

Co-self-assembly of cationic microparticles to deliver pEGFP-ZNF580 for promoting the transfection and migration of endothelial cells

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Abstract: The gene transfection efficiency of polyethylenimine (PEI) varies with its molecular weight. Usually, high molecular weight of PEI means high gene transfection, as well as high cytotoxicity in gene delivery in vivo. In order to enhance the transfection efficiency and reduce the cytotoxicity of PEI-based gene carriers, a novel cationic gene carrier was developed by co-self-assembly of cationic copolymers. First, a star-shaped copolymer poly(3(S)-methylmorpholine-2,5-dione-co-lactide) (P(MMD-co-LA)) was synthesized using D-sorbitol as an initiator, and the cationic copolymer (P(MMD-co-LA)-g-PEI) was obtained after grafting low-molecular weight PEI. Then, by co-self-assembly of this cationic copolymer and a diblock copolymer methoxy-poly(ethylene glycol) (mPEG)-b-P(MMD-co-LA), microparticles (MPs) were formed. The core of MPs consisted of a biodegradable block of P(MMD-co-LA), and the shell was formed by mPEG and PEI blocks. Finally, after condensation of pEGFP-ZNF580 by these MPs, the plasmids were protected from enzymatic hydrolysis effectively. The result indicated that pEGFP-ZNF580-loaded MP complexes were suitable for cellular uptake and gene transfection. When the mass ratio of mPEG-b-P(MMD-co-LA) to P(MMD-co-LA)-g-PEI reached 3/1, the cytotoxicity of the complexes was very low at low concentration (20 $\mu\text{g mL}^{-1}$). Additionally, pEGFP-ZNF580 could be transported into endothelial cells (ECs) effectively via the complexes of MPs/pEGFP-ZNF580. Wound-healing assay showed that the transfected ECs recovered in 24 h. Cationic MPs designed in the present study could be used as an applicable gene carrier for the endothelialization of artificial blood vessels.

Keywords: star-shaped copolymer, microparticles, gene carrier, endothelial cells, transfection, migration

Introduction

Atherosclerotic vascular diseases have become the major cause of morbidity and mortality in recent years all over the world.¹ Coronary bypass surgery is one of the most vital procedures for treating occluded coronary arteries.^{2,3} However, restenosis of artificial blood vessels usually leads to the failure of the surgery. To decrease or prevent the formation of thrombus, hydrophilic modification of the artificial blood vessels has been used and demonstrated to be an efficient strategy. Various strategies including hydrophilic surface modification have been explored by us and other researchers.^{4,5} For example, silk fiber, collagen, heparin, poly(ethylene glycol), gelatin, zwitterionic polynorborene and 2-methacryloyloxyethyl phosphorylcholine have been widely used in surface modification of artificial blood scaffolds.⁶⁻¹⁴ The hydrophilicity, hemocompatibility and biocompatibility of artificial blood vessels have been improved greatly after surface modification.

For preventing thrombosis, another effective strategy is to form endothelium on the luminal surface of artificial vascular grafts. Up to date, many strategies have been reported to promote the proliferation and migration of endothelial cells (ECs).¹⁵ For example, Mattix et al designed and manufactured magnetic vascular nanoparticles for rapid endothelialization to reduce the risk of restenosis.¹⁶ Moreover, after modification with anti-CD34 antibody on the surface of a stent, the attachment of ECs was promoted greatly.¹⁷

As a future therapeutic strategy, gene therapy has been bloomed over years. It has great potential in treating cancers, atherosclerotic vascular diseases and genetic disorders.^{18,19} Gene therapy is also an attractive method to realize rapid endothelialization, which has attracted wide interests of researchers.²⁰ For example, to enhance the proliferation of ECs, both vascular endothelial growth factor and platelet-derived growth factor have been encapsulated in electrospun membranes with dual-delivery function.²¹ Furthermore, in the process of intervention for atherosclerosis and proliferation of ECs, *ZNF580* gene plays an important role in promoting the proliferation of ECs and inhibiting the proliferation of smooth muscles.²² In our previous studies, several gene carriers to condense with pEGFP-ZNF580 gene (pEGFP-ZNF580 gene is the recombinant plasmid of enhanced GFP plasmid and *ZNF580* gene) were prepared with different block copolymers for the proliferation of ECs.²³⁻²⁹

As the “gold” standard nonviral gene carrier, polyethylenimine (PEI) is widely used as an effective gene carrier, which exhibits a good performance in condensing DNA due to its high positive charge density.³⁰ However, the drawbacks of high-molecular weight PEI, including nondegradability and high cytotoxicity, limit its application in vivo.³¹⁻³³

In recent decades, the excellent properties of star-shaped polymers have attracted researchers' attention. Compared with linear counterparts, star-shaped polymers with the same molecular weight have showed higher transfection efficiency.³⁴⁻³⁶ Moreover, compared with linear polymers, the amphiphilic star-shaped polymers show relatively small hydrodynamic radius, and low solution and melt viscosities.³⁵ Plenty of star-shaped polycations containing a biocompatible core display excellent performance in gene therapy. For example, Yang et al synthesized a bioreducible star-shaped gene carrier consisting of a biocompatible polyhedral oligomeric silsesquioxane core and eight disulfide-linked poly(2-dimethylamino)ethyl methacrylate arms.³⁶ The eight corner organic groups could be easily used to immobilize functional groups, which could

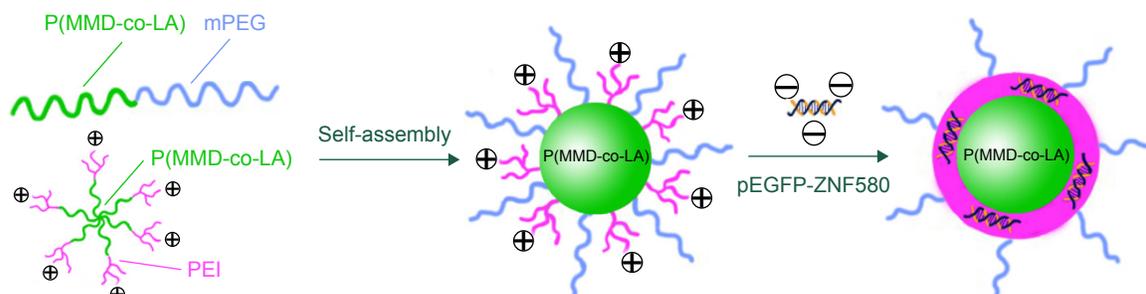
lower cytotoxicity and increase transfection efficiency. By group transfer polymerization, Georgiou et al synthesized a series of 2-(dimethylamino) ethyl methacrylate-b-methoxy hexa(ethylene glycol) methacrylate star-shaped copolymers which showed low toxicity and high transfection efficiency compared to that of homopolymer.³⁷ Considering the excellent properties of star-shaped polymers and the good performance of *ZNF580* gene for rapid endothelialization, it is interesting to investigate and develop a potential star-shaped polymeric gene carrier aiming at accelerating the proliferation of human ECs.

In the present investigation, low-molecular weight PEI was grafted onto the end of star-shaped hydrophobic polymers to reduce the cytotoxicity of high-molecular weight PEI. As a result, a cationic copolymer poly(3(S)-methylmorpholine-2,5-dione-co-lactide) (P(MMD-co-LA))-grafted PEI (P(MMD-co-LA)-g-PEI) was synthesized. Then, by co-self-assembly of P(MMD-co-LA)-g-PEI and methoxy-poly(ethylene glycol) (mPEG) (mPEG-b-P(MMD-co-LA)), a series of microparticles (MPs) were prepared (Scheme 1). Moreover, pEGFP-ZNF580 was condensed with PEI located at the surface of the MPs. The properties of MPs and pEGFP-ZNF580-loaded MPs were evaluated. The cytotoxicity could be varied easily by changing the ratios of mPEG-b-P(MMD-co-LA) to P(MMD-co-LA)-g-PEI in mixed shell. The proliferation rate of ECs was characterized by transfection efficiency and wound-healing assay.

