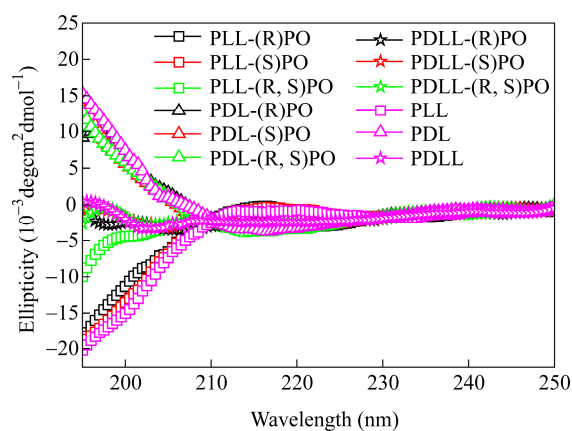


Fig. 1 CD spectra of PL-POs and PLs at the concentration of 0.1 mg/mL in PBS (pH7.4)

Characterization of PL-POs Polyplexes and PLs Polyplexes

To study the complexation of PL-POs and PLs with pDNA, gel electrophoresis assay was performed. As shown in Fig. 2, all the PLs and PL-POs could completely retard pDNA migration at the N/P ratio of 8 and 16. The results showed that at the N/P ratio of 8 and 16, all the polymers could bind well with pDNA and form polyplexes with pDNA. From the gel electrophoretic analysis, no differential behavior was observed among the PL-POs in their capability to condense pDNA.

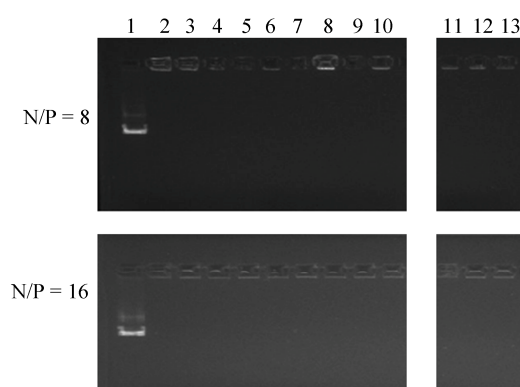


Fig. 2 Gel retardation assay: Lane 1, free pDNA; lane 2, PLL-(R)PO/pDNA; lane 3, PLL-(S)PO/pDNA; lane 4, PLL-(R,S)PO/pDNA; lane 5, PDL-(R)PO/pDNA; lane 6, PDL-(S)PO/pDNA; lane 7, PDL-(R,S)PO/pDNA; lane 8, PDLL-(R)PO/pDNA; lane 9, PDLL-(S)PO/pDNA; lane 10, PDLL-(R,S)PO/pDNA; Lane 11, PLL/pDNA; lane 12, PDL/pDNA; lane 13, PDLL/pDNA

The sizes of PLs and PL-POs polyplexes were determined using DLS, and the results are shown in Fig. 3(a). The sizes of all the polyplexes ranged approximately from 50 nm to 100 nm in diameter at the N/P ratio of 8 and 16. It has been recommended that the optimal particle size for nanocarrier delivering to cancer is 20–100 nm^[31]. For all the polyplexes, it was found that the chirality of main chain or side chain had no significant effect on their particle size at the N/P ratio of 8 and 16. The particle sizes of PLs polyplexes were basically the same and they were slightly smaller than those of PL-POs polyplexes.

The zeta potentials of the PL-POs polyplexes and PLs polyplexes are shown in Fig. 3(b). The zeta potentials of the polyplexes were ranged from 8 mV to 15 mV at the N/P ratio of 8 and 16, which were measured at pH 7.4. All the polyplexes had no significant difference in zeta potential. The positively charged polyplexes were favorable for adhesion to the negatively charged cell membrane^[32–34].

To further study the complexation of PL-POs and PLs with pDNA, ethidium bromide (EB) exclusion assay was used to quantitate their binding ability. EB, a competitive with polycations to complex with pDNA, has the ability to disrupt the polyplexes and form EB/pDNA complexes with fluorescence. The fluorescent intensity indicates the binding ability of cationic polymers with pDNA, in which high fluorescent intensity means weak binding ability. From Fig. 3(c), it was found that all the PL-POs or the three PLs had the similar binding ability with pDNA in each group. The chirality of the polymer main and side chain had no obvious effect on their binding ability with pDNA. PLs had apparently higher binding ability than that of PL-POs, which can be attributed to the higher charge density of primary amine than tertiary amine.

In vitro Cytotoxicity and Gene Transfection

In vitro cytotoxicity of polymers (PL-POs and PLs) used in transfection experiment was evaluated with MTT assay on Hela cells. All the PL-POs showed no inhibitory effect on cell proliferation at the working concentration for gene transfection. PLL, PDL and PDLL showed similar cytotoxicity (Supporting Fig. S4). At the transfection working concentration of N/P ratio of 8, PLs did not show any toxicity. At the transfection working concentration of N/P ratio of 16, PLs killed about 40% of the cells. The gene transfection efficiencies of PL-POs in Hela cells were determined at the N/P ratio of 8 (Fig. 4a) and 16 (Fig. 4b). The transfection

efficiencies of PLL-POs group were much higher than those of PDL-POs and PDLL-POs group, especially at the N/P ratio of 8. The results showed the chirality of polymer main chain had a significant effect on their transfection efficiencies. PL-POs with L configuration had higher transfection efficiencies than those of PL-POs with D or DL configuration. However, in PLL-POs group, the transfection efficiencies of PLL-(R)PO, PLL-(S)PO and PLL-(R,S)PO were very close and had no significant difference among each other, which indicated the chirality of side chain had no effect on the transfection efficiency. This result was also concluded in PDL-POs and PDLL-POs groups.

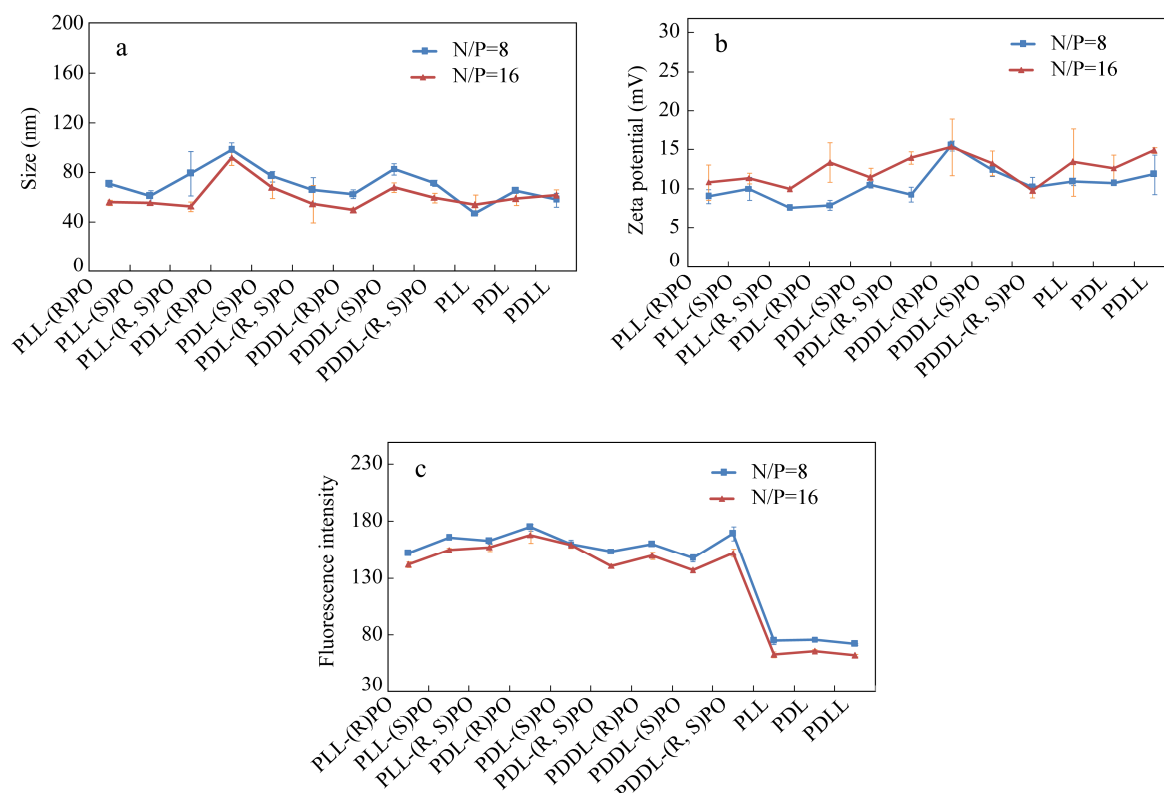


Fig. 3 (a) Average size of PL-POs polyplexes and PLs polyplexes at the N/P ratio of 8 and 16; (b) Zeta potential of PL-POs polyplexes and PLs polyplexes at the N/P ratio of 8 and 16; (c) Ability of polymers to form complexes with pDNA at the N/P ratio of 8 and 16, analyzed using an ethidium bromide (EB) exclusion assay. All the experiments were repeated for three times and error bars represent standard deviation.

The gene transfections of PLL, PDL and PDLL were also studied to further examine the effect of the chirality of main chain on their transfection efficiency. The experiments were performed at the N/P ratio of 8 and 16, and the results are shown in Fig. 4(c). PLL polyplexes had the highest transfection efficiency among the three polyplexes and the transfection efficiencies of PDL and PDLL polyplexes had no significant difference between each other. Compared with PL-POs polyplexes, PLs polyplexes had relatively higher transfection efficiencies. This may be ascribed to the extreme hydrophilicity of PL-POs containing a lot of hydroxyl groups, which was hard for cellular uptake, because proper hydrophobicity of polymers can be more easily for cellular uptake^[35–38].

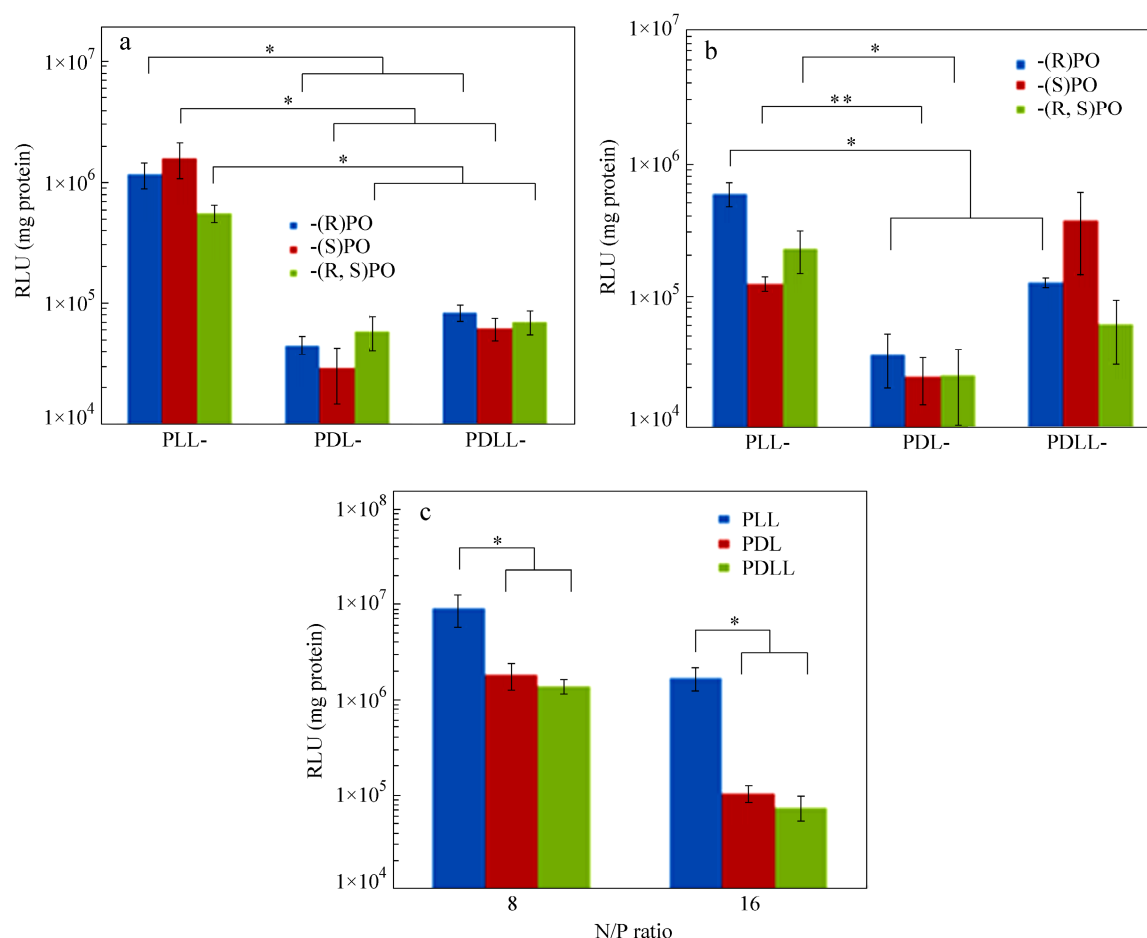


Fig. 4 (a) *In vitro* transfection of PL-POs after 48 h in HeLa cells at the N/P ratio of 8; (b) *In vitro* transfection of PL-POs in HeLa cells after 48 h at the N/P ratio of 16; (c) *In vitro* transfection of PLs after 48 h in HeLa cells at the N/P ratio of 8 and 16 (Data are expressed as mean $\pm$ SD ($n = 3$). Significance: *, $p < 0.05$; **, $p < 0.01$)

Cellular Uptake

The cellular uptake of PLL/pDNA, PDL/pDNA and PDLL/pDNA polyplexes was studied by confocal microscopy (Fig. 5 and Supporting Figs. S5–S10). HeLa cells were cultured with each polyplex (N/P ratio = 8) for 2 h or 6 h. pDNA in polyplex was labeled with Cy5.0 and expressed as red. Hoechst was used to stain cell nuclei and displayed as blue. With 2 h of incubation, red fluorescence in cells cultured with PLL/pDNA was much higher than those cultured with PDL/pDNA and PDLL/pDNA. With 6 h of incubation, more red fluorescence was observed in the cells treated with PLL/pDNA and those were still few in the cells treated with PDL/pDNA and PDLL/pDNA. The results indicated that PLL/pDNA had higher cellular uptake than PDL/pDNA and PDLL/pDNA, which may account for the higher transfection efficiency of PLL group. Most amino acids found in natural proteins occur in the L configuration. Cell membrane also contains a number of natural proteins. The higher cellular uptake of polyplex with L configuration than that of D configuration may attribute to its favorable interacts with cell membrane^[39, 40].

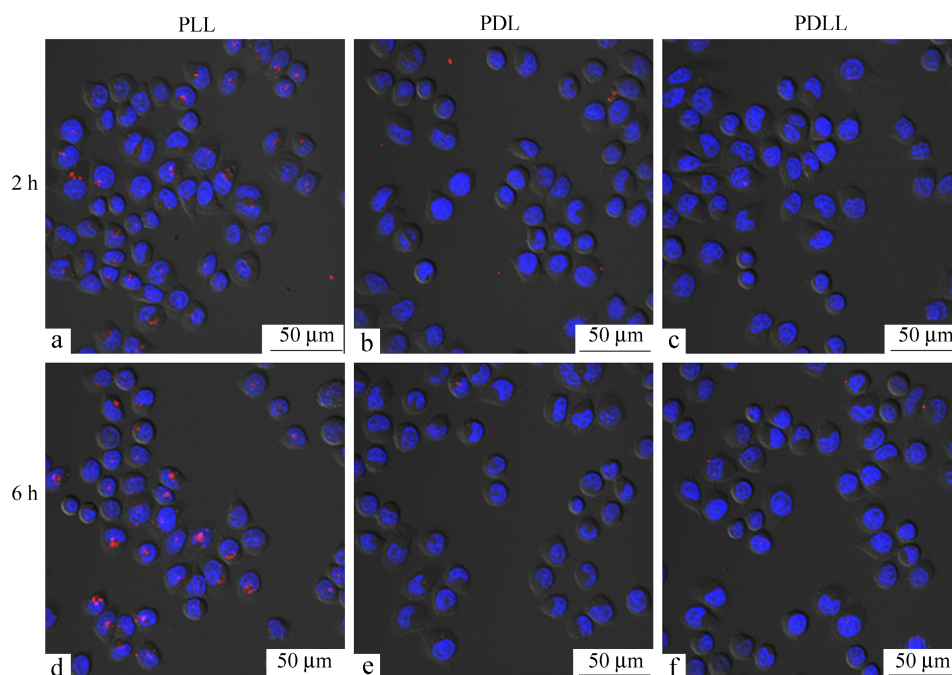


Fig. 5 Confocal laser scanning microscopy images of HeLa cells co-cultured with PLs/pDNA polyplex (N/P = 8) for 2 h: (a) PLL/pDNA, (b) PDL/pDNA, (c) PDLL/pDNA, or 6 h: (d) PLL/pDNA, (e) PDL/pDNA, (f) PDLL/pDNA (pDNA was labeled with Cy5 (red) and cell nuclei were stained with Hoechst (blue).)

CONCLUSIONS

In this work, we prepared nine PL-POs with similar molecular weight and different chiralities in main and side chains to study the chirality effect on gene transfection efficiency. We found that the nine PL-POs had same pDNA bind ability but different transfection efficiencies. Polylysine with L configuration of main chain had obviously higher transfection efficiency than those with D and DL configuration due to its higher cellular uptake rate. The side chain chirality had no effect on transfection efficiency. This study offers guidance on engineering polymer structures to improve their transfection efficiency and an insight into the mechanisms involved in their different transfection profiles.

SUPPORTING INFORMATION

^1H -NMR spectrum of PLL in D_2O (Supporting Fig. S1), GPC measurements of Z-PLL, Z-PDL and Z-PDLL in DMF (Supporting Fig. S2), ^1H -NMR spectrum of PLL-(R)PO in D_2O (Supporting Fig. S3), MTT assay of PLs (Supporting Fig. S4) and additional confocal microscopy images (Supporting Figs. S5–S10).

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