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CREDVW-Linked Polymeric Micelles As a Targeting Gene Transfer Vector for Selective Transfection and Proliferation of Endothelial Cells

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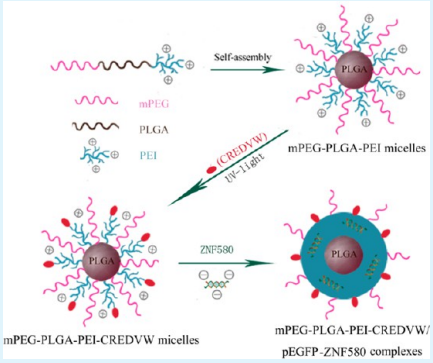
ABSTRACT: Nowadays, gene transfer technology has been widely used to promote endothelialization of artificial vascular grafts. However, the lack of gene vectors with low cytotoxicity and targeting function still remains a pressing challenge. Herein, polyethylenimine (PEI, 1.8 kDa or 10 kDa) was conjugated to an amphiphilic and biodegradable diblock copolymer poly(ethylene glycol)-*b*-poly(lactide-*co*-glycolide) (mPEG-*b*-PLGA) to prepare mPEG-*b*-PLGA-*g*-PEI copolymers with the aim to develop gene vectors with low cytotoxicity while high transfection efficiency. The micelles were prepared from mPEG-*b*-PLGA-*g*-PEI copolymers by self-assembly method. Furthermore, Cys-Arg-Glu-Asp-Val-Trp (CREDVW) peptide was linked to micelle surface to enable the micelles with special recognition for endothelial cells (ECs). In addition, pEGFP-ZNF580 plasmids were condensed into these CREDVW-linked micelles to enhance the proliferation of ECs. These CREDVW-linked micelle/pEGFP-ZNF580 complexes exhibited low cytotoxicity by MTT assay. The cell transfection results demonstrated that pEGFP-ZNF580 could be transferred into ECs efficiently by these micelles. The results of Western blot analysis showed that the relative ZNF580 protein level in transfected ECs increased to 76.9%. The rapid migration of transfected ECs can be verified by wound healing assay. These results indicated that CREDVW-linked micelles could be a suitable gene transfer vector with low cytotoxicity and high transfection efficiency, which has great potential for rapid endothelialization of artificial blood vessels.



KEYWORDS: *peptide, micelles, targeting, gene vector, polyethylenimine, endothelial cells*

1. INTRODUCTION

In modern society, vascular diseases have become one of the main troubles in people's common lives.[1] Artificial blood vessels have been developed and widely used to treat vascular diseases. Although artificial blood vessels with diameter >6 mm have been successfully used in clinical applications, the small diameter artificial vascular grafts usually demonstrate poor clinical performance. To address this issue, many strategies have been developed to improve surface hemocompatibility,[2−7] adhesion and proliferation of endothelial cells as well as rapid endothelialization of artificial blood vessels.[1,8−12]

In recent years, gene therapy for vascular diseases has received great attention.[13,14] Despite encouraging results from clinical trials, the lack of safe and effective gene vectors is still a major barrier for gene therapy in clinic applications.[15] Over the past several decades, numerous efforts have been dedicated to develop the safe and efficient gene vectors.[16−18] Lately, polymeric micelles, which are prepared by self-assembly from amphiphilic and cationic copolymers, have been developed as a kind of gene vectors for DNA or siRNA delivery.[19] Moreover, the micelles of multiple block polymers can act as effective gene vectors with various special physical and biological functions.[20,21]

Among synthetic cationic homopolymers for gene delivery, polyethylenimine (PEI) offers high positive charge density for strong DNA affinity and exhibits a unique proton sponge effect over a broad pH range, which allows gene transfer to cytoplasm without endosome disruptive reagents.[22−24] It should be noted that PEI with high molecular weight exhibits high transfection efficiency. Unfortunately, this kind PEI usually shows high cytotoxicity in vitro, as well as acute and long-term toxicity in vivo because of the excessive positive charge.[25] On the other

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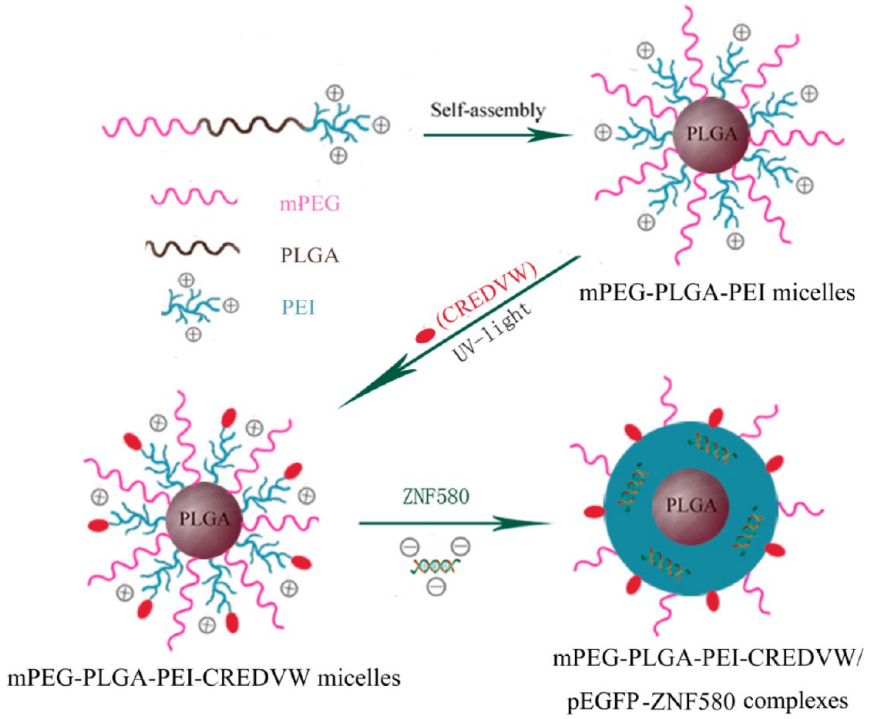
Scheme 1. Self-Assembly of mPEG-*b*-PLGA-*g*-PEI-CREDVW Micelles and the Formation of mPEG-*b*-PLGA-*g*-PEI-CREDVW/pEGFP-ZNF580 Complexes



hand, low-molecular-weight PEI, such as 1.8 kDa branched PEI, possesses acceptable cytotoxicity but displays much lower transfection efficiency than 25 kDa PEI.[26] To prepare nontoxic and efficient gene vectors, researchers have made tremendous efforts to modify PEI.[27−31]

Cross-linking low molecular weight PEI by biodegradable disulfide linkages can prepare cross-linked PEI (high molecular weight) with high gene transfection efficiency and low cytotoxicity,[32] but it is difficult to control the reaction degree and avoid side reactions. Alternatively, poly(ethylene glycol) (PEG) has often been used to modify PEI through forming the shell of gene delivery micelles to decrease cytotoxicity, meanwhile increase the long circulation and stability. Recently, amphiphilic block copolymers comprising PEG, poly(ε-caprolactone) (PCL) and PEI segments have been demonstrated to be a kind of effective, biodegradable and biocompatible transfer vectors.[27,33,34] In particular, we have grafted PEG and PEI onto the biodegradable polymers, and used them as gene vectors to enhance the proliferation of endothelial cells (ECs).[35,36] These gene vectors exhibit good gene transfection efficiency and low cytotoxicity. In order to prepare gene vectors by a convenient method, we have used self-assembly of two block copolymers to form the complex micelles, which consist of a biodegradable poly(lactide-*co*-glycolide) (PLGA) core and a mixed PEG/PEI shell. The cytotoxicity and proliferation of ECs could be tuned by changing the ratio of mPEG to PEI in the mixed shell.[37]

To further enhance the rapid endothelialization in coculture systems, it is necessary to develop targeting gene vectors with the specific recognition of ECs. Arg-Gly-Asp (RGD) peptide derived from extracellular matrix (ECM) components has been widely used as cell recognition site.[38,39] RGD modified biomaterial surfaces promote cell adhesion, but without cell selectivity, because RGD predominantly interacts with the integrin β-subunit, whereas a various integrins possess the same β-subunit, such as αIIbβ3 and αvβ3. RGD is insufficient to selectively adhere ECs over other cells. On the other hand, Arg-Glu-Asp-Val (REDV) peptide is a fibronectin-derived peptide which is known for its ability to selectively bind ECs over smooth muscle cells (SMCs).[40,41] The REDV sequence is one of the main recognition sites for integrin α4β1 binding, which leads to specific interaction between fibronectin and ECs.[42] Usually, REDV is used to modify stent and graft surfaces to induce selective adhesion of ECs.[43,44] For example, Liu et al. conjugated REDV to the surface of titanium stent for selective ECs attachment.[39] Ji et al. demonstrated that the competitive adhesion and growth of ECs over SMCs could be enhanced through the specific recognition of REDV peptide.[40]

For re-endothelialization, some genes and growth factors, such as ZNF580 gene, VEGF and PDGF factors, have been used.[45−47] In particular, our group has demonstrated that the proliferation and migration of ECs could be promoted by ZNF580 gene significantly.[35,36] If REDV and PEG are conjugated with PEI, the gene vectors will have the advantages of REDV (specific recognition) and PEG (low cytotoxicity, long circulation, and stability). To the best of our knowledge, these special gene vectors and their complexes with ZNF580 plasmid are still unexplored.