Materials and methods

Materials

mPEG (weight-average molecular weight [M_w] =5,000 Da and polydispersity index =1.05), PEI (branched PEI, M_w =10 kDa), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), N-hydroxy succinimide (NHS), 4-dimethylamino pyridine (DMAP), D-sorbitol and stannous octoate ($\text{Sn}(\text{Oct})_2$) were purchased from Sigma-Aldrich (St Louis, MO, USA). Lactide (LA) was purchased from Foryou Medical Device Co., Ltd. (Huizhou, People's Republic of China). 1,4-Dioxane, succinic anhydride triethylamine (Et_3N), toluene and dimethyl sulfoxide (DMSO) were obtained from Institute of Jiangtian Chemical (Tianjin, People's Republic of China) and were dried by reflux over CaH_2 and distilled. pEGFP-ZNF580 was preserved by the Department of Physiology and Pathophysiology, Logistics University of Chinese People's Armed Police Force. 3(S)-Methyl-morpholine-2,5-dione (MMD) was prepared using our previously reported method.³



Scheme 1 Process of co-self-assembly of MPs and the formation of MPs/pEGFP-ZNF580 complexes.

Notes: The co-self-assembly of cationic copolymers P(MMD-co-LA)-g-PEI and mPEG-b-P(MMD-co-LA) could form a complex MPs system with a biodegradable P(MMD-co-LA) core and a mixed mPEG/PEI shell. Then, ZNF580 gene was condensed with PEI located at the interface between P(MMD-co-LA) core and mPEG shell.

Abbreviations: MPs, microparticles; P(MMD-co-LA), poly(3(S)-methyl-morpholine-2,5-dione-co-lactide); PEI, polyethylenimine; mPEG, methoxy-poly(ethylene glycol).

Synthesis of diblock copolymer

Diblock copolymer mPEG-b-P(MMD-co-LA) was synthesized by ring-opening polymerization (ROP) as reported previously.^{27,31} First, 5.0 g of mPEG was placed into a Schlenk under nitrogen atmosphere. Second, 0.50 g of MMD, 4.5 g of LA and 400 μL of 0.25 mol L^{-1} Sn(Oct)₂ solution were added successively into the Schlenk, which was full of nitrogen. Finally, the Schlenk was immersed into an oil bath (120°C, 24 h). The copolymer was purified and dried at room temperature in vacuum for 24 h afterward.

Synthesis of P(MMD-co-LA)-g-PEI cationic copolymer

Synthesis of star-shaped copolymer

Using D-sorbitol as the initiator, P(MMD-co-LA) was prepared by ROP of MMD and LA as reported previously.^{27,31} Briefly, 0.03 g of D-sorbitol, 4.50 g of LA and 0.50 g of MMD were added into the Schlenk full of nitrogen. Then, the Schlenk was immersed in an oil bath at 125°C for 24 h. The copolymer was purified and dried at room temperature in vacuum for 24 h. The yield was 79.4%.

Synthesis of star-shaped P(MMD-co-LA)-COOH

Star-shaped P(MMD-co-LA)-COOH copolymer was synthesized as reported previously.³¹ P(MMD-co-LA) (3.0 g) was dissolved in dry 1,4-dioxane in a Schlenk flask (25 mL). Then, succinic anhydride (1.2 g), Et₃N (400 μL) and DMAP (1.5 g) were added in a three-necked flask. The reaction was performed at 25°C for 24 h under a dry nitrogen atmosphere. The copolymer was purified, and the chloroform solution was washed with hydrochloric acid aqueous solution (10%, v/v), saturated sodium bicarbonate solution and saturated sodium chloride solution three times, respectively. Finally, after rotary evaporation, star-shaped P(MMD-co-LA)-COOH

copolymer was obtained. The copolymer was dried at room temperature in vacuum.

Synthesis of cationic copolymer P(MMD-co-LA)-g-PEI

Cationic copolymer P(MMD-co-LA)-g-PEI was synthesized as reported previously.³¹ P(MMD-co-LA)-COOH (0.30 g), EDC (0.06 g) and NHS (0.03 g) were dissolved into 10 mL of DMSO and reacted at 25°C for 2 h. The solution was dripped into 5.0 mL of PEI solution (1.5 g, 10 kDa in DMSO). The mixture was stirred (25°C, 24 h) and dialyzed. Finally, the solution was lyophilized to obtain the cationic copolymer P(MMD-co-LA)-g-PEI.

Characterization of copolymers

Copolymer structures were characterized by Fourier transform infrared (FT-IR) spectrometer (FTS-6000; Bio-Rad Laboratories Inc., Hercules, CA, USA) and proton nuclear magnetic resonance (¹H NMR) spectrum (AV-400; Bruker Corporation, Billerica, MA, USA). The number-average molecular weight (M_n) of copolymers was obtained by gel permeation chromatography (GPC; Viscotek; Malvern Instruments, Malvern, UK; tetrahydrofuran was used as the eluent).

Preparation of MPs with different ratios of cationic copolymer and diblock copolymer mPEG-b-P(MMD-co-LA)

MPs with different ratios of cationic copolymer and diblock copolymer mPEG-b-P(MMD-co-LA) were prepared as reported previously.²⁴ Briefly, P(MMD-co-LA)-g-PEI and mPEG-b-P(MMD-co-LA) were separately dissolved in DMSO (5.0 mg mL^{-1}). Different volumes of copolymer solutions were blended to form suspension with various mass ratios. To 5.0 mL of phosphate-buffered saline (PBS), 1.0 mL of the blended polymer solution was added dropwise to form MPs.

Preparation of MPs/pEGFP-ZNF580 complexes

The concentration of pEGFP-ZNF580 was 0.10 mg mL⁻¹. To prepare MPs/pEGFP-ZNF580 complexes at different molar ratios of N/P (N/P = 1, 5, 10, 15, 20; [N/P molar ratios were calculated from weight of polymer and pDNA, N content in the polymer and P content in pDNA]), the solution was added dropwise into above MPs suspension under stirring. The complexes were allowed to stand for 25–30 minutes at room temperature before characterization.

Size distribution and zeta potential

The size distribution and zeta potential of MPs and MPs/pEGFP-ZNF580 complexes were determined using a Zetasizer 3000HS (Malvern Instruments) at the wavelength of 677 nm with a constant angle of 90°.

Biological characterization

Agarose gel electrophoresis

Agarose gel electrophoresis was performed as described in a previous work to assess the pEGFP-ZNF580 condensation ability of complex MPs.²⁷ Briefly, the MPs/pEGFP-ZNF580 with various N/P molar ratios were formed. Electrophoresis was used to observe the retarded location of DNA.

In vitro cytotoxicity

The cytotoxicity of MPs and MPs/pEGFP-ZNF580 complexes was determined by MTT assay using PEI 10 kDa as control as reported in previous research.^{24,27} Briefly, EA.hy926 cells (ATCC, Manassas, VA, USA) were seeded in serum-free medium for 12 h. Afterward, the medium was replaced by fresh growth medium. Then, MPs/pEGFP-ZNF580 complexes and MPs solution were added. After 48 h, an enzyme-linked immunosorbent assay reader (Titertek Multiscan MC) measured the optical density at the wavelength of 490 nm.

