Original

Yang, J.; Liu, W.; Lv, J.; Feng, Y.; Ren, X.; Zhang, W.:

REDV–polyethyleneimine complexes for selectively enhancing gene delivery in endothelial cells

In: Journal of Materials Chemistry B (2016) Royal Society of Chemistry

DOI: 10.1039/C6TB00686H

Cite this: *J. Mater. Chem. B*, 2016, 4, 3365

REDV–polyethyleneimine complexes for selectively enhancing gene delivery in endothelial cells

Jing Yang,^{ab} Wen Liu,^a Juan Lv,^{ab} Yakai Feng,^{*abcd} Xiangkui Ren^{*ad} and Wencheng Zhang^{*e}

Gene therapy provides a new strategy for promoting endothelialization, and rapid endothelialization has attracted increasing attention for inhibiting thrombosis and restenosis in artificial vascular implants. However, the low transfection efficiency and high cytotoxicity of gene delivery systems prevent their *in vivo* application. In this study, an endothelial cell (EC)-specific gene carrier with relatively high transfection efficiency and low cytotoxicity was prepared successfully. Using bifunctional hydroxylsuccinimide–poly(ethylene glycol)–maleimide (NHS–PEG–MAL) as the linker, an EC-specific REDV peptide was conveniently grafted onto polyethyleneimine-*b*-poly(lactide-co-3(S)-methyl-morpholine-2,5-dione)-*b*-polyethyleneimine (PEI–PLMD–PEI). By varying the molar ratios of REDV to PEI, a series of REDV modified copolymers REDV–PEG-*g*-PEI–PLMD–PEI-*g*-PEG–REDV (REDV–PPP) were prepared. Then these copolymers were self-assembled into nanoparticles (NPs) as gene carriers. These NPs could easily condense the EGFP-ZNF580 plasmid (pZNF580) to form REDV peptide functionalized NP/pZNF580 complexes with low cytotoxicity. The fluorescence images, Western blot analysis, and quantitative real-time RT-PCR results verified that the effective transfection of REDV peptide functionalized NP/pZNF580 complexes in ECs was comparable with the positive control of PEI (25 000 Da)/pZNF580 complexes. The high transfection efficiency was attributed to the enhanced cell uptake by the REDV peptide and relied on the quantity of the peptide. Furthermore, the rapid migration of the transfected ECs showed the active function of the expressed ZNF580 protein and further demonstrated that the REDV peptide functionalized NP/pZNF580 complexes could improve the transfection of pZNF580 in ECs. These results provided a useful platform to design EC-specific gene carriers and use gene therapy to enhance endothelialization.

Received 17th March 2016,
Accepted 21st April 2016

DOI: 10.1039/c6tb00686h

www.rsc.org/MaterialsB

1. Introduction

Nowadays cardiovascular diseases continue to be the leading cause of death in modern society. The vascular stents or grafts are applied for the treatment of these coronary vascular diseases.¹ However, the risks of inadequate hemocompatibility, thrombosis and restenosis limit their ultimate success in clinical applications.

Various bioinert and bioactive molecules such as poly(ethylene glycol) (PEG), zwitterionic polymers, heparin, silk-fibroin and gelatin have been used to improve the hemocompatibility of scaffolds.^{2–11} The modification can improve the hydrophilicity and reduce their interaction with proteins and platelets, thus significantly enhancing the hemocompatibility.^{12–15} In order to prevent thrombosis and restenosis in implants, rapid endothelialization which can form a robust and confluent endothelial cell (EC) monolayer has been considered to be another promising method. Surface functionalization and gene engineering are two main strategies for endothelialization.¹ The extracellular matrix (ECM) proteins, antibodies and growth factors have been explored for the surface functionalization of vascular scaffolds.^{16–19} Among them, EC binding ligands such as RGD, REDV and CAG peptides which were isolated from the ECM have attracted great attention. In particular, the tetrapeptide REDV can specifically bind the integrin receptors of $\alpha_4\beta_1$ which are abundant on ECs.