In the present study, we synthesized the gene transfer vectors with both low cytotoxicity and high targeting function to ECs. Polymeric micelles were prepared by self-assembly from block copolymers of methoxy-poly(ethylene glycol)-*b*-poly(lactide-*co*-glycolide)-*g*-polyethylenimine (mPEG-*b*-PLGA-*g*-PEI(1.8 kDa, or 10 kDa)), and then CREDVW peptide was linked to PEI to obtain the CREDVW-linked micelles (mPEG-*b*-PLGA-*g*-PEI-CREDVW). Based on the positive charged surface, CREDVW-linked micelles can compact pEGFP-ZNF580 plasmids to form CREDVW-linked micelle/pEGFP-ZNF580 complexes

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(Scheme 1). The CREDVW-linked micelles as gene transfer vectors were characterized by DNA condensation and cytotoxicity. Transfection efficiency, wound healing assay and Western blot analysis were used to evaluate the transfected ECs by these mPEG-*b*-PLGA-*g*-PEI-CREDVW/pEGFP-ZNF580 complexes in vitro.

2. EXPERIMENTAL SECTION

2.1. Materials. Poly(ethylene glycol) monomethyl ether (mPEG, M_w = 1.9 kDa), stannous octoate ($Sn(Oct)_2$), polyethylenimine (branched PEI, M_w = 1.8 kDa, and 10 kDa), 4-dimethylamino pyridine (DMAP), *N*-hydroxy succinimide (NHS), 1-ethyl-3-(3-(dimethylamino)propyl)-carbodiimide hydrochloride (EDC), diallylcarbamyl chloride, and 2,2-dimethoxy-2-phenylacetophenone (DMPA) were purchased from Sigma-Aldrich (St. Louis, USA). L-Lactide (L-LA) and glycolide (GA) were obtained from Foryou Medical Device Co., Ltd. (Huizhou, China). Succinic anhydride, triethylamine (Et_3N), 1,4-dioxane, 1-methyl-2-pyrrolidinone (NMP) and toluene were purchased from Institute of Guangfu Fine Chemical Research (Tianjin, China). The solvents were dried by reflux over Na and distilled just before use. Dimethyl sulfoxide (DMSO) was purchased from Sigma (St. Louis, MO). Lipofectamine 2000 reagent was purchased from Invitrogen (Grand Island, USA). BCA protein assay kit was purchased from Solarbio Science and Technology Co., Ltd. (Beijing, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), rabbit antihuman ZNF580 polyclonal antibody and goat antirabbit IgG were purchased from Abcam (HK) Ltd. (Hong Kong, China). pEGF-ZNF580 plasmids (pDNA) were preserved by department of physiology and pathophysiology, logistics university of Chinese People's Armed Police Force. Other chemicals were analytically pure from Jiangtian Chemicals (Tianjin, China).

2.2. Synthesis of mPEG-*b*-PLGA-*g*-PEI. *2.2.1. Synthesis of mPEG-b-PLGA−OH.* Monohydroxy-terminated block copolymers were synthesized by ring-opening polymerization of LA and GA using mPEG as a macroinitiator and $Sn(Oct)_2$ as a catalyst. LA (4.5 g, 31 mmol), GA (0.50 g, 4.3 mmol), $Sn(Oct)_2$ toluene solution (0.40 mL, 0.25 M), and mPEG (1.9 g, 1.0 mmol) were added into a flame-dried and nitrogen-purged Schlenk flask. The flask was sealed and maintained at 110 °C for 24 h. The product was purified by twice precipitating into cold *n*-hexane from chloroform solution, and was then vacuum-dried at 37 °C until constant weight.

2.2.2. Synthesis of mPEG-b-PLGA-COOH. The synthesis of mPEG-*b*-PLGA-COOH was accomplished by hydroxyl esterification with succinic anhydride, according to a similar method reported previously for esterification of monohydroxy terminated PEG (mPEG−OH).[48] mPEG-*b*-PLGA-OH (5.0 g, 0.72 mmol) dissolved in 20 mL of 1,4-dioxane, Et_3N 200 μL, DMAP (0.89 g, 7.2 mmol), and succinic anhydride (0.73 g, 7.2 mmol) were added into the nitrogen-purged Schlenk flask, sealed, and maintained at 25 °C for 24 h. The reaction mixture was precipitated in cold hexane to obtain the crude polymer. It was redissolved in dichloromethane (DCM). The DCM solution was washed twice with sodium bicarbonate solution, three times with aqueous hydrochloric acid (10% in v/v) and then four times with a saturated NaCl solution. The organic phase was then isolated, dried over anhydrous sodium sulfate, and filtered. MPEG-*b*-PLGA-COOH recovered by precipitation into cold hexane was vacuum-dried to a constant weight at 37 °C to eliminate any water and succinic anhydride residues.

2.2.3. Synthesis of mPEG-b-PLGA-g-PEI. MPEG-*b*-PLGA-COOH (0.50 g, 0.070 mmol) diblock copolymer was dissolved in 5.0 mL of DMSO, then the solution was transferred into a dried Schlenk flask under nitrogen atmosphere. EDC (0.067 g, 0.35 mmol) and NHS (0.040 g, 0.35 mmol) were added into the Schlenk flask, and stirred at 25 °C for 2 h. Then PEI 1.8 kDa (0.38 g, 0.21 mmol) dissolved in DMSO was also added and reacted at 25 °C for 24 h under a nitrogen atmosphere. mPEG-*b*-PLGA-*g*-PEI(1.8 kDa) copolymer was then collected by dialyzing and lyophilization.

MPEG-*b*-PLGA-*g*-PEI(10 kDa) copolymer was prepared by analogous method.

2.3. Synthesis of mPEG-*b*-PLGA-*g*-PEI-DA. MPEG-*b*-PLGA-*g*-PEI(1.8 kDa) (0.20 g, 0.015 mmol) and Et_3N (32 μL) were dissolved into 5.0 mL of NMP in a dried a three-necked flask. Then diallylcarbamyl chloride (34 μL) was dissolved in NMP (5 mL) in a dried constant pressure drop funnel (10 mL) and the mixed solution was added to the three-necked flask slowly for 2.0 h at 0 °C. The reaction was carried out at 0 °C overnight. The product was collected by filtering, dialyzing and lyophilization.

2.4. Characterization of the Copolymers. ^{1}H NMR spectra of the copolymers were recorded with a Bruker Avance spectrometer (AV-400, Bruker, Karlsruhe, Germany) operating at 400 MHz using deuterated chloroform ($CDCl_3$) as a solvent and tetramethylsilane (TMS) as the internal standard. The average molecular weight (M_w, M_n) and molecular weight polydispersity index (M_w/M_n, PDI) of the products were measured by a Waters 1515 gel permeation chromatographer (GPC, Waters company, Milford, USA). The measurements were performed using a series of narrow polystyrene standards for the calibration of the columns and THF as the eluent at a flow rate of 1.0 mL min^{-1} at 25 °C. The elemental analysis was performed by elemental analyzer (vario EL CUBE, elementar, Germany).

2.5. Preparation and Characterization of Micelles. *2.5.1. Preparation of mPEG-b-PLGA-g-PEI-CREDVW micelles.* MPEG-*b*-PLGA-*g*-PEI-DA was dissolved in DMSO (5.0 mg mL^{-1}), and 2.0 mL of the solution was added dropwise to 20 mL of phosphate buffer saline (PBS, pH 7.4) in a beaker. The mixture solution was sonicated for 0.5 h to acquire uniform diameter. Then 1.0 mg DMPA dissolved in 1.0 mL DMSO, 14 mg CREDVW peptide dissolved in 2.0 mL PBS (pH 7.4) were added to the mixture solution, and the solution was treated by UV-light for 10 min. The micelles of mPEG-*b*-PLGA-*g*-PEI-CREDVW were obtained by dialyzing for further study.

The micelles of mPEG-*b*-PLGA-*g*-PEI-REVD as control were prepared by analogous method by using CREVDW peptide (without targeting function) instead of targeting CREDVW peptide.