Wound-healing assay

The migration capability of cells treated with ZNF580 gene was measured as reported previously.^{24,27,31} Cells were transfected at the N/P molar ratio of 20. After 48 h, a “wound” scratch was formed by plastic pipette tip. The migration process was recorded using an inverted microscope.³⁸

Transfection in vitro

EA.hy926 cells were transfected as previously reported.²⁴ Before transfection, cells were incubated with serum-free medium for 12 h. The complexes were added into wells. After 4 h, the medium was replaced. The expression of GFP in cells was analyzed.

Results

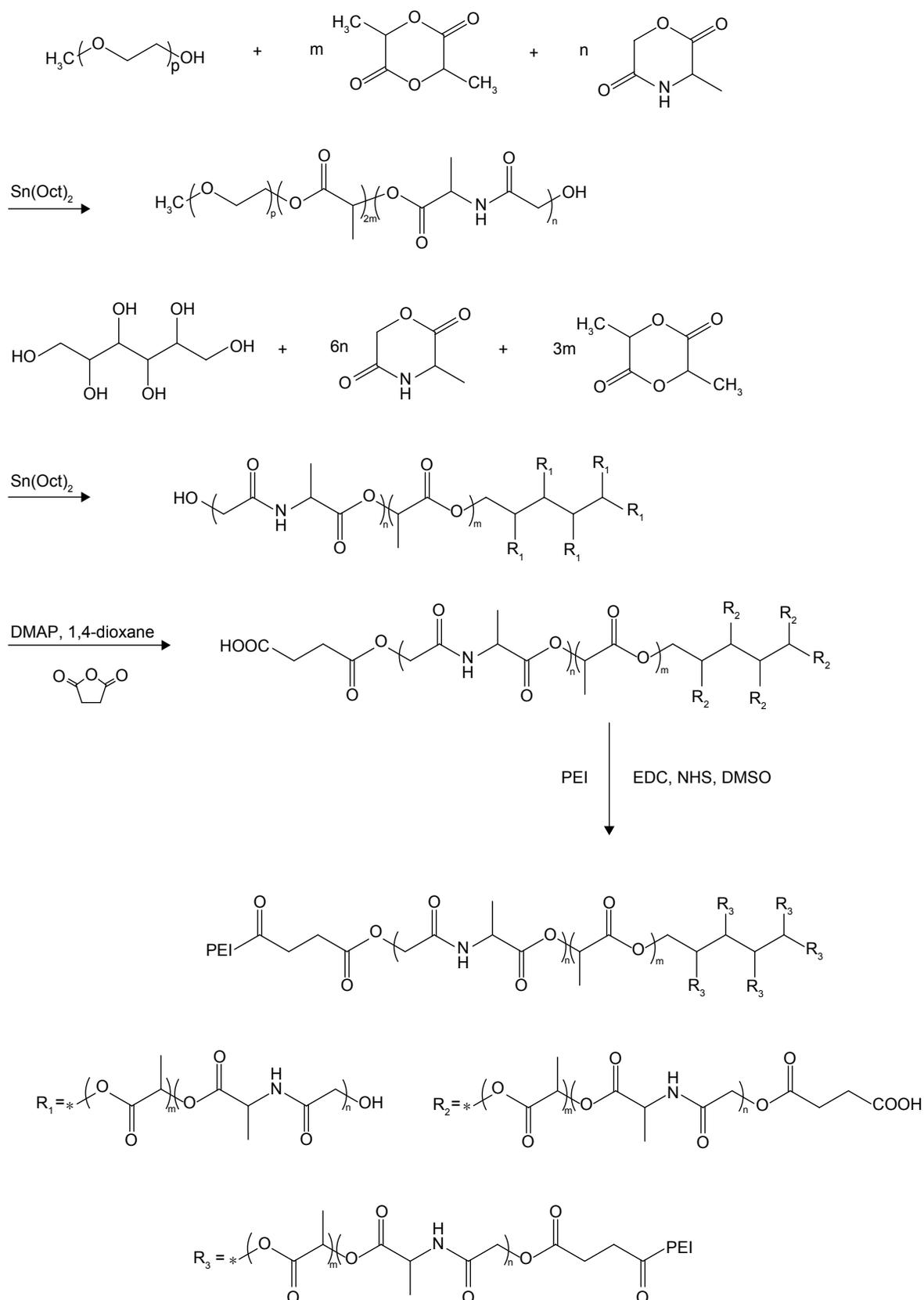
Synthesis of copolymer

The diblock copolymer mPEG-b-P(MMD-co-LA) was synthesized as shown in Scheme 2. Equipped with a hydrophilic corona, MPs presented high hydrophilicity and flexibility. Cationic copolymer P(MMD-co-LA)-g-PEI was synthesized through the following three steps. First, a hexahydroxyl-terminated star-shaped copolymer P(MMD-co-LA)-OH was prepared. Then, the star-shaped P(MMD-co-LA)-COOH was obtained by the reaction between succinic anhydride and P(MMD-co-LA)-OH. Finally, after the amidation reaction between PEI (10 kDa) and P(MMD-co-LA)-COOH, the cationic P(MMD-co-LA)-g-PEI copolymer was formed.

The structure of copolymer was characterized using FT-IR. A peak at 1,096 cm⁻¹ was observed for mPEG-b-P(MMD-co-LA) and star-shaped P(MMD-co-LA)-OH copolymers, which indicated that the ester groups (–O–CO–) existed in P(MMD-co-LA) blocks and mPEG. The absorption peak at 1,753 cm⁻¹ indicated the carboxylate group (–O–CO–). The peak at 1691 cm⁻¹ indicated the secondary amide (–NH–) in P(MMD-co-LA). The absorption peak at 2890 cm⁻¹ was assigned to the methylene group (–CH₂–) in mPEG-b-P(MMD-co-LA) copolymer (Figure 1).^{3,27}

The ¹H NMR spectra of mPEG-b-P(MMD-co-LA), star-shaped P(MMD-co-LA)-OH, the star-shaped carboxyl-terminated copolymer P(MMD-co-LA)-COOH and the cationic copolymer P(MMD-co-LA)-g-PEI are shown in Figure 2. The characteristic peaks of mPEG, LA and MMD residues were assigned as follows: 3.67 ppm (CH₂CH₂O in mPEG), 5.18 ppm (OCHCH₃CO in LA residue) and 4.6 ppm (COCH₂O in MMD residue). The spectrum of carboxyl-terminated star-shaped P(MMD-co-LA)-COOH (Figure 2C) showed a peak at 2.73 ppm, which was attributed to the terminal group (COCH₂CH₂COOH). Figure 2D demonstrates characteristic resonances of NHCH₂CH₂ protons in PEI at 2.4 ppm to 3.2 ppm.^{3,29} The content of LA and MMD was almost same in P(MMD-co-LA) blocks of mPEG-b-P(MMD-co-LA) and star-shaped P(MMD-co-LA)-OH calculated according to ¹H NMR. These results suggested that mPEG-b-P(MMD-co-LA) and cationic P(MMD-co-LA)-g-PEI copolymers were synthesized successfully.

The average molecular weight and the distribution of molecular weight of the synthesized copolymers were determined by GPC (Table 1). The M_n of the copolymer mPEG-b-P(LA-co-MMD) and star-shaped P(MMD-co-LA)-OH was calculated to be 14,943 and 20,638, respectively. The molecular weight distribution of copolymers is shown in Figure 3.