^a School of Chemical Engineering and Technology, Tianjin University, Weijin Road 92, Tianjin 300072, China. E-mail: yakai.feng@tju.edu.cn

^b Collaborative Innovation Center of Chemical Science and Chemical Engineering (Tianjin), Weijin Road 92, Tianjin 300072, China

^c Key Laboratory of Systems Bioengineering of Ministry of Education, Tianjin University, Weijin Road 92, Tianjin 300072, China

^d Tianjin University-Helmholtz-Zentrum Geesthacht, Joint Laboratory for Biomaterials and Regenerative Medicine, Weijin Road 92, Tianjin 300072, China

^e Department of Physiology and Pathophysiology, Logistics University of Chinese People's Armed Police Force, Tianjin 300162, China

Therefore, the REDV peptide has been used to modify different stents and grafts for enhancing the adhesion of ECs and promoting endothelialization.^{20–24} What is more, the GREDV-modified alginate/CaCO₃ hybrid hydrogel scaffolds induced an angiogenic potential by stimulating new vessel formation and showed the highest blood vessel density after three weeks of implanting *in vivo*.²⁵

Gene therapy, which has been widely applied in anti-tumor agents and bone tissue engineering, could also provide an alternative and promising option to promote endothelialization from the transfected ECs.^{1,26–35} Besides the commonly used vascular endothelial growth factor (VEGF) gene, the ZNF580 gene is also a good choice due to the fact that the overexpression of the ZNF580 gene in EA.hy926 cells leads to the enhanced expression of the VEGF protein, and the migration and proliferation of the cells.³⁶ As the half-time of therapeutic DNAs could be limited to a few minutes resulting from potential degradation by endonucleases in physiological fluids and the extracellular space,²⁰ gene carriers play an important role for successful gene transfection, especially for ECs which are one of the most difficult cell types to be transfected. Hence, a range of synthetic delivery carriers have been developed.^{33,37–42} Among them, nanoparticles (NPs) generated from amphiphilic copolymers by self-assembly have gradually attracted increasing attention in gene delivery systems because of the fact that the hydrophobic core and the hydrophilic shell significantly improve their stability and biocompatibility.^{43,44} For example, we have synthesized a series of NPs with poly(depsipeptide-co-cyclic diester), polyethyleneimine (PEI) and poly(ethylene glycol) (PEG).^{45–48} The corresponding NP/DNA complexes highly reduced cytotoxicity, increased the transfection efficiency and improved the proliferation of ECs. Actually, the growth and proliferation of ECs are in competition with other types of cells *in vivo*, especially smooth muscle cells. So for rapid endothelialization, it is very important to design gene delivery systems which can be specific for ECs. As mentioned above, the REDV peptide could selectively interact with ECs. Therefore, it is of great interest to develop a convenient method to link the REDV peptide with a precise immobilization amount onto gene carriers in order to investigate whether this gene delivery system could be specifically uptaken by ECs and the mechanism for mediating gene transfection.^{49,50} However, to the best of our knowledge, rare references have been reported on these.

In this work, the REDV peptide was conveniently grafted onto polyethyleneimine-*b*-poly(lactide-co-3(S)-methyl-morpholine-2,5-dione)-*b*-polyethyleneimine (PEI-PLMD-PEI) *via* the bifunctional hydroxylsuccinimide-poly(ethylene glycol)-maleimide (NHS-PEG-MAL) spacer. Then the copolymers of REDV-PEG-*g*-PEI-PLMD-PEI-*g*-PEG-REDV (REDV-PPP) were self-assembled into NPs (Scheme 1). By varying the molar ratios of REDV to PEI, a series of REDV modified gene carriers were produced. The properties of these NPs in condensing the therapeutic gene (such as pZNF580) and in gene delivery were systematically investigated *in vitro*. In particular, the specific interaction of REDV and ECs was studied using the flow cytometer, which provided us with useful information in the design of EC specific gene carriers.

Scheme 1 REDV peptide functionalized REDV-PPP NP/pZNF580 complexes for effective and safe delivery of pZNF580: (1) self-assembly of REDV-PPP copolymers into NPs, (2) condensation of pZNF580 with the REDV peptide functionalized NPs, and (3) specifically selective adhesion of ECs, (4) cell internalization of the complexes, (5) endosomal escape of the complexes as well as (6) nucleus accumulation of the complexes.

2. Experimental section

2.1. Materials

Branched PEI with an average molecular weight of 25 000 Da (PEI25) was purchased from Sigma-Aldrich (Beijing, China). PEI-PLMD-PEI was gifted by our colleague. NHS-PEG-MAL (3500 Da) was purchased from JenKem Technology Co., Ltd. The solvents were dried by refluxing over CaH₂ and distilled just before use. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from DingGuo ChangSheng Biotech. Co., Ltd (Beijing, China). A BCA protein assay kit was purchased from Solarbio Science and Technology Co., Ltd (Beijing, China). Rabbit anti-human ZNF580 polyclonal antibody and goat anti-rabbit IgG were purchased from Abcam (HK) Ltd (Hong Kong, China). The pEGFP-ZNF580 plasmid (pZNF580) was preserved by Department of Physiology and Pathophysiology, Logistics University of Chinese People's Armed Police Force. CREVDW and CREVDW peptides were supplied by GL Biochem. (Shanghai) Ltd. Human umbilical vein endothelial cells (HUVECs) were obtained from the Allcells (Shanghai, China). Other chemicals and reagents were all analytically pure and were obtained from Jiangtian Chemicals (Tianjin, China).