2.5.2. Characterization of mPEG-b-PLGA-g-PEI-CREDVW Micelles. In order to verify CREDVW peptide linking to the micelles successfully and determine the quantity of peptide, a series of peptide solutions with concentrations varying from 1.0 to 10 mg L^{-1} were prepared in PBS (pH 7.4) to acquire the standard curve. Then the fluorescence emission spectrum of mPEG-*b*-PLGA-*g*-PEI and mPEG-*b*-PLGA-*g*-PEI-CREDVW (mPEG-*b*-PLGA-*g*-PEI-REDV) micelles solution were measured by Cary Eclipse (VARIAN) fluorescence spectrophotometer. The fluorescence emission spectrum of the mixed solution of CREDVW and mPEG-*b*-PLGA-*g*-PEI after dialysis was also measured. The excitation wavelength was chosen as 280 nm and the fluorescent intensity at 350 nm was monitored.

2.5.3. Preparation of pEGFP-ZNF580-Loaded Micelles. The plasmid of pEGFP-ZNF580 was diluted to 50 μg mL^{-1} with PBS (pH 7.4). The complexes were prepared by adding plasmid solution at various N/P molar ratios (5, 10, 15 and 20) to micelles solution (0.25 mg mL^{-1}). N/P molar ratios were calculated from weight of polymer and plasmid, N content in the polymer, and P content in plasmid. Before characterization and further experiments, the complexes solution was stirred for 3 h at room temperature.

2.5.4. Hydrodynamic Diameter and Zeta Potential of Blank Micelles and Complexes. The diameter and zeta potential of micelles and complexes were measured using a Zetasizer 3000HS (Malvern Instrument, Inc., Worcestershire, UK) at the wavelength of 677 nm with a constant angle of 90°.

2.5.5. Morphology of Complexes. The morphology of the dried complexes of mPEG-*b*-PLGA-*g*-PEI(1.8 kDa, 10 kDa)-CREDVW/pDNA at N/P 20 was characterized by a Japanese model JEM-2100F at 200 kV accelerating voltages.

2.6. Agarose Gel Electrophoresis. Agarose gel electrophoresis was performed to investigate the effectiveness of micelles condensing pDNA. The complexes with various N/P molar ratios varying from 5 to 20 were prepared as described above. The complexes solution was mixed with 6x loading buffer (2 μL) and loaded into a agarose gel (0.80 wt %) containing 0.50 μg mL^{-1} ethidium bromide. Electrophoresis was performed in 1x TAE buffer at 120 V for 30 min. DNA

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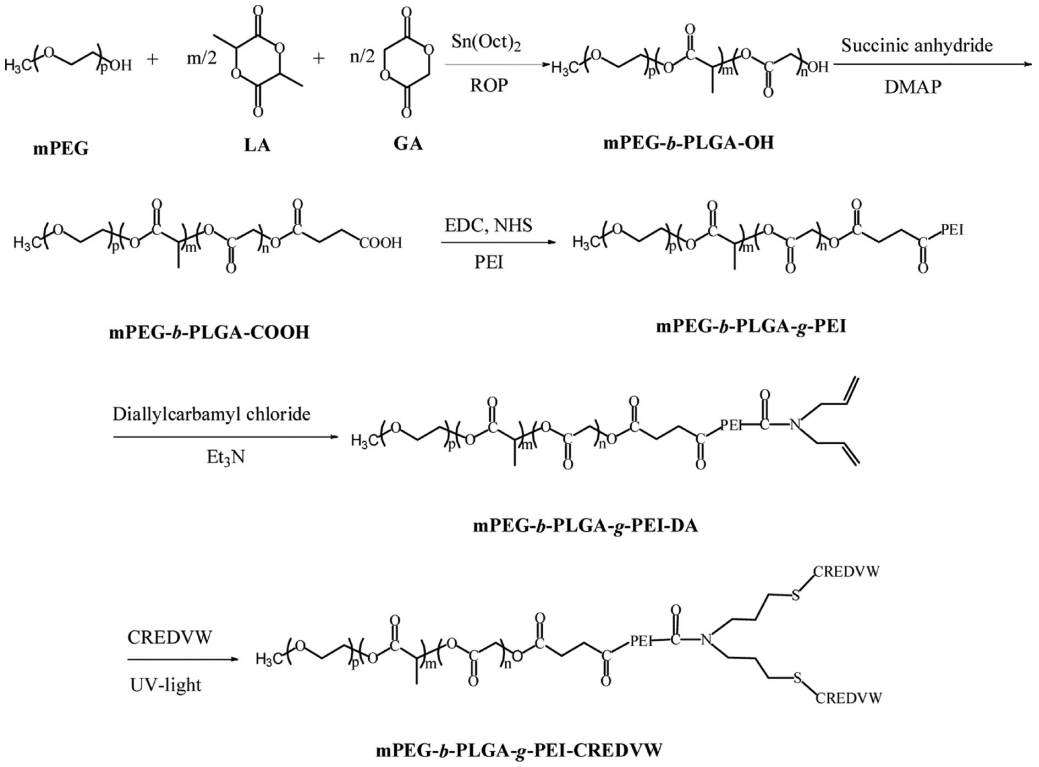
Scheme 2. Synthesis Route of mPEG-*b*-PLGA-*g*-PEI-CREDVW Amphiphilic Triblock Copolymers



retardation was analyzed on UV illuminator to indicate the location of the pDNA.

2.7. In Vitro Cytotoxicity. The cytotoxicity of micelles and complexes was evaluated by MTT assay using PEI 1.8 kDa and 10 kDa as controls. EA.hy926 cells (1.0×10^4 cell/well) were seeded in 96-well plate, and cultured for 24 h until 80−90% confluence. Then, the medium was replaced by serum-free medium. After 12 h, the medium was changed again for fresh growth medium (10 wt % FBS DMEM). Micelles solution and complexes at various N/P molar ratios were added into the medium. After 48 h, the supernatant was discarded, 20 μL of MTT solution (5.0 mg mL^{-1}) was added to each well and formazan crystals were allowed to form for another 4.0 h. Then, the medium was removed carefully, 150 μL of DMSO was added to each well, the plate was oscillated at low speed on volatility instrument for 10 min. Optical density (OD) was measured by an ELISA reader (Titertek multiscan MC) at the wavelength of 490 nm. The relative cell viability (%) was calculated using the following formula: (OD490′: the absorbance value of experimental wells minus zero wells, avg (OD490C′): the average absorbance value of corrected control wells).

$$\text{relative cell viability} = \frac{\text{OD490}'}{\text{avg(OD490C}')} 100\%$$

2.8. In Vitro Transfection. EA.hy926 cells and rat vascular SMCs were seeded in 24-well plate at a density of 1.0×10^4 cell/well and cultured for 24 h until 80−90% confluence. The cells were incubated with serum-free medium for 12 h before transfection. Complexes at N/P molar ratio of 20 (1.0 μg pEGFP-ZNF580 per well) were added into wells. After 4 h, the medium was changed with fresh growth medium (10% FBS DMEM). Then, cells incubated and the expression of green fluorescence protein (GFP) in cells was observed under an inverted fluorescent microscope at 12 and 24 h time points.

2.9. Wound Healing Assay. The migration capability of EA.hy926 cells transfected by complexes was assessed using a scratch wound healing assay. EA.hy926 cells were transfected by the complexes at the N/P molar ratio 20. After 48 h, the transfected cells were incubated to produce a nearly confluent cell monolayer in a 6-well plate. A liner wound was subsequently generated in the monolayer using a sterile 200 μL plastic pipet tip. Cellular debris was removed by washing with D-hanks buffer (pH 7.4). Then, the migration process at different time points (0, 6, and 12 h) was monitored using inverted microscope and the migration area was calculated using ImageJ 2.1 based on the images after 12 h. The percentage of migration area was calculated by the following equation.

$$\text{migration area (\%)} = \frac{\text{wounded area} - \text{nonrecovered area}}{\text{wounded area}} 100\%$$

2.10. Western Blot Analysis. Western blot analysis was performed to test the expression of ZNF580 gene at the level of protein. Cells were washed twice with 0.1 M PBS and then were lysed in RIPA lysis buffer. The protein concentration of the lysate was determined using a BCA protein assay kit. Cell lysates containing 50 μg of protein were subjected to SDS-PAGE using 10 wt % polyacrylamide resolving gels. After electrophoresis, proteins were transferred onto polyvinylidene fluoride (PVDF) membranes and incubated with rabbit anti-ZNF580 polyclonal antibody, followed by incubation with horseradish peroxidase conjugated to goat antirabbit IgG to assess the loading levels of protein, then incubated with enhanced chemiluminescence reagents (300 μL) for 3 min and was exposed to film. The belt was analyzed using ImageJ 2.1 and β-actin antibody was used as a control.