Scheme 2 Synthesis of diblock copolymer mPEG-b-P(MMD-co-LA) and cationic copolymer P(MMD-co-LA)-g-PEI.

Abbreviations: mPEG, methoxy-poly(ethylene glycol); P(MMD-co-LA), poly(3(S)-methyl-morpholine-2,5-dione-co-lactide); PEI, polyethylenimine; Sn(Oct)₂, stannous octoate; DMAP, 4-dimethylamino pyridine; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride; NHS, N-hydroxy succinimide; DMSO, dimethyl sulfoxide.

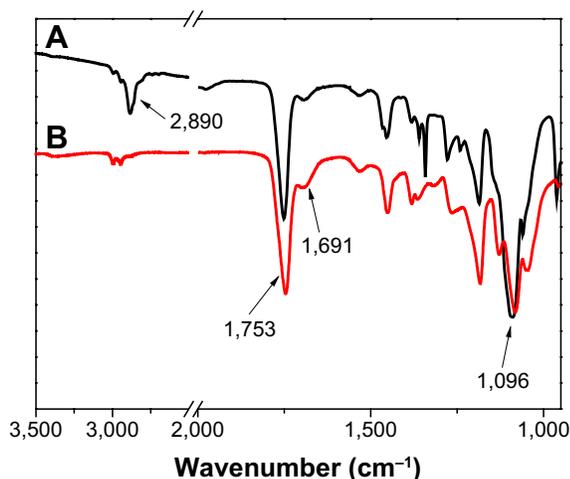


Figure 1 FT-IR spectra (A: copolymer mPEG-b-P(MMD-co-LA); B: star-shaped P(MMD-co-LA)-OH).

Abbreviations: FT-IR, Fourier transform infrared; mPEG, methoxy-poly(ethylene glycol); P(MMD-co-LA), poly(3(S)-methyl-morpholine-2,5-dione-co-lactide).

Zeta potential and hydrodynamic diameter of MPs and MPs/pEGFP-ZNF580 complexes

The size and zeta potential are important parameters that affect endocytosis. The particle size is an important factor for cellular uptake.³⁹ MPs with suitable diameter (below 200 nm) are much easily endocytosed and are less impressionable to be cleared by the reticuloendothelial system.²⁹ The positive-charged surface is more favorable for endocytosis. The co-self-assembly of copolymer mPEG-b-P(MMD-co-LA) and cationic copolymer P(MMD-co-LA)-g-PEI at different mass ratios could lead to form MPs with P(MMD-co-LA) core and mixed mPEG/PEI shell. Three kinds of MPs were prepared from mPEG-b-P(MMD-co-LA) and cationic copolymer P(MMD-co-LA)-g-PEI. The mass ratios

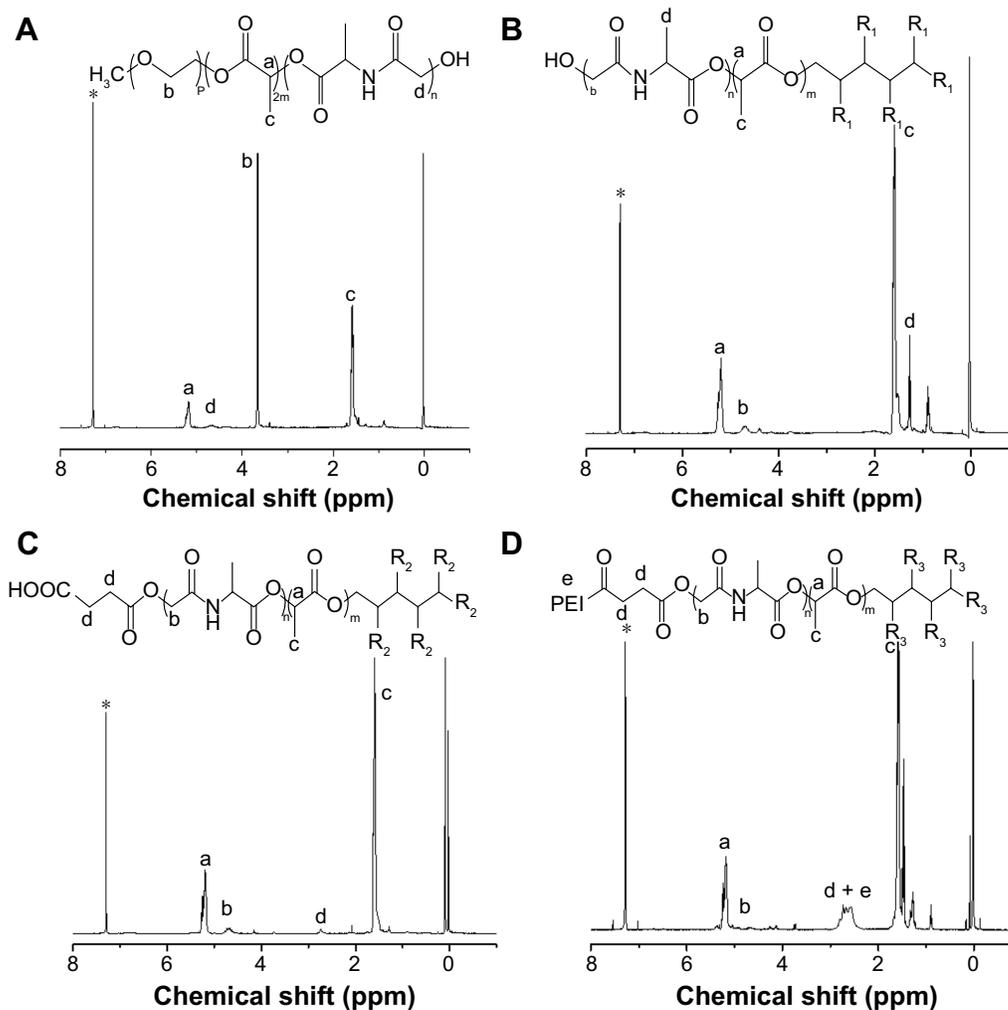


Figure 2 ¹H NMR spectra of copolymers.

Notes: (A) mPEG-b-P(MMD-co-LA). (B) Star-shaped P(MMD-co-LA)-OH. (C) Star-shaped P(MMD-co-LA)-COOH. (D) P(MMD-co-LA)-g-PEI. *CDCl₃ peaks. In Figure 2C, a: peak at 5.18 ppm attribute to (-OCHCH₂CO-, 1H) in LA residue; b: peak at 4.6 ppm attribute to (-COCH₂O-, 2H) in MMD residue; c: peak at 1.59 ppm attribute to (-COCH(CH₂)O-, 3H) in LA residue; d: peak at 2.73 ppm attributed to (-COCH₂CH₂COOH, 4H), the terminal group of P(MMD-co-LA)-COOH.

Abbreviations: ¹H-NMR, proton nuclear magnetic resonance; mPEG, methoxy-poly(ethylene glycol); P(MMD-co-LA), poly(3(S)-methyl-morpholine-2,5-dione-co-lactide); PEI, polyethylenimine.