2.2. Preparation of REDV peptide functionalized NPs and NP/pZNF580 complexes

2.2.1. Preparation of REDV peptide functionalized NPs and dysfunctional REDV NPs. REDV peptide functionalized copolymers

(REDV-PPP) were conveniently synthesized from a PEI-PLMD-PEI triblock copolymer using a heterobifunctional NHS-PEG-MAL spacer and the cysteine-terminated peptide CREVDW with molar ratios of 1:1:1 and 1:0.5:0.5 (PEI/PEG/REDV). Briefly, the PEI-PLMD-PEI copolymer (5.0 mg) and MAL-PEG-NHS were first dissolved in DMSO (2.0 mL), and after 2 h, the CREVDW peptide was added. The reaction mixture was stirred for 12 h at room temperature. Then they were dialyzed in distilled water for 2 days and lyophilized to obtain REDV-PPP copolymers. As controls, the dysfunctional peptide CREVDW (without targeting function) linked copolymer of REVD-PPP was also generated by a similar process using the CREVDW peptide.

REDV peptide functionalized and dysfunctional REVD peptide linked core-shell NPs were prepared as follows: 1 mL of the above copolymers in DMSO (5 mg mL^{-1}) was added dropwise to 20 mL of PBS buffer (pH = 7.4) under intensive magnetic stirring. Then the solutions were dialyzed for 2 days to obtain REDV-PPP1 NPs ($n(\text{PEI/PEG/REDV}) = 1:1:1$), REVD-PPP1 NPs ($n(\text{PEI/PEG/REVD}) = 1:1:1$), REDV-PPP2 NPs ($n(\text{PEI/PEG/REDV}) = 1:0.5:0.5$) and REVD-PPP2 NPs ($n(\text{PEI/PEG/REVD}) = 1:0.5:0.5$), respectively.

2.2.2. Preparation of NP/pZNF580 complexes. The NP/pZNF580 ratio was expressed as the nitrogen/phosphate (N/P) molar ratio, which is the number of nitrogen atoms in NPs compared to phosphate groups in pZNF580. NP/pZNF580 complexes were obtained by adding NP solutions (0.5 mg mL^{-1}) to pZNF580 solutions ($200 \text{ } \mu\text{g mL}^{-1}$) at predetermined N/P molar ratios with gentle vortexing and incubated at room temperature for 30 min before characterization. In the following studies, all NP/pZNF580 complexes were freshly prepared before use.

2.3. Physical-chemical characterization of NPs and NP/pZNF580 complexes

2.3.1. Determination of peptide immobilization on NPs. The CREVDW peptide and dysfunctional CREVDW peptide were expected to immobilize on the NPs *via* MAL-functionalized PEG by the Michael addition reaction. A fluorescence spectrometer (Varian Cary Eclipse fluorescence spectrometer) was used to verify the conjugation of the peptide. The fluorescence intensities of all NPs after dialysis were measured based on the emission wavelength at 350 nm with a slit width of 5 nm and an excitation wavelength at 280 nm with a slit width of 5 nm. PEI-PLMD-PEI without the peptide was used as a negative control. A series of peptide solutions with different concentrations (from 0.040 to 0.063 mg mL^{-1}) in PBS (pH = 7.4) were also detected under the same conditions to obtain the standard curve, and thus the conjugated peptide could be quantitatively calculated.

2.3.2. Size distribution and zeta potential of NPs and NP/pZNF580 complexes. The hydrodynamic diameter and zeta potential of NPs and NP/pZNF580 complexes with different N/P molar ratios (5, 10, 15, 20 and 25) were measured using a Zetasizer Nano ZS (Malvern Instrument, Inc., Worcestershire, UK) based on the dynamic light scattering principle (DLS). The detection angle was set to 173° to avoid the “multiple scattering effects” as well as the impact of dust.