2.11. Statistical Analysis. Each experiment was repeated three times, and all data were expressed as the mean ± SD (standard deviation). Statistical analysis was performed using analysis of variance (ANOVA), and P values <0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Synthesis of mPEG-*b*-PLGA-*g*-PEI. The synthesis route of mPEG-*b*-PLGA-*g*-PEI and mPEG-*b*-PLGA-*g*-PEI-REDV amphiphilic block copolymers is shown in Scheme 2. Herein, amphiphilic diblock copolymer mPEG-*b*-PLGA was synthesized by ring-opening polymerization of LA and GA in the presence of mPEG macroinitiator and $Sn(Oct)_2$ catalyst. GA was used to enhance the degradation ability of PLGA, and thus the suitable degradation rate of the gene transfer vectors

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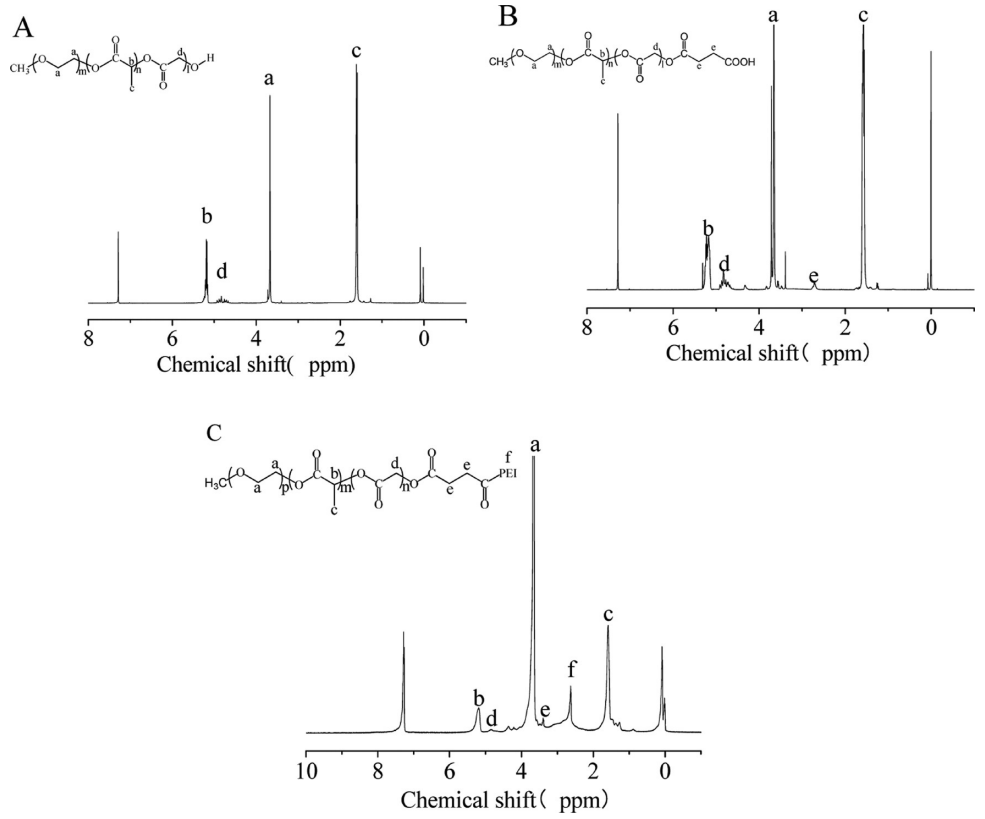

Figure 1. ^{1}H NMR spectra of amphiphilic copolymers in $CDCl_3$. (A) mPEG-*b*-PLGA-OH, (B) mPEG-*b*-PLGA-COOH, (C) mPEG-*b*-PLGA-*g*-PEI(1.8 kDa).

can be acquired. The average molecular weight of the diblock copolymer was estimated by GPC as 6500, which is slight lower than the designed molecular weight value of 6900. In addition, we calculated the molecular weight from ^{1}H NMR as 6600.

^{1}H NMR spectra of the copolymers of mPEG-*b*-PLGA−OH, mPEG-*b*-PLGA-COOH, and mPEG-*b*-PLGA-*g*-PEI are shown in Figure 1. The characteristic peaks corresponding to mPEG, LA, GA and PEI were assigned as below: 3.65 ppm (CH_2CH_2O, 4H), 5.19 ppm ($OOCCHCH_3$, 1H), 4.58−4.98 ppm ($OOCCH_2$, 2H), 2.00−3.20 ppm (−NH-CH_2-CH_2-, PEI). In addition, the peak of 2.70 ppm ($OCCH_2CH_2COO$, 4H) proved the successful synthesis of mPEG-*b*-PLGA-COOH. The contents of mPEG, LA, and GA in the copolymer were 27.5, 65.2, and 7.3% calculated from ^{1}H NMR, respectively. In order to determine chemical compositions of mPEG-*b*-PLGA-*g*-PEI, elemental analysis was performed and the results are shown in Table 1. The found N contents in mPEG-*b*-PLGA-*g*-PEI(1.8 kDa) and mPEG-*b*-PLGA-*g*-PEI(10 kDa) were 9.94 and 16.09%, respectively. These results demonstrated that PEI(1.8 kDa, 10 kDa) had been grafted onto mPEG-*b*-PLGA copolymer to obtain mPEG-*b*-PLGA-*g*-PEI copolymers successfully.

Table 1. Contents of C, H, and N in mPEG-*b*-PLGA-*g*-PEI(1.8 kDa) and mPEG-*b*-PLGA-*g*-PEI(10 kDa)

	content (wt %)		
sample ID	C	H	N
mPEG-*b*-PLGA-*g*-PEI(1.8 kDa)	49.07	5.29	9.94
mPEG-*b*-PLGA-*g*-PEI(10 kDa)	41.94	5.36	16.09

3.2. Characterization of mPEG-*b*-PLGA-*g*-PEI-CREDVW Micelles. To improve the transfection efficiency and promote the proliferation of ECs, we linked REDV peptides onto the micelles to obtain a kind of high-efficiency gene transfer vector targeting to ECs. For the convenience of reaction and quantification, we used CREDVW containing REDV sequence. W is tryptophan residue in CREDVW peptide sequence, whose indolyl group shows specific fluorescence emission spectrum at 350 nm with the excitation spectrum at 280 nm owing to the large conjugated π bond.[49] As shown in Figure 2, mPEG-*b*-

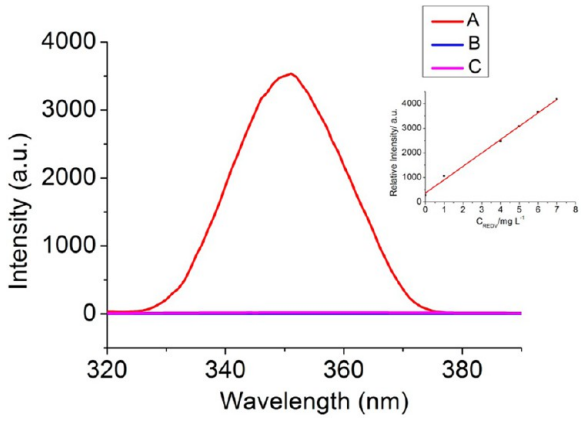

Figure 2. Fluorescence emission spectrum of micelles solution and the curve in the upper right of the figure was the standard curve of CREDVW peptide. (A) mPEG-*b*-PLGA-*g*-PEI(1.8 kDa)-CREDVW, (B) mPEG-*b*-PLGA-*g*-PEI(1.8 kDa), (C) the mixed solution of mPEG-*b*-PLGA-*g*-PEI micelles and CREDVW peptide after dialysis.

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PLGA-*g*-PEI(1.8 kDa)-CREDVW micelles had a specific emission spectrum at 350 nm compared to mPEG-*b*-PLGA-*g*-PEI(1.8 kDa) micelles. To address whether the peptides were linked to the micelles or adsorbed to the micelles, we determined the fluorescence emission spectrum (Figure 2) of the mixed solution of mPEG-*b*-PLGA-*g*-PEI micelles and CREDVW peptide after dialysis. No emission spectrum at 350 nm was observed, indicating that dialysis can fully dialyze CREDVW peptide. The concentration of mPEG-*b*-PLGA-*g*-PEI(1.8 kDa)-CREDVW was 10 mg L^{-1} and the concentration of peptides calculated by the standard cure was 5.63 mg L^{-1} (The curve in the upper right of Figure 2 was the standard curve of CREDVW peptide, $y = 363.78 + 543.87$(mg L^{-1}), $R^2 = 0.996$).