Table 1 Characterization of diblock copolymer mPEG-b-P(MMD-co-LA) and star-shaped copolymer P(MMD-co-LA)-OH

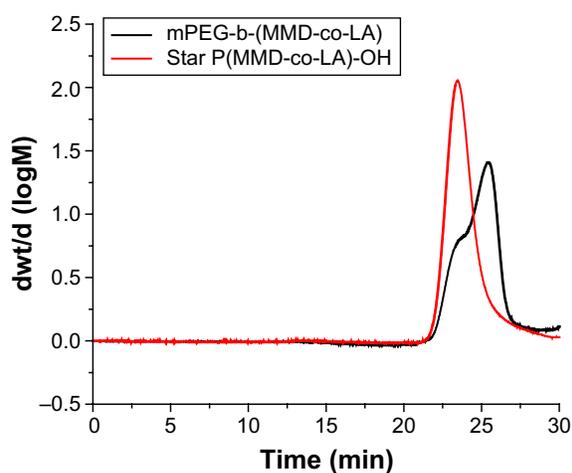
Sample ID	F_{LA}^a (%)	M_n^b	M_n^c	M_w^c	PDI ^c
mPEG-b-P(MMD-co-LA)	90.4	12,200	14,943	24,508	1.6401
P(MMD-co-LA)-OH	91.1	–	20,638	37,455	1.8148

Notes: ^aWeight content of LA in P(MMD-co-LA) block, calculated from ¹H NMR spectroscopy. ^bCalculated according to ¹H NMR. ^cDetermined by means of GPC using THF as eluent and polystyrene standards for calibration.

Abbreviations: mPEG, methoxy-poly(ethylene glycol); P(MMD-co-LA), poly(3(S)-methyl-morpholine-2,5-dione-co-lactide); M_n , number-average molecular weight; M_w , weight-average molecular weight; PDI, polydispersity index; LA, lactide; ¹H NMR, proton nuclear magnetic resonance; GPC, gel permeation chromatography; THF, tetrahydrofuran.

of mPEG-b-P(MMD-co-LA) to P(MMD-co-LA)-g-PEI were 3/1, 2/2 and 1/3. Complexes were prepared by mixing the MPs suspension and pEGFP-ZNF580 in PBS to form the MPs/pEGFP-ZNF580 complexes.

As shown in Figure 4, the hydrodynamic diameter (D_h) was measured by dynamic light scattering (DLS). The D_h values of these three kinds of MPs were 83.7 ± 0.3 nm, 83.1 ± 5.4 nm and 82.5 ± 6.5 nm, respectively. In addition, the size of MPs/pEGFP-ZNF580 complexes was affected by the N/P ratio. When the mass ratio of mPEG-b-P(MMD-co-LA) to P(MMD-co-LA)-g-PEI was 3:1, the increasing size of MPs/pEGFP-ZNF580 at N/P molar ratio of 5 was mainly due to the introduction of plasmid, while the reducing trend of size at higher N/P ratios may result from better plasmid-condensing effect and more compact structure of the complexes compared with that at N/P = 5.⁴⁰ The complexes with other mass ratios (2:2 and 1:3) of mPEG-b-P(MMD-co-LA) to P(MMD-co-LA)-g-PEI also showed the same tendency. More importantly, all of the MPs and the complexes were less than 120 nm and beneficial for endocytosis.

**Figure 3** GPC traces of diblock copolymer mPEG-b-P(MMD-co-LA) and star-shaped P(MMD-co-LA)-OH.

Abbreviations: GPC, gel permeation chromatography; mPEG, methoxy-poly(ethylene glycol); P(MMD-co-LA), poly(3(S)-methyl-morpholine-2,5-dione-co-lactide).

As shown in Figure 4, the MPs possessed positive charge because the grafted cationic PEI maintained relatively high zeta potential. With the increasing PEI content in shell (the mass ratios of mPEG-b-P(MMD-co-LA) to P(MMD-co-LA)-g-PEI from 3/1 to 1/3), the zeta potential of MPs increased from 19.6 ± 4.2 mV to 24.0 ± 0.6 mV. When the ratios of mPEG-b-P(MMD-co-LA) to P(MMD-co-LA)-g-PEI were same, the zeta potential of the complexes increased with the increasing molar ratio of N/P. These results indicated that the MPs/pEGFP-ZNF580 complexes may be suitable for cell adhesion, cell uptake and transfection.⁴¹

Agarose gel electrophoresis

Through electrostatic interaction, well-designed gene carriers can effectively condense negatively charged pEGFP-ZNF580 into MPs.⁴² In the present investigation, agarose gel electrophoresis assays were performed to confirm the successful binding between MPs and pEGFP-ZNF580.

As shown in Figure 5, the MPs/pEGFP-ZNF580 complexes could condense pEGFP-ZNF580 efficiently by changing N/P molar ratio. MPs with 3/1, 2/2 and 1/3 ratios of mPEG-b-P(MMD-co-LA) to P(MMD-co-LA)-g-PEI could retard the pEGFP-ZNF580 migration at N/P ratios of 15, 15 and 10, respectively. This tendency can be explained by that gene carriers help in efficient condensation of pEGFP-ZNF580 with high positive potential. Furthermore, when the content of PEI in the mixed shell increased, the condensation ability of MPs was enhanced gradually. However, when the content of mPEG in the mixed shell was high, pEGFP-ZNF580 could not be bound totally.

In vitro cytotoxicity

The cytotoxicity of MPs and MPs/pEGFP-ZNF580 complexes was evaluated. The concentration ranged from 10 to 100 mg L⁻¹. After 24 h, the relative viability of cells treated with the complexes was measured. Results showed that relative cell viability of MPs/pEGFP-ZNF580 complexes and MPs was much higher than high-molecular weight PEI at the same concentration (Figure 6). The cytotoxicity of MPs/pEGFP-ZNF580 complexes was lower after loading ZNF580 at the same concentration. This phenomenon could be attributed to the surface positive charge density, which reflected the cell compatibility of these polycationic MPs. High positive charges on the surface of MPs usually result in the death of cells.⁴³ At the same concentration, the cell viability presented a descending trend of MPs/pEGFP-ZNF580 complexes gradually along with the decrease in mass ratios of mPEG-b-P(MMD-co-LA) to P(MMD-co-LA)-g-PEI from 3/1 to 0/4. For example, the cell

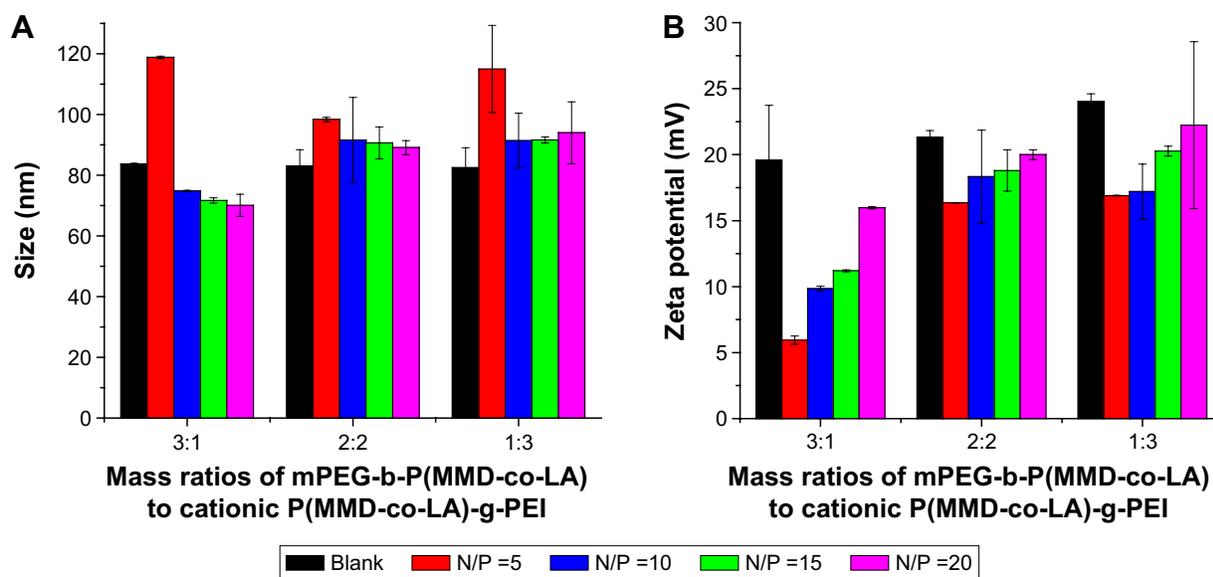


Figure 4 Hydrodynamic diameter (D_n) and zeta potential of MPs and MPs/pEGFP-ZNF580 complexes measured by DLS.

Notes: (A) Hydrodynamic diameter (D_n), (B) Zeta potential by DLS. N/P molar ratios were calculated from weight of polymer and pDNA, N content in the polymer and P content in pDNA.

Abbreviations: MPs, microparticles; DLS, dynamic light scattering; mPEG, methoxy-poly(ethylene glycol); P(MMD-co-LA), poly(3(S)-methyl-morpholine-2,5-dione-co-lactide); PEI, polyethylenimine.

viability of MPs/pEGFP-ZNF580 complexes ($60 \mu\text{g mL}^{-1}$) with the 3/1, 2/2, 1/3 and 0/4 mass ratios was $78.5\% \pm 0.4\%$, $72.6\% \pm 0.4\%$, $72.5\% \pm 0.2\%$ and $53.9\% \pm 0.2\%$, respectively. The main reason could be as follows: when the N/P was same, the zeta potential of MPs/pEGFP-ZNF580 complexes was improved as the content of positively charged PEI increased. High positive charges could be fatal to the cells. The chains of PEG could shield PEI and buffer the adsorption of polymers and cell membrane.²⁸ The increased PEG content enhanced the shielding effect greatly, thus decreasing the cytotoxicity. When the concentration was below $30 \mu\text{g mL}^{-1}$, MPs and complexes showed perfect cell viability. The corresponding value of the

relative cell viability was above 75%. These results manifested that the concentration of $30 \mu\text{g mL}^{-1}$ was the suitable standard for the MPs/pEGFP-ZNF580 complexes system used as low-cytotoxic gene carriers.

Cell migration

The migration and proliferation of different cell lines were evaluated via the scratch assay.⁴⁴ It is a relatively simple and straightforward approach for analyzing cell migration in vitro.⁴⁵ In the present study, an artificial scratch with parallel borders was mechanically created as shown in Figure 7. The cells migrated to close this “wound” scratch

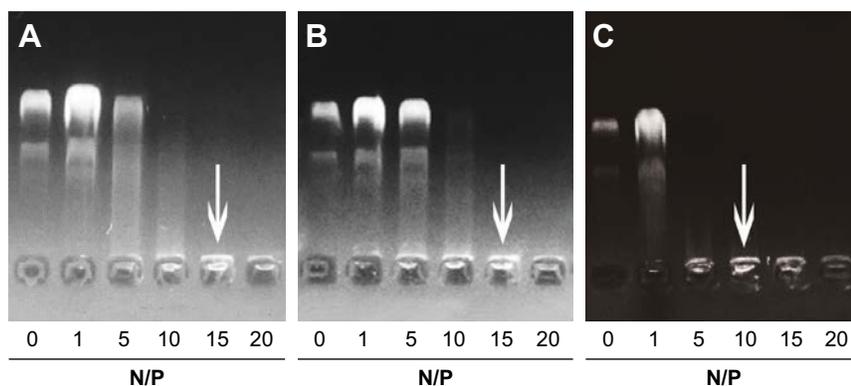


Figure 5 Agarose gel electrophoresis of MPs/pEGFP-ZNF580 complexes at various N/P ratios (0, 1, 5, 10, 15 and 20).

Notes: MPs and MPs/pEGFP-ZNF580 complexes at various N/P ratios (0, 1, 5, 10, 15 and 20). (A) MPs/pEGFP-ZNF580 complexes were prepared from MPs with 3/1 mass ratio of mPEG-b-P(MMD-co-LA) to P(MMD-co-LA)-g-PEI at various N/P ratios. (B) MPs/pEGFP-ZNF580 complexes were prepared from MPs with 2/2 mass ratio of mPEG-b-P(MMD-co-LA) to P(MMD-co-LA)-g-PEI at various N/P ratios. (C) MPs/pEGFP-ZNF580 complexes were prepared from MPs with 1/3 mass ratio of mPEG-b-P(MMD-co-LA) to P(MMD-co-LA)-g-PEI at various N/P ratios. The arrows indicate the N/P ratios where the DNA mobility is completely retarded. N/P molar ratios were calculated from weight of polymer and pDNA, N content in the polymer and P content in pDNA.

Abbreviations: MPs, microparticles; mPEG, methoxy-poly(ethylene glycol); P(MMD-co-LA), poly(3(S)-methyl-morpholine-2,5-dione-co-lactide); PEI, polyethylenimine.

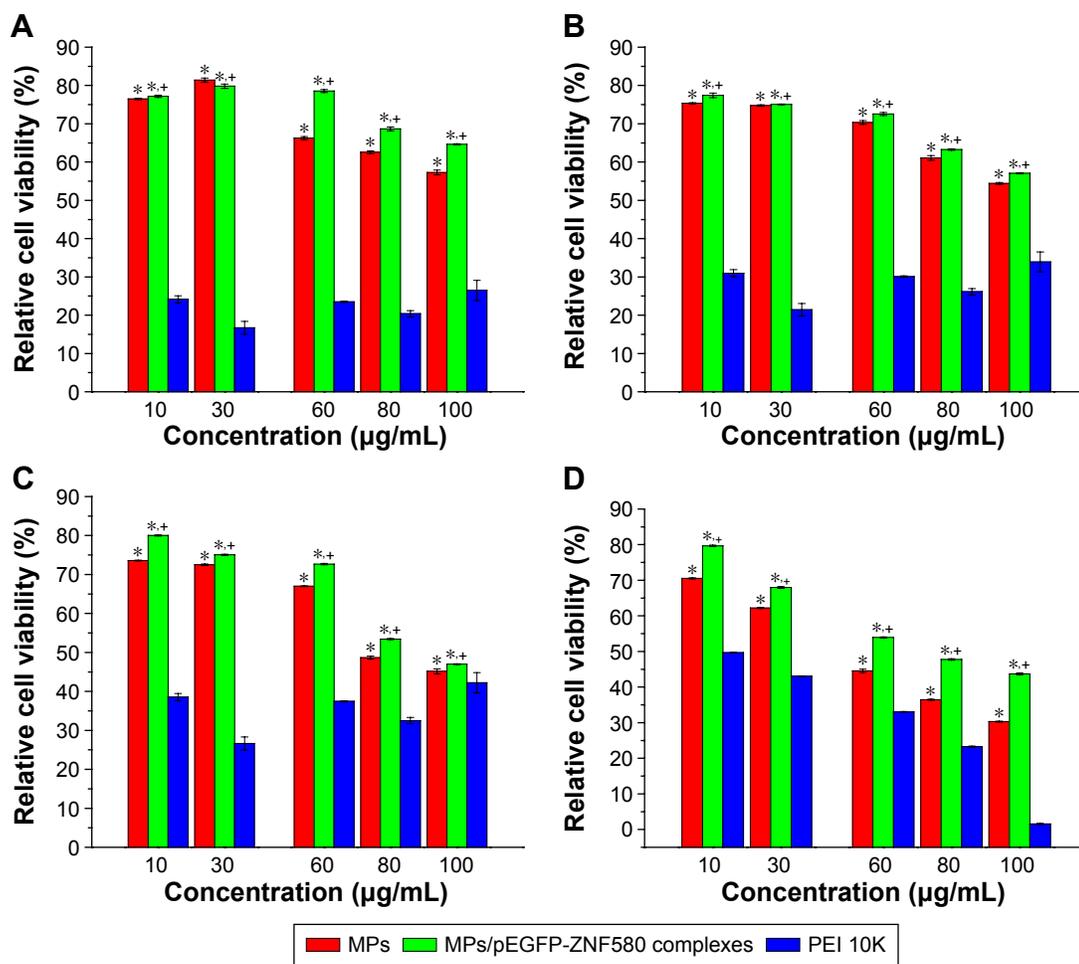


Figure 6 Relative cell viability of EA.hy926 cells with different concentrations of MPs and MPs/pEGFP-ZNF580 complexes at N/P molar ratio of 20. **Notes:** Cells treated with PEI ($M_w = 10$ kDa) served as the control group. (A) 3/1 mass ratio of mPEG-b-P(MMD-co-LA) to P(MMD-co-LA)-g-PEI. (B) 2/2 mass ratio of mPEG-b-P(MMD-co-LA) to P(MMD-co-LA)-g-PEI. (C) 1/3 mass ratio of mPEG-b-P(MMD-co-LA) to P(MMD-co-LA)-g-PEI. (D) 0/4 mass ratio of mPEG-b-P(MMD-co-LA) to P(MMD-co-LA)-g-PEI. (\pm SD, $n=3$, $*P<0.05$ as compared with the PEI 10k group; $^{+}P<0.05$ as compared with the MPs group). **Abbreviations:** MPs, microparticles; PEI, polyethylenimine; M_w , weight-average molecular weight; mPEG, methoxy-poly(ethylene glycol); P(MMD-co-LA), poly(3(S)-methyl-morpholine-2,5-dione-co-lactide); SD, standard deviation.

after cells treated with MPs/pEGFP-ZNF580 complexes which were prepared from 3/1, 2/2 and 1/3 ratios of mPEG-b-P(MMD-co-LA) to P(MMD-co-LA)-g-PEI.

Compared with cells without any treatment, cells treated with MPs/pEGFP-ZNF580 complexes showed a high migration rate (more than 90%). The migration rate of cells treated with complexes with the 3/1, 2/2 and 1/3 ratios of mPEG-b-P(MMD-co-LA) to P(MMD-co-LA)-g-PEI was slightly higher than the group treated with the complex with the ratio of 0/4. This phenomenon is mainly due to the high cytotoxicity of the complexes with ratio of 0/4. The 2/2 and 1/3 ratio groups showed relatively high cell migration.

In vitro transfection

The pEGFP-ZNF580 could be transported into EA.hy926 cells by MP gene carriers. After escaping from lysosome,

pEGFP-ZNF580 entered into the nucleus, and expressed effectively. In order to determine qualitatively whether the pEGFP-ZNF580 had been transfected into the EA.hy926 cells, the expression of GFP in ECs was observed under the fluorescent microscope. The concentration of MPs/pEGFP-ZNF580 complexes suspension was $30 \mu\text{g mL}^{-1}$ (N/P = 20). Both cells treated with pEGFP-ZNF580 and complexes with 0/4 ratio of mPEG-b-P(MMD-co-LA) to P(MMD-co-LA)-g-PEI served as the control groups.

As shown in Figure 8, cells labeled with green fluorescence were observed obviously in test groups, which indicated that the plasmids had transfected and expressed successfully in cells. Furthermore, the MPs/pEGFP-ZNF580 complexes with 1/3 mass ratio of mPEG-b-P(MMD-co-LA) to P(MMD-co-LA)-g-PEI showed high transfection efficiency. This phenomenon could be attributed to the high positive charge

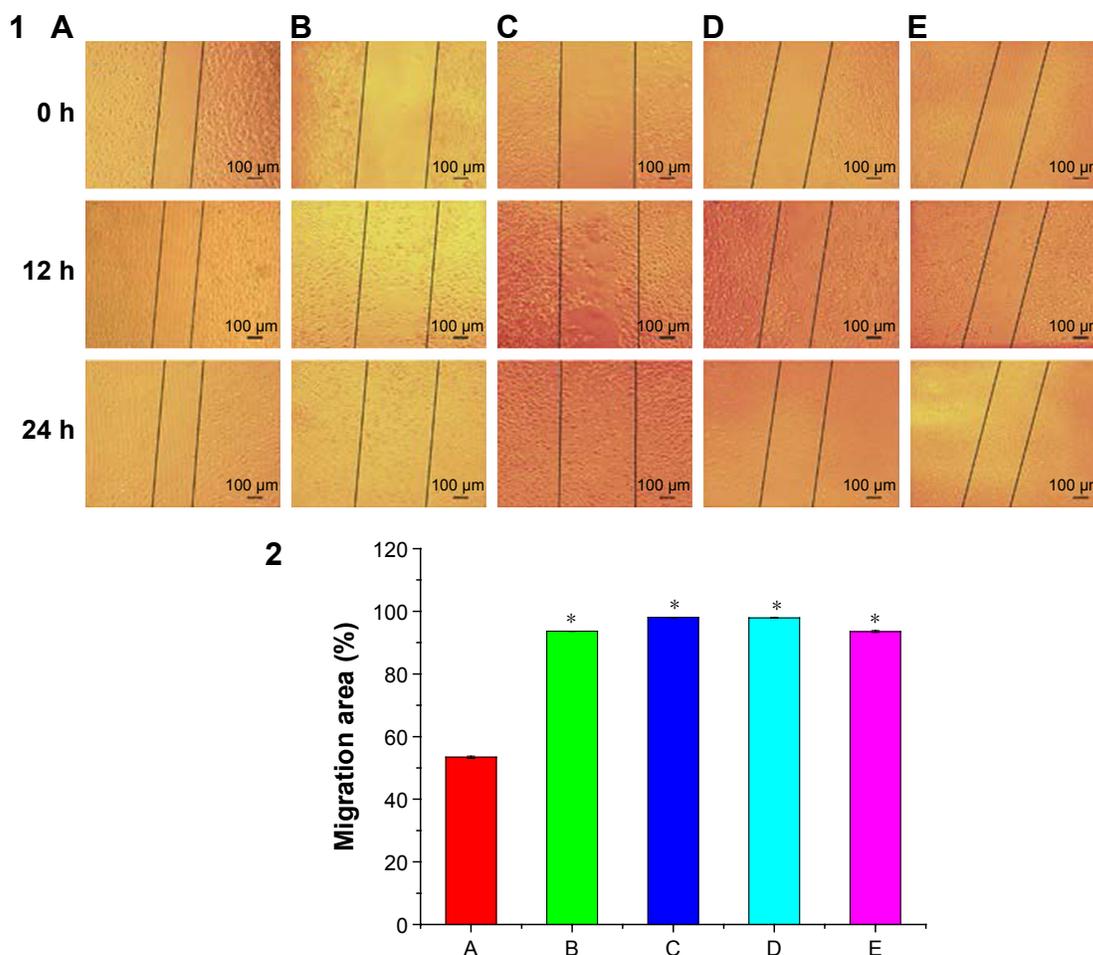


Figure 7 Migration process of transfected EA.hy926 cells by MPs/pEGFP-ZNF580 complexes at N/P molar ratio of 20 at different (1) time points and (2) migration area after 24 h calculated by Image-Pro Plus (version 6.0).

Notes: Cells without any treatment served as the control. (A) Cells without any treatment. (B) Cells treated with MPs/pEGFP-ZNF580 complexes which were prepared from MPs with 3/1 mass ratio of mPEG-b-P(MMD-co-LA) to P(MMD-co-LA)-g-PEI. (C) Cells treated with MPs/pEGFP-ZNF580 complexes which were prepared from MPs with 2/2 mass ratio of mPEG-b-P(MMD-co-LA) to P(MMD-co-LA)-g-PEI. (D) Cells treated with MPs/pEGFP-ZNF580 complexes which were prepared from MPs with 1/3 mass ratio of mPEG-b-P(MMD-co-LA) to P(MMD-co-LA)-g-PEI. (E) Cells treated with MPs/pEGFP-ZNF580 complexes which were prepared from MPs with 0/4 mass ratio of mPEG-b-P(MMD-co-LA) to P(MMD-co-LA)-g-PEI (\pm SD, $n=3$, $*P<0.05$ vs control group).

Abbreviations: MPs, microparticles; mPEG, methoxy-poly(ethylene glycol); P(MMD-co-LA), poly(3(S)-methyl-morpholine-2,5-dione-co-lactide); PEI, polyethylenimine; SD, standard deviation.

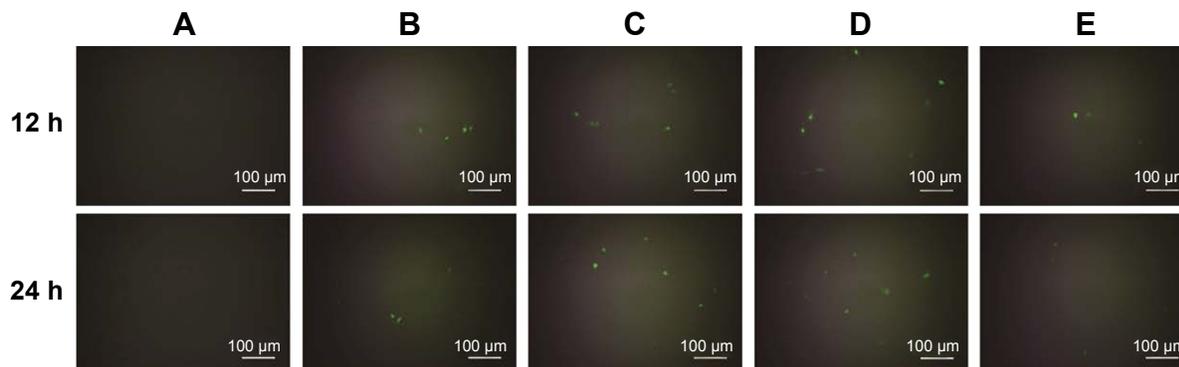


Figure 8 Fluorescence images of EA.hy926 cells transfected by complexes at different ratios of mPEG-b-P(MMD-co-LA) to P(MMD-co-LA)-g-PEI at the N/P molar ratio of 20 and time intervals of 12 and 24 h.

Notes: (A) Cells treated with pEGFP-ZNF580. (B) Cells treated with MPs/pEGFP-ZNF580 complexes which were prepared from 3/1 mass ratio of mPEG-b-P(MMD-co-LA) to P(MMD-co-LA)-g-PEI. (C) Cells treated with MPs/pEGFP-ZNF580 complexes which were prepared from 2/2 mass ratio of mPEG-b-P(MMD-co-LA) to P(MMD-co-LA)-g-PEI. (D) Cells treated with MPs/pEGFP-ZNF580 complexes which were prepared from 1/3 mass ratio of mPEG-b-P(MMD-co-LA) to P(MMD-co-LA)-g-PEI. (E) Cells treated with MPs/pEGFP-ZNF580 complexes which were prepared from 0/4 mass ratio of mPEG-b-P(MMD-co-LA) to P(MMD-co-LA)-g-PEI.

Abbreviations: mPEG, methoxy-poly(ethylene glycol); P(MMD-co-LA), poly(3(S)-methyl-morpholine-2,5-dione-co-lactide); PEI, polyethylenimine; MPs, microparticles.

density of PEI, which had unique proton sponge effect without additional disruptive reagents of endosome.^{46–49}

Discussion

To obtain gene carriers with good transfection efficiency and less cytotoxicity for the proliferation of ECs, amphiphilic copolymers P(MMD-co-LA)-g-PEI and mPEG-b-P(MMD-co-LA) were prepared in the present study. To increase the transfection efficiency, PEI with low molecular weight was grafted onto the end of star-shaped copolymer P(MMD-co-LA)-COOH, which has the dense molecular architecture and flexible tailored structure. The MPs consisting of a P(MMD-co-LA) core and a mixed mPEG/PEI shell could be formed by co-self-assembly, in which the hydrophilic mPEG preferentially stretches at the outmost surface of the MPs. It can shield PEI to decrease its cytotoxicity and increase the circulation time and stability in the shell of gene delivery micelles.⁵⁰ The pEGFP-ZNF580 could be condensed and loaded by these MPs to form complexes. In the present study, to testify whether the different mPEG/PEI ratios of complexes influence the transfection efficiency of complexes dramatically or not, a series of MPs consisting of different mass ratios of mPEG-b-P(MMD-co-LA) to P(MMD-co-LA)-g-PEI were prepared.

The results of gel electrophoresis showed that MPs could condense and load pEGFP-ZNF580 efficiently by changing N/P molar ratios. The cytotoxicity of gene carriers demonstrated that these shielded gene transfer systems have lower cytotoxicity and prolong circulation times in vivo. Due to the shielding effect of PEG, when the mass ratio of mPEG-b-P(MMD-co-LA) to P(MMD-co-LA)-g-PEI was 3/1, the MPs co-self-assembled by these two kinds of copolymers showed the lowest cytotoxicity. Moreover, the special structure of MPs could also contribute to reduce the cytotoxicity of MPs/pEGFP-ZNF580 complexes.

The transfection efficiency of MPs/pEGFP-ZNF580 complexes was further evaluated. With the increasing content of PEI, the transfection of ECs could be improved gradually. The star-shaped structure of P(MMD-co-LA) carries high content of PEI which helps to enhance transfection efficiency, through which the endocytosis could be improved. These results showed that gene carrier with 1/3 ratio of mPEG-b-P(MMD-co-LA) to P(MMD-co-LA)-g-PEI was the most suitable for gene transfection among the groups. Furthermore, because of the low cytotoxicity, the transfection efficiency of complexes with 3/1, 2/2 and 1/3 ratios was higher than the complexes with 0/4 ratio of mPEG-b-P(MMD-co-LA) to P(MMD-co-LA)-g-PEI. In addition, the migration of ECs treated with MPs/pEGFP-ZNF580

complexes was much better than untreated cells, and the difference between three kinds of complexes was not obvious. According to the above results, the gene carriers formed via co-self-assembly had low cytotoxicity and high transfection efficiency and enhanced the property of cell migration and proliferation.

Conclusion

We synthesized a cationic copolymer P(MMD-co-LA)-g-PEI and a diblock copolymer mPEG-b-P(MMD-co-LA). A series of MPs with suitable diameter and positive charge were prepared. pEGFP-ZNF580 was effectively delivered into ECs by these MP complexes. The MPs as gene carriers exhibited low cytotoxicity and facilitated enhanced proliferation as well as improved migration of ECs. This kind of gene carrier might have great application for modifying artificial blood vessels in order to accomplish endothelialization.

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Disclosure

The authors report no conflicts of interest in this work.

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