2.3.3. Agarose gel retardation assay. Agarose gel electrophoresis was performed to evaluate the binding ability of the

NPs with pZNF580. NP/pZNF580 complexes with different N/P molar ratios (0, 1, 5, 10, 15 and 20) were freshly prepared and used in this assay. $10 \text{ } \mu\text{L}$ of each NP/pZNF580 complex suspensions containing $3 \text{ } \mu\text{g}$ plasmids was mixed with $2 \text{ } \mu\text{L}$ of $6\times$ loading buffer containing the tracking dye. The mixtures were loaded into the wells of 0.8% agarose gels ($30 \text{ mL } 1\times$ TAE buffer containing $2.5 \text{ } \mu\text{g}$ ethidium bromide) and run with $1\times$ TAE buffer at 100 V for 30 min. The resulting gels were imaged under a UV illuminator to show the location of the pZNF580 band and to analyze the binding of NPs with pZNF580.

2.4. Cell culture

HUVECs were cultured in complete medium supplemented with FBS and growth factors in an incubator (37°C , 5% CO_2). The culture medium was changed every 3 days until the cells reached 70–90% confluence. Then HUVECs were trypsinized to subculture according to the standard techniques and the densities of the cells were calculated using a hemacytometer.

2.5. *In vitro* transfection of HUVECs by the NP/pZNF580 complexes

HUVECs were seeded in a 6-well tissue culture plate with a density of 4×10^5 cell per well and cultured overnight at 37°C until a 50–70% confluence was obtained. Before transfection, the cells were starved using serum-free medium for 12 h. NP/pZNF580 complexes with a N/P molar ratio of 15 and $2 \text{ } \mu\text{g}$ pZNF580 per well were added into each well and incubated for 4 h. Then the culture medium was exchanged with complete medium, and the cells were further incubated. The expression of green fluorescence protein (GFP) in cells was imaged using an inverted fluorescence microscope (Fluorescence OLYMPUS U-RFLT50, microscopy Olympus DP72) after 12, 24 and 48 h of incubation. The transfection efficiency was defined as the number of fluorescent cells counted/total cells counted *via* the Image-Pro Plus 6.0 software. A minimum of 9 visual fields from 3 wells were counted for each sample to yield transfection efficiencies. PEI25/pZNF580 complexes were used in the positive control groups and only pZNF580 without any carriers were used in the negative control groups.

2.6. Protein extraction and Western blot assay

The gene transfection efficiencies of NP/pZNF580 complexes, PEI25/pZNF580 complexes and only pZNF580 in HUVECs were evaluated at the protein level by Western blot assay. The transfection process was performed as described in Section 2.5. After 24 h transfection, the total proteins were collected by a $60 \text{ } \mu\text{L}$ RIPA lysis buffer (Beyotime) with $0.6 \text{ } \mu\text{L}$ PSMF per well, quantified by a BCA Protein Assay Kit (Solarbio Science and Technology Co., Ltd), and denatured with $5\times$ SDS (Beijing Leagene Biotechnology Co., Ltd) by boiling for 8 min.

The expressed ZNF580 protein was determined by Western blot analysis. 60 mg of the total proteins per lane was electrophoretically separated by 10% SDS-PAGE. Then the proteins were transferred to a PVDF membrane (Millipore, $0.22 \text{ } \mu\text{m}$) which was then blocked in TBST containing 5% defat milk and 0.1% Tween 20 in Tris-buffered saline for 1 h at room temperature.

The transferred proteins immunoreacted with rabbit anti-human pZNF580 polyclonal antibodies (1 : 1000 Abcam ab59015) overnight at 4 °C. Blots were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1 : 2500, Cell Signaling Technology, Inc., MA, USA) for 1 h and visualized by the standard enhanced chemiluminescence ECL kit (Western Bright Quantum, Advantia). Housekeeping endogenous protein β -actin was used as a control. All blots were quantitatively analyzed using Image J software.

2.7. Quantitative real-time RT-PCR assay

The gene transfection efficiency of NP/pZNF580 complexes, PEI25/pZNF580 complexes and only pZNF580 in HUVECs was evaluated at the transcriptional aspect by quantitative real-time RT-PCR analysis. The transfection process was performed as described in Section 2.5. After 24 h, the total RNA was extracted with a 1 mL TRIzol reagent (Invitrogen) and was spectrometrically quantified. First strand cDNAs were reversely transcribed from 1 μ g total RNA in a 20 μ L reaction system (CWBIO, CW2569) according to the manufacturer's protocol. The resulting cDNAs were used as templates for quantitative real-time RT-PCR using a SYBR Green on ABI 7300 stepone sequence detection PCR system (Applied Biosystems). The reaction mixture was prepared according to the manufacturer's protocol (TransStartTM Top Green qPCR SuperMix AQ 131-02). The sequences of the sense and antisense PCR primers were 5'-AAAAAGCTTGTGGAGGCG CACGTGCTG-3', 5'-AAAAAGATCTTGCCCGAGTGCGCCCGTG-3', respectively. Relative expression levels were normalized based on the expression of housekeeping GAPDH gene with the sequences of the sense and antisense PCR primers 5'-AGGT GAAGGTCGGAGTCAAC-3', 5'-CGTCCTGGAAGATGGTGAT-3', respectively. PCR parameters are 95 °C for 10 min, 40 cycles of amplification at 95 °C for 15 s, 60 °C for 1 min, and 72 °C for 20 s, followed by a melt curve analysis: 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s, and 60 °C for 15 s. The specificity of amplification was detected and collected using StepOne software v2.1. The relative expression values of ZNF580 mRNA were calculated based on the amplification efficiencies using a threshold curve value ($2^{-\Delta\Delta Ct}$).⁵¹