3.3. Hydrodynamic Diameter, Zeta Potential, and Morphology of Blank Micelles and Complexes. It is well-known that particle size and zeta potential are important factors for endocytosis. Nanoparticles less than 200 nm are much easier for endocytosis and less impressionable to clearance by the reticuloendothelial system (RES).[50] Also, the positive charged surface is more favorable for endocytosis. The diameter and zeta potential of blank micelles were summarized in Table 2. The micelles without peptides showed smaller diameter and a little higher potential compared with the micelles with peptides. The targeting REDV and nontargeting REVD-linked micelles showed similar diameter and zeta potential. Figure 3a, b and Table 2 demonstrated that all of the micelles and complexes were less than 150 nm. Furthermore, the diameter of complexes decreased with the increasing of N/P ratio, which was similar to the earlier works.[51,52] Figure 3c, d showed that the zeta potential presented an increasing tendency with the increasing of N/P ratio, mainly because of the increasing content of mPEG-*b*-PLGA-*g*-PEI-REDV.[53] The mPEG-*b*-PLGA-*g*-PEI(1.8 kDa)-REDV/pDNA complexes were positively charged when the N/P ratio was 15 or 20. While the mPEG-*b*-PLGA-*g*-PEI(10 kDa)-REDV/pDNA complexes were positively charged when the N/P ratio was above 10. The above results showed that the mPEG-*b*-PLGA-*g*-PEI(10 kDa)-REDV/pDNA complexes may be more suitable for cell adhesion owing to their high positive charges. Furthermore, the mPEG-*b*-PLGA-*g*-PEI(1.8 kDa or 10 kDa)-REVD/pDNA complexes were found to be similar to corresponding REVD complexes in diameter and zeta potential.

Table 2. Size and Zeta Potential of Micelles of mPEG-*b*-PLGA-*g*-PEI, mPEG-*b*-PLGA-*g*-PEI-REDV, and mPEG-*b*-PLGA-*g*-PEI-REVD

sample ID	size (nm)	PDI[a]	Z.P. (mV)
mPEG-*b*-PLGA-*g*-PEI (1.8 kDa)	82.7 ± 1.2	0.22 ± 0.05	13.7 ± 0.5
mPEG-*b*-PLGA-*g*-PEI (10 kDa)	92.9 ± 0.9	0.38 ± 0.02	34.5 ± 3.2
mPEG-*b*-PLGA-*g*-PEI(1.8 kDa)-REDV	111.5 ± 1.9	0.29 ± 0.01	13.3 ± 0.3
mPEG-*b*-PLGA-*g*-PEI(1.8 kDa)-REVD	113.2 ± 0.8	0.31 ± 0.02	13.2 ± 0.3
mPEG-*b*-PLGA-*g*-PEI(10 kDa)-REDV	144.9 ± 2.6	0.36 ± 0.01	30.6 ± 3.8
mPEG-*b*-PLGA-*g*-PEI(10 kDa)-REVD	143.6 ± 2.4	0.27 ± 0.03	31.1 ± 2.7

[a]PDI: Polydispersity index.

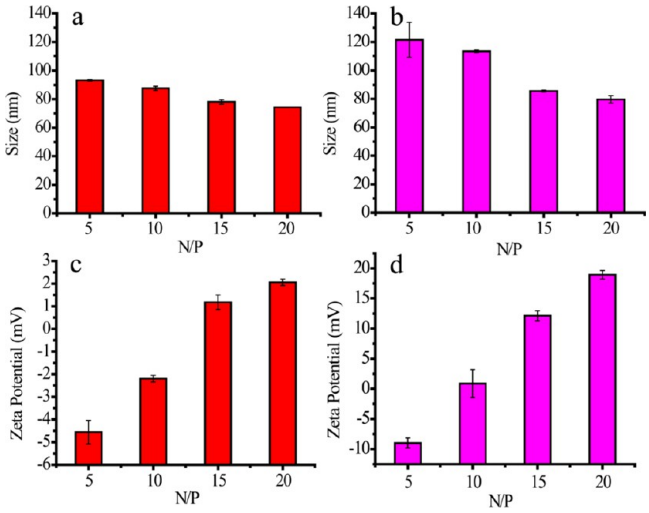

Figure 3. Hydrodynamic diameter and zeta potential of complexes at various N/P molar ratios. (a) Diameter of mPEG-*b*-PLGA-*g*-PEI(1.8 kDa)-REDV/pDNA, (b) diameter of mPEG-*b*-PLGA-*g*-PEI(10 kDa)-REDV/pDNA, (c) zeta potential of mPEG-*b*-PLGA-*g*-PEI(1.8 kDa)-REDV/pDNA, (d) zeta potential of mPEG-*b*-PLGA-*g*-PEI(10 kDa)-REDV/pDNA.

Besides the hydrodynamic diameter and zeta potential of complexes, the morphology of mPEG-*b*-PLGA-*g*-PEI(1.8 kDa)-REDV/pDNA and mPEG-*b*-PLGA-*g*-PEI(10 kDa)-REDV/pDNA complexes was also characterized by TEM (Figure 4). The complexes showed irregular spherical particles with different sizes, which may be caused by the drying process.

3.4. Agarose Gel Electrophoresis. Condensing pDNA sufficiently into complexes is a prerequisite to protect pDNA from the effect of serum and enzyme. Therefore, agarose gel electrophoresis was performed to evaluate whether the micelles can condense pDNA to form complexes successfully. As shown in Figure 5a, mPEG-*b*-PLGA-*g*-PEI(1.8 kDa) micelles could completely inhibit pDNA migration at N/P 10, whereas mPEG-*b*-PLGA-*g*-PEI(1.8 kDa)-REDV or mPEG-*b*-PLGA-*g*-PEI(1.8 kDa)-REVD micelles condense pDNA efficiently at N/P 20 (Figure 5 b, c). This tendency can also be observed in mPEG-*b*-PLGA-*g*-PEI(10 kDa), mPEG-*b*-PLGA-*g*-PEI(10 kDa)-REDV, and mPEG-*b*-PLGA-*g*-PEI(10 kDa)-REVD micelles (Figure 5d–f). These micelles could completely inhibit pDNA migration at N/P 5, N/P 10, and N/P 10, respectively. High positive potential of gene vectors benefits for efficient condensation of pDNA, however, it can also lead to high cytotoxicity. According to the above results, all gene vectors can inhibit pDNA migration at N/P 20. Therefore, the complexes at N/P 20 were chosen for further study.

3.5. In Vitro Cytotoxicity. The cytotoxicity of the micelles and complexes was evaluated by MTT using EA.hy926 cells. The MTT results were presented in Figure 6. PEI(1.8 kDa) and PEI(10 kDa) were used as controls. We designed the micelles with PEG and PEI mixed shells, both of which influenced the cytotoxicity of micelles and complexes.[54−56] The relative cell viability decreased with the increasing concentration of micelles or complexes. Evidently, under the same conditions, mPEG-*b*-PLGA-*g*-PEI(10 kDa)-REDV and mPEG-*b*-PLGA-*g*-PEI(10 kDa)-REVD micelles or complexes had significantly lower relative cell viability than mPEG-*b*-PLGA-*g*-PEI(1.8 kDa)-REDV and mPEG-*b*-PLGA-*g*-PEI(1.8 kDa)-REVD micelles or complexes, which was caused by high positive charges of high

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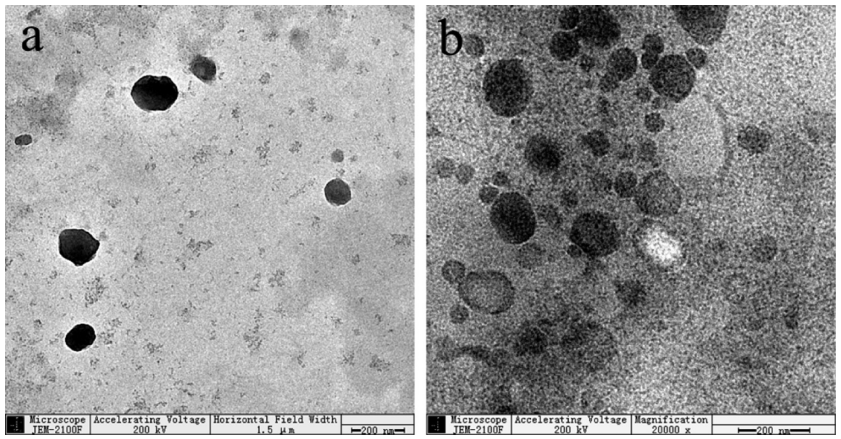

Figure 4. TEM images of complexes at N/P 20. (a) mPEG-*b*-PLGA-*g*-PEI(1.8 kDa)-REDV/pDNA complexes, (b) mPEG-*b*-PLGA-*g*-PEI(10 kDa)-REDV/pDNA complexes.

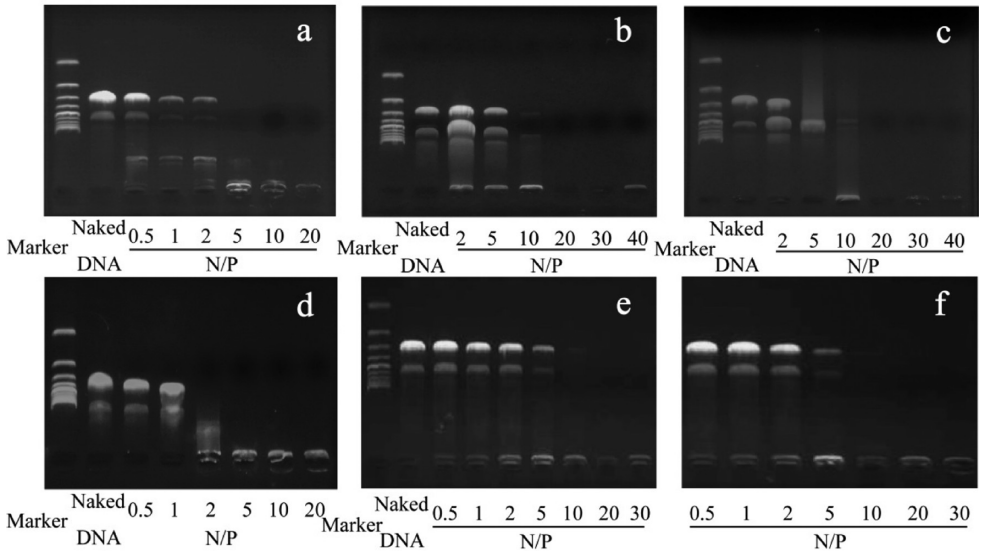

Figure 5. Agarose gel electrophoresis of complexes at various N/P molar ratios. (a) mPEG-*b*-PLGA-*g*-PEI(1.8 kDa)/pDNA, (b) mPEG-*b*-PLGA-*g*-PEI(1.8 kDa)-REDV/pDNA, (c) mPEG-*b*-PLGA-*g*-PEI(1.8 kDa)-REVD/pDNA, (d) mPEG-*b*-PLGA-*g*-PEI(10 kDa)/pDNA, (e) mPEG-*b*-PLGA-*g*-PEI(10 kDa)-REDV/pDNA, (f) mPEG-*b*-PLGA-*g*-PEI(10 kDa)-REVD/pDNA.

molecular weight PEI in them.[57] The high relative cell viability of micelles and complexes comprising PEI 1.8 kDa resulted from the low cytotoxicity of low molecular weight PEI 1.8 kDa and the shielding effect of PEG (1.9 kDa). We used PEG (1.9 kDa) here for the reason that the micelles could exert targeting function effectively as well as shielding effect. For the micelles and complexes comprising PEI 10 kDa, the higher cytotoxicity resulted from the high molecular weight[58,59] and the insufficient shielding effect of PEG (1.9 kDa). In addition, the relative cell viability of complex groups was much higher than the micelle groups and PEI groups at the same concentration. This phenomenon can be explained from two aspects: pEGFP-ZNF580 gene and peptides. When the concentration was ≤40 μg mL^{-1}, the relative cell viability of micelles and complexes was higher than 75%. Therefore, the concentration of 40 μg mL^{-1} was selected for further study.

3.6. In Vitro transfection. The in vitro transfection of targeting gene vector mPEG-*b*-PLGA-*g*-PEI(1.8 kDa, 10 kDa)-REDV and nontargeting gene vector mPEG-*b*-PLGA-*g*-PEI(1.8 kDa, 10 kDa)-REVD was assessed in EA.hy926 cells using pEGFP-ZNF580 as a reporter gene.[30] Cells treated with pEGFP-ZNF580 plasmid or Lipofectamine 2000/pEGFP-ZNF580 complexes were used as the negative control and the positive control, respectively. In our previous study, we used complexes without peptides as control to verify the targeting ability of REDV peptides. Here, to further address targeted gene delivery effect of mPEG-*b*-PLGA-*g*-PEI(1.8 kDa, 10 kDa)-REDV, the inactive REVD-linked mPEG-*b*-PLGA-*g*-PEI micelle/pEGFP-ZNF580 complexes were prepared and used to transfect EA.hy926 cells as a nontargeting control.[42,60] When the complexes contained the same molecular weight PEI(1.8 kDa or 10 kDa), the transfection efficiency of complexes with targeting REDV peptide (Figure 7D, F) was much better than the complexes with REVD (Figure 7C, E) owing to the REDV specific selectivity to ECs. More importantly, the mPEG-*b*-PLGA-*g*-PEI(10 kDa)-REDV complexes displayed significant higher transfection efficiency than mPEG-*b*-PLGA-*g*-PEI(1.8 kDa)-REDV, as well as higher than Lipofectamine 2000 complexes. The results can be explained by different molecular weight of PEI and the shielding effect of PEG. The high molecular weight (10 kDa) and insufficient shielding effect of PEG resulted in the high surface potential of

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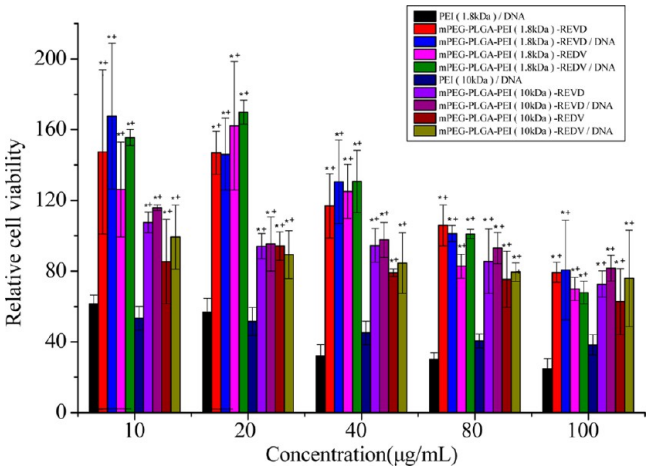

Figure 6. Relative cell viability of EA.hy926 cells after 48 h of treatment with different PEI concentrations of micelles and complexes at N/P molar ratio of 20. ($\bar{x} \pm$ SD, $n = 3$, *statistically different from PEI (1.8 kDa) group ($p < 0.05$), $^{+}$statistically different from PEI (10 kDa) group ($p < 0.05$)).

complexes comprising PEI 10 kDa, which further led to high transfection efficiency, and these results were consistent with previous studies.[58] In conclusion, the introducing of REDV peptide endowed the mPEG-*b*-PLGA-*g*-PEI(1.8 kDa, 10 kDa)-REDV micelles with targeting ability to ECs, which was proved by the inactive REVD peptide. Hence, pEGFP-ZNF580 loaded by mPEG-*b*-PLGA-*g*-PEI(1.8 kDa, 10 kDa)-REDV micelles expressed successfully in EA.hy926 cells, especially the mPEG-*b*-PLGA-*g*-PEI(10 kDa)-REDV/pDNA complexes showed much higher transfection efficiency. Besides inactive REVD method, we further proved the selectivity of REDV peptide to ECs by the transfection efficiency of rat vascular SMCs treated with mPEG-*b*-PLGA-*g*-PEI(10 kDa)-REDV/pDNA complexes. The transfection results (Figure 7G, G′) showed very low transfection efficiency of SMCs, which highlighted the target ability of mPEG-*b*-PLGA-*g*-PEI(10 kDa)-REDV/pDNA complexes to ECs.

3.7. Wound Healing Assay. The wound healing assay was performed to evaluate the migration and proliferation ability of cells, and the results are shown in Figure 8. Cells treated with Lipofectamine 2000/pDNA were used as positive control, and cells treated with pEGFP-ZNF580 as the negative control. Compared with the controls, the complexes showed much higher rate of migration. The migration rate of the cells in complexes-group with active REDV peptide was a little higher than that of complexes-group with inactive REVD peptide, but it was not significant. In addition, mPEG-*b*-PLGA-*g*-PEI(1.8 kDa)-REDV/pDNA complexes and mPEG-*b*-PLGA-*g*-PEI(10 kDa)-REDV/pDNA complexes showed little difference in the rate of migration, and the values of migration area were 90.6 and 86.4%, respectively. The REDV-linked mPEG-*b*-PLGA-*g*-PEI(1.8 kDa, 10 kDa)/pDNA complexes enhanced the migration and proliferation of ECs.

3.8. Protein Extraction and Western Blot Analysis. Western blotting is a powerful analytical method to detect a specific protein. Herein, it was used to study the expression of ZNF580 gene. According to the results of transfection in vitro, mPEG-*b*-PLGA-*g*-PEI(10 kDa)-REDV/pDNA complexes were used to transfect EA.hy926 cells. Relative protein level (%) was calculated by the expression of ZNF580 gene and *β*-actin gene. pDNA and Lipofectamine 2000/pDNA treated cells were used as negative and positive control, respectively. Figure 9 showed that relative protein ZNF580 was expressed successfully by the delivery and release of REDV-linked complexes, i.e., mPEG-*b*-PLGA-*g*-PEI(10 kDa)-REDV/pDNA, in EA.hy926 cells compared with the controls. The relative protein level in transfected EA.hy926 cells by these complexes was 76.9% (Figure 9), which was apparently the highest expression of ZNF580, whereas that of mPEG-*b*-PLGA-*g*-PEI(10 kDa)-REVD/pDNA complexes (prepared from nontargeting gene vector) was 45.6%, and Lipofectamine 2000 group was 33.6%. These results demonstrated the outstanding contribution of REDV peptide with targeting ability for the endocytosis of complexes and further contributed to the expression of ZNF580 compared with mPEG-*b*-PLGA-*g*-PEI(10 kDa)-REVD/pDNA complexes.

4. DISCUSSION

To obtain safe and efficient gene transfer vectors for the proliferation of ECs, we prepared mPEG-*b*-PLGA-*g*-PEI(1.8 kDa, 10 kDa) amphiphilic and biodegradable copolymers by a two-step synthesis route, and then mPEG-*b*-PLGA-*g*-PEI(1.8 kDa, 10 kDa)-DA were obtained through amidation reaction. Thus, the mPEG-*b*-PLGA-*g*-PEI(1.8 kDa, 10 kDa)-DA micelles with double bonds could be formed by self-assembling method. In order to endow the micelles with targeting ability, CREDVW peptide containing REDV sequence was covalently linked to the micelles by thiol−ene Click-reaction, and the mPEG-*b*-PLGA-*g*-PEI(1.8 kDa, 10 kDa)-CREDVW micelles could be acquired as gene transfer vectors with targeting ability to ECs. The pEGFP-ZNF580 plasmid could be condensed and loaded by these micelles to form complexes. In the present study, to testify whether the introduction of REDV peptide endowed the complexes with special targeting ability to ECs and improved the transfection efficiency of complexes dramatically or not, the inactive REVD-linked complexes were also prepared as nontargeting control.

According to the results of agarose gel electrophoresis, both of mPEG-*b*-PLGA-*g*-PEI(1.8 kDa, 10 kDa) micelles with and without peptides could condense and load pEGFP-ZNF580 plasmids efficiently by changing N/P molar ratio. To reduce the cytotoxicity of gene transfer vectors and prolong the circulation time in vivo, PEGylation is a kind of method that has been often used to modify cationic polymers owing to its stealth behavior. Studies have demonstrated that these shielded gene transfer systems have lower cytotoxicity and prolonged circulation times in vivo.[54−56] In the present study, PEG (1.9 kDa) was used to synthesize safe and effective gene transfer vectors with low cytotoxicity and long circulation time in vivo. The lower cytotoxicity of micelles and complexes comprising PEI 1.8 kDa than that of micelles and complexes comprising PEI 10 kDa resulted from the lower molecular weight of PEI 1.8 kDa and the shielding effect of PEG. Moreover, the introducing of REDV peptide could also contribute to reducing the cytotoxicity of micelles and complexes.

The transfection efficiency of mPEG-*b*-PLGA-*g*-PEI(1.8 kDa, 10 kDa)-REDV/pEGFP-ZNF580 complexes with targeting function was evaluated. The introducing of REDV can increase the recognition of these complexes to ECs, and thus, the endocytosis of these complexes could be improved, which can improve the transfection efficiency. The results showed that the transfection efficiency of complexes comprising mPEG-*b*-PLGA-*g*-PEI(10 kDa)-REDV was much higher than the complexes comprising mPEG-*b*-PLGA-*g*-PEI(1.8 kDa)-REDV.

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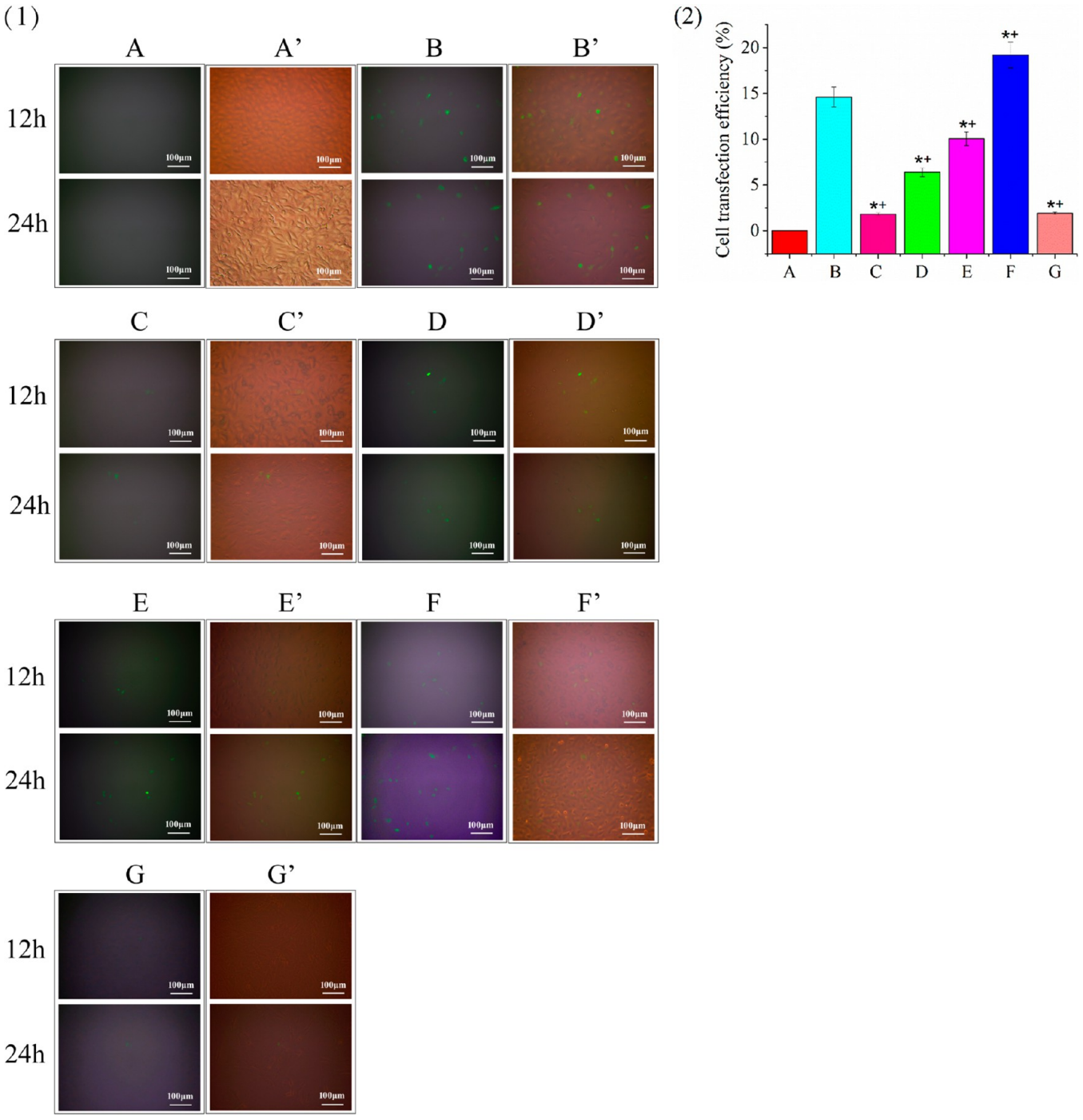

Figure 7. (1) Fluorescence images of EA.hy926 cells and rat vascular SMCs (as a control) transfected by complexes with the N/P molar ratio of 20 with concentration of 40 μg mL^{-1} at time intervals of 12 and 24 h and (2) the transfection efficiency at 24 h. (A) EA.hy926 cells treated with pDNA served as the negative control, (B) EA.hy926 cells treated with Lipofectamine 2000/pDNA served as positive control group, (C) EA.hy926 cells treated with mPEG-*b*-PLGA-*g*-PEI(1.8 kDa)-REVD/pDNA complexes, (D) EA.hy926 cells treated with mPEG-*b*-PLGA-*g*-PEI(1.8 kDa)-REDV/pDNA complexes, (E) EA.hy926 cells treated with mPEG-*b*-PLGA-*g*-PEI(10 kDa)-REVD/pDNA complexes, (F) EA.hy926 cells treated with mPEG-*b*-PLGA-*g*-PEI(10 kDa)-REDV/pDNA complexes, G: rat vascular SMCs treated with mPEG-*b*-PLGA-*g*-PEI(10 kDa)-REDV/pDNA complexes. (A′, B′, C′, D′, E′, F′, and G′ are the bright-field images, and A, B, C, D, E, F, and G are the corresponding dark-field images, respectively) ($\overline{x} \pm$ SD, n = 3, *statistically different from cells treated with pDNA group ($p < 0.05$), $^{+}$statistically different from cells treated with Lipofectamine 2000/pDNA group ($p < 0.05$)).

It is consistent with the results from earlier studies.[58] Also, the transfection efficiency of complexes with targeting REDV peptide was obviously higher than the complexes with REVD. The high selectivity of mPEG-*b*-PLGA-*g*-PEI(10 kDa)-REDV for ECs was further proved by using SMCs as a control. The targeting ability of REDV could also be demonstrated by the result of Western blot analysis. MPEG-*b*-PLGA-*g*-PEI(10 kDa)-REDV micelle/pEGFP-ZNF580 complexes showed very high expression of ZNF580 compared to the complexes with inactive REVD.

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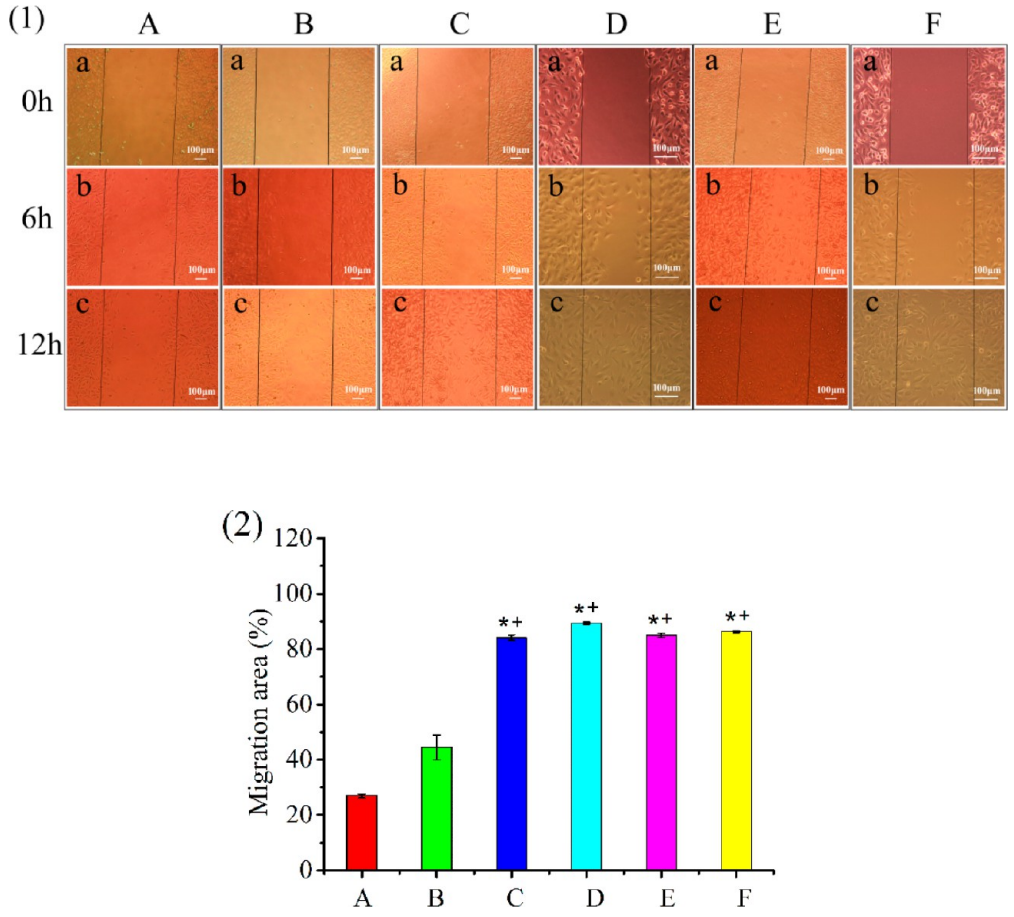

Figure 8. (1) Migration process of EA.hy926 cells at different time points and (2) migration area after 12 h calculated by Image-Pro Plus (6.0). (A) Cells without any treatment served as the negative control, (B) cells treated with Lipofectamine 2000/pDNA as the positive control, (C) cells treated with mPEG-*b*-PLGA-*g*-PEI(1.8 kDa)-REVD/pDNA complexes, (D) cells treated with mPEG-*b*-PLGA-*g*-PEI(1.8 kDa)-REDV/pDNA complexes, (E) cells treated with mPEG-*b*-PLGA-*g*-PEI(10 kDa)-REVD/pDNA complexes, (F) cells treated with mPEG-*b*-PLGA-*g*-PEI(10 kDa)-REDV/pDNA complexes. ($\bar{x} \pm$ SD, $n = 3$, *statistically different from cells without any treatment group ($p < 0.05$), $^{+}$statistically different from cells treated with Lipofectamine 2000/pDNA group ($p < 0.05$)).

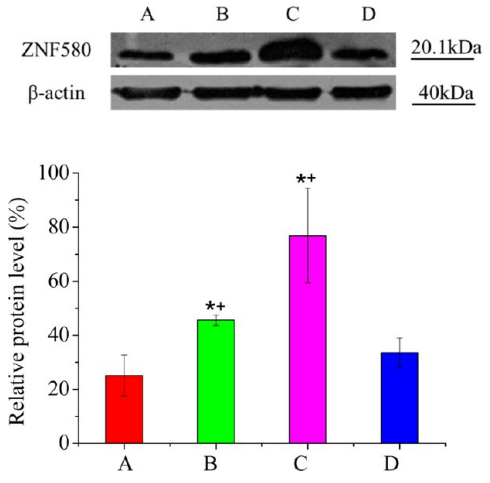

Figure 9. Western blot analysis for ZNF580 protein expression in EA.hy926 cells transfected by different complexes, pDNA and Lipofectamine 2000/pDNA after 48 h. (A) pDNA, (B) mPEG-*b*-PLGA-*g*-PEI(10 kDa)-REVD/pDNA complexes, (C) mPEG-*b*-PLGA-*g*-PEI(10 kDa)-REDV/pDNA complexes, (D) Lipofectamine 2000/pDNA. ($\bar{x} \pm$ SD, $n = 3$, *statistically different from pDNA group ($p < 0.05$), $^{+}$statistically different from cells treated with Lipofectamine 2000/pDNA group ($p < 0.05$)).

In addition, the migration of ECs treated with REDV or REVD-linked mPEG-*b*-PLGA-*g*-PEI(1.8 kDa, 10 kDa)/pEGFP-ZNF580 complexes was much better than that of pEGFP-ZNF580 or Lipofectamine 2000/pEGFP-ZNF580 complexes treated ECs. However, the results of wound healing assay did not feature the significant function of REDV peptide over REVD peptide. The reason is still not clear and needs to be investigated in detail.

According to above results, the targeting gene vectors have been demonstrated to have many advantages over nontargeting gene vectors (Table 3). They have low cytotoxicity, high

Table 3. Advantages and Disadvantages of Targeting Gene Vectors over Nontargeting Vectors

	targeting gene vectors	nontargeting vectors
vector name	REDV-linked mPEG-*b*-PLGA-*g*-PEI	REVD-mPEG-*b*-PLGA-*g*-PEI
EC targeting ligand	REDV (targeting function)	REVD (no targeting function)
cytotoxicity	low	low
transfection efficiency	high	low
cell migration and proliferation	high	low
protein expression	high	low

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transfection efficiency, and enhanced cell migration and proliferation. They can effectively transfer gene to ECs and induce high ZNF580 protein expression.

5. CONCLUSIONS

We have synthesized the biodegradable gene transfer vectors with low cytotoxicity and favorable targeting function. On the basis of these gene vectors, pEGFP-ZNF580 plasmid was efficiently transported into EA.hy926 cells and expressed into corresponding protein. The proliferation and migration of ECs were improved greatly as a result of the outstanding targeting ability of these gene vectors. With low cytotoxicity, high transfection efficiency and favorable target ability, this kind of gene transfer vectors may have great potential for endothelialization of artificial blood vessels. We believe that this method will open a new avenue to design and synthesize new targeting gene vectors for ECs, and will be more widely used in rapid endothelialization of biomaterial surfaces in the future.

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Notes

The authors declare no competing financial interest.

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