2.8. Cell uptake

In order to evaluate cell uptake, the NPs were covalently labeled with Cy5 fluorescence dye according to the instructions and the labeled NPs condensed pZNF580 to perform the cell uptake process. Briefly, HUVECs were seeded in a 6-well tissue culture plate (4×10^5 cell per well) and cultured to 70–80% confluence. After they were starved overnight, Cy5 labeled NP/pZNF580 complexes were added into the culture medium with a N/P molar ratio of 15 and 2 μ g pZNF580 per well. After 4 h incubation, the cells were washed with PBS three times to completely remove residual complex suspensions, and collected. After centrifugation and re-suspension with PBS several times, the cells were suspended in 400 μ L PBS. The cell uptake of the NP/pZNF580 complexes was analyzed using a flow cytometer (Beckman MoFlo XDP).

2.9. *In vitro* cytotoxicity of the NP/pZNF580 complexes

MTT assay was performed to evaluate the cytotoxicity of the NP/pZNF580 complexes. HUVECs were seeded in a 96-well tissue culture plate with a density of 1×10^4 cells per well in 200 μ L culture medium and incubated overnight at 37 °C. The cells were treated in serum-free medium for 12 h before NP/pZNF580 complexes (at the N/P ratio of 15) with designated pZNF580 plasmid concentrations (1, 3, 5, 8 and 10 μ g mL⁻¹) were added. After 4 h incubation with NP/pZNF580 complexes, the culture medium was exchanged (removal of the complexes) and the cells were further incubated for 24 h. Then 20 μ L filtered MTT (5 mg mL⁻¹ in 0.01 M PBS) solution was added to each well and 4 h later the medium was removed from the wells. 150 μ L DMSO was added to sufficiently dissolve the insoluble formazan crystals for 10 min. The absorbance of the DMSO solutions was measured at 590 nm using a microplate reader (Bio-Rad, IMARKTM) and the relative cell viability was calculated as a percentage relative to the non-transfected blank control group. PEI10/pZNF580 complexes and PEI25/pZNF580 complexes with the corresponding pZNF580 concentrations were used as controls.

2.10. Cell migration assays

The wound healing assay and transwell migration assay were used to evaluate the migration capability of the HUVECs transfected by NP/pZNF580 complexes, and using PEI25/pZNF580 complexes or only pZNF580 in the control groups. The transfection process was performed by the procedure described in Section 2.5.

For the wound healing assay, a linear wound was scratched at the middle of each well with a sterile 200 μ L pipette tip after 24 h transfection. The cells were washed with D-Hanks 3 times to remove the cell debris, and incubated in culture medium without serum at 37 °C. The migration of the cells were monitored at pre-determined time points (0, 6, and 12 h) and imaged using an inverted microscope (OLYMPUS U-RFLT50, microscopy Olympus DP72). The relative recovered area (%) of the cells after 12 h was measured using Image J software and calculated using the following formula:

$$\text{Relative recovered area (\%)} = \frac{\text{The recovered area}}{\text{The total wounded area}} \times 100\%$$

The migration capability of the transfected HUVECs was also evaluated in transwell chambers using filter membranes with 8 μ m pores (Corning Life Sciences, USA). After 24 h transfection, the cells were subjected to serum-free medium for 12 h, and then seeded into the upper chamber (2×10^5 cells per well in 200 μ L serum-free medium). The lower chamber was filled with 500 μ L complete medium. After further incubation at 37 °C for 6 h, the upper chamber was washed with D-Hanks 3 times and fixed with 4% paraformaldehyde. Then the cells on the inside surface of the upper chamber were removed using a cotton swab, and the cells that migrated to the lower surface of the transwell filter were stained with eosin for 5 min and imaged using a microscope (Fluorescence OLYMPUS U-RFLT50, microscopy Olympus DP72). Migration activity was quantified by

counting in three random fields from each well with triplicate samples *via* the Image-Pro Plus 6.0 software.

2.11. Statistical analysis

Each experiment was repeated three times and the data were represented as the mean $\pm$ SD (standard deviation) unless indicated otherwise. SPSS 11.5 software was used to compare the differences using one-way ANOVA tests and *P* values less than 0.05 were considered as statistically significant differences.

3. Results

REDV peptide functionalized copolymers were conveniently synthesized by simply grafting the REDV peptide onto PEI-PLMD-PEI *via* the bifunctional spacer of NHS-PEG-MAL. The reactive NHS group reacted with the primary amino group of PEI-PLMD-PEI to form the amide bond and then conjugated the CREVDW peptide with the MAL group to obtain the REDV-PPP copolymers (Fig. 1). By varying the molar ratio of PEI/NHS-PEG-MAL (1:1 and 1:0.5), the immobilization amount of the functional REDV peptide can be well controlled. Additionally, the dysfunctional CREVDW peptide was used to prepare REVD-PPP copolymers as controls in the following biological evaluation.

3.1. Grafting of the selective REDV peptide and dysfunctional REVD peptide onto PEI-PLMD-PEI

The targeting peptide CREVDW and dysfunctional peptide CREVDW were immobilized onto the PEI-PLMD-PEI copolymer *via* the heterobifunctional NHS-PEG-MAL spacer. In order to identify the grafting ratio, the peptide sequence was designed to contain a tryptophan (W) residue in each sequence, which could be quantitatively detected by fluorescence spectroscopy at 350 nm with the excitation spectrum at 280 nm. As clearly illustrated in Fig. 2, the negative control of PEI-PLMD-PEI aqueous solution showed no emission at 350 nm, while peptide functionalized NPs exhibited a characteristic emission peak at 350 nm indicating the successful grafting of the peptide onto the amphiphilic block copolymers of PEI-PLMD-PEI. According to the standard fluorescence curve ($y = 5429.3C \text{ (mg mL}^{-1}) + 12.3$, ($R^2 = 0.999$)), the contents of the peptide in the NPs of REDV-PPP1, REVD-PPP1, REDV-PPP2 and REVD-PPP2 were

Fig. 2 Fluorescence emission spectra of the prepared NP solutions after dialysis: REDV-PPP1 NPs (A), REVD-PPP1 NPs (B), REDV-PPP2 NPs (C), REVD-PPP2 NPs (D) and PEI-PLMD-PEI (E). The inserted figure was the standard curve of the CREVDW peptide for quantitative analysis.

quantitatively calculated to be 2.11 wt%, 2.09 wt%, 1.20 wt% and 1.17 wt%, respectively.

3.2. Characterization of NPs and NP/pZNF580 complexes

The amphiphilic REDV-PPP copolymers self-assembled into core-shell structure NPs in aqueous solution. Here, the hydrophobic PLMD block formed the core of the NPs and the hydrophilic PEI, PEG and REDV were preferentially located at the surface to create the shell. All NP suspensions presented a bright "pathway" due to a beam of red light ($\lambda = 650 \text{ nm}$) indicating the successful self-assembly of the copolymers. After the condensation of NPs with pZNF580, the cationic PEI collapsed with pZNF580 around the core and the hydrophilic PEG and REDV stretched out. Thus the NPs presented the core-shell-corona structure in aqueous solution.

The hydrodynamic diameters and zeta potentials of four NPs (REDV-PPP1, REVD-PPP1, REDV-PPP2 and REVD-PPP2 NPs) and their corresponding NP/pZNF580 complexes were measured by DLS using a Zetasizer Nano ZS and the results are shown in Fig. 3. For the NPs of REDV-PPP1, REVD-PPP1, REDV-PPP2 and REVD-PPP2 without pZNF580, the hydrodynamic diameters were $286.2 \pm 3.4 \text{ nm}$, $286.0 \pm 12.9 \text{ nm}$, $202.5 \pm 7.6 \text{ nm}$, and $205.5 \pm 14.8 \text{ nm}$, respectively. Upon increasing the N/P molar ratios from 5 to 25, the size of NP/pZNF580 complexes decreased gradually due to the strong interaction of positively charged NPs

Fig. 1 Preparation route of REDV-PPP copolymers by grafting REDV targeting peptide onto copolymers.

Fig. 3 Hydrodynamic diameters (the column chart) and zeta potentials (the line chart) of NPs and NP/pZNF580 complexes at various N/P molar ratios (5, 10, 15, 20 and 25): (A) REDV-PPP1/pZNF580 complexes, (B) REVD-PPP1/pZNF580 complexes, (C) REDV-PPP2/pZNF580 complexes and (D) REVD-PPP2/pZNF580 complexes.

and negatively charged pZNF580, which were in agreement with the previously reported work.⁵⁰ The zeta potential measurements were performed to evaluate the condensation capability of NPs with pZNF580 *via* electrostatic interaction. For the NPs without pZNF580, the zeta potential of REDV-PPP1 (18.6 ± 0.8 mV) and REVD-PPP1 (18.3 ± 1.2 mV) was lower than REDV-PPP2 (35.2 ± 3.8 mV) and REVD-PPP2 (35.6 ± 2.5 mV), which was probably because the conjugation of a neutral PEG linker could shield the surface charges; the more the PEG content, the more the shield efficiency. After the condensation of pZNF580, the zeta potential increased upon increasing the N/P molar ratio. At a low N/P molar ratio (N/P = 5), the surface positive charges of NPs were neutralized. Upon increasing the N/P molar ratios from 10 to 25, the abundant positive charges resulted in relatively high zeta potentials as well as the strong condensation of pZNF580. The small size and positive zeta-potential of NP/pZNF580 complexes would facilitate cell uptake and subsequently effective transfection.

3.3. Agarose gel retardation assay

For well-designed gene carriers, the positively charged NPs should effectively condense the negatively charged DNA and protect DNA from degradation and dysfunction. Here, agarose gel retardation assay was performed to investigate the condensation capability of NPs with pZNF580.

NP/pZNF580 complexes containing 3 μ g DNA with N/P molar ratios of 0, 1, 5, 10, 15 and 20 were loaded into the lanes of agarose gel. As shown in Fig. 4, the naked pZNF580 (N/P = 0) containing the negative charges migrated toward the positive electrode.

The migration of pZNF580 was retarded with the increasing N/P molar ratio and complete retardation was reached with the N/P molar ratio of 5 for all of the four NPs groups. This may be because the low positive charge in the complexes as well as the PEG blocks in the shell hindered the interactions between NPs

Fig. 4 Agarose gel retardation assay of the NP/pZNF580 complexes at various N/P molar ratios (0, 1, 5, 10, 15 and 20): (A) REDV-PPP1/pZNF580 complexes, (B) REVD-PPP1/pZNF580 complexes, (C) REDV-PPP2/pZNF580 complexes and (D) REVD-PPP2/pZNF580 complexes.

and pZNF580 at a low N/P molar ratio (such as N/P = 1). The pZNF580 condensation could be achieved through increasing the N/P molar ratio.

In the following biological tests, NP/pZNF580 complexes at the N/P molar ratio of 15 were selected because of their relatively small hydrodynamic diameters and positive zeta potentials which are favorable for cell uptake and gene transfection.

3.4. *In vitro* transfection of HUVECs

The *in vitro* transfection of HUVECs was performed to evaluate the delivery efficiency of the NP/pZNF580 complexes with PEI25/pZNF580 complexes treated as the positive control and only pZNF580 treated as the negative control. The recombinant pZNF580 encoding the interest gene ZNF580 as well as the reporter gene GFP was used to trace the transfection by visually observing the green fluorescence of the expressed GFP (Fig. 5(1)). First of all, we optimized the transfection conditions by varying the concentrations of pZNF580 and found that 2 μ g pZNF580 per well could perform the good transfection for NP/pZNF580 complexes. While for PEI25/pZNF580 complexes, this amount was too high to be tolerated, thus 1 μ g pZNF580 per well was used as the control for the best option. The following experiments were conducted under the optimized conditions. The transfection was enhanced for all groups from 12 h to 24 h, while a decrease of green fluorescence was observed upon further extending the time. So we presented the fluorescence images of 12 h and 24 h, the longer time images were not shown. We detected the successful delivery of pZNF580 into HUVECs for REDV-PPP1/pZNF580 complexes and REDV-PPP2/pZNF580 complexes, which is demonstrated by the statistical results of the transfection efficiencies of the green fluorescence analysis in Fig. 5(2). Furthermore, the transfection efficiency of the REDV peptide functionalized NPs groups was comparable with the positive control of PEI25/pZNF580 complexes, which may be attributed to the targeting REDV peptide inducing the complexes specifically adhered by ECs. For a further verification of the targeting function of the REDV peptide for ECs, the dysfunctional REDV peptide substituting the REDV peptide was grafted onto NPs and the transfection results are also shown in Fig. 5. Only few cells in the REDV-PPP/pZNF580 groups exhibited green fluorescence in the fluorescence images compared to the corresponding active REDV peptide functionalized NP/pZNF580 complexes and the PEI25/pZNF580 complex control group. These results indicated that the REDV peptide functionalized REDV-PPP NPs could effectively deliver pZNF580 into HUVECs.

3.5. Western blot assay of the ZNF580 protein in transfected cells

Western blot assay was used to evaluate the expression of the ZNF580 protein after 24 h transfection (Fig. 6). The relative pZNF580 protein levels of HUVECs transfected by REDV-PPP1/pZNF580 complexes, REDV-PPP2/pZNF580 complexes and PEI25/pZNF580 complexes were $40.3 \pm 2.4\%$, $36.7 \pm 1.7\%$ and $40.0 \pm 1.2\%$, respectively, showing the good transfection efficiency of the REDV peptide functionalized NPs. These results corresponded to the transfection results obtained by

Fig. 5 Fluorescence (left image) and corresponding bright-field (right image) images of HUVECs transfected by REDV-PPP1/pZNF580 complexes (A), REDV-PPP1/pZNF580 complexes (B), REDV-PPP2/pZNF580 complexes (C), REDV-PPP2/pZNF580 complexes (D), PEI25/pZNF580 complexes (E) as the positive control and only pZNF580 (F) as the negative control at time intervals of 12 h and 24 h (1), and the transfection efficiency at 24 h (2) based on the numbers of fluorescent cells counted dividing the total cells counted via the Image-Pro Plus 6.0 software (mean $\pm$ SD, $n = 9$, * statistically different from (F) the group with $P < 0.05$).

fluorescence images, indicating that the targeting adhesion of REDV peptide for ECs and the REDV peptide functionalized NPs could be a promising strategy and good candidates for successful delivery of pZNF580 into HUVECs.

3.6. *In vitro* quantitative real-time RT-PCR assay

The gene transfection efficiency of NP/pZNF580 complexes in HUVECs was also evaluated at the transcriptional aspect by

Fig. 6 The Western blot image (1) of the ZNF580 protein expression in HUVECs transfected by PEI25/pZNF580 complexes (A), only pZNF580 (B), REDV-PPP1/pZNF580 complexes (C) and REDV-PPP2/pZNF580 complexes (D) after 24 h and the statistical result (2) via the Image-J software (mean $\pm$ SD, $n = 3$, * statistically different from (B) the group with $P < 0.05$).

quantitative real-time RT-PCR analysis of measuring the expression of ZNF580 mRNA after 24 h transfection (Fig. 7). The relative ZNF580 mRNA level transfected by REDV-PPP1/pZNF580 complexes (1.83 ± 0.2 fold) and REDV-PPP2/pZNF580 complexes (1.60 ± 0.3 fold) showed comparable results with the positive control of the PEI25/pZNF580 complex group (1.73 ± 0.1 fold).

The results of the transcriptional aspect (quantitative real-time RT-PCR results), the protein aspect (western blot results), and the cell aspect (transfection fluorescence images) were consistent with each other indicating that the targeting of REDV peptide for ECs and further demonstrating that the REDV peptide functionalized NPs are promising candidates as gene carriers for transfection in ECs.

Fig. 7 Quantitative transfection efficiency of pZNF580 at the transcriptional aspect in HUVECs transfected by PEI25/pZNF580 complexes (A), only pZNF580 (B), REDV-PPP1/pZNF580 complexes (C) and REDV-PPP2/pZNF580 complexes (D) after 24 h via the StepOne software v2.1 (mean $\pm$ SD, $n = 3$, * statistically different from (B) the group with $P < 0.05$).

3.7. Cell uptake of the complexes

The internalization of the complexes was evaluated using a flow cytometer and the cell uptake results are shown in Fig. 8. We could see that there is not much difference in the percentages of cell uptake among the complexes; they were all above 97%. However, the mean fluorescence intensities of REDV peptide functionalized groups were significantly stronger than the dysfunctional REDV groups, and even comparable with the positive control of the PEI/pZNF580 group. This indicated that the REDV peptide showed positive effects on cell uptake in ECs which should be the reason for the improved transfection efficiency. Higher zeta potentials are thought to facilitate cell uptake, while in this study, the REDV-PPP/pZNF580 complexes exhibited higher zeta potential than the REDV-PPP1/pZNF580 complexes, but weaker mean fluorescence intensity in the cell uptake and transfection efficiency, demonstrating that the quantity of the REDV peptide influences its targeting effect on ECs.

3.8. In vitro cytotoxicity assay

The cytotoxicity of REDV-PPP1/pZNF580 complexes, REDV-PPP2/pZNF580 complexes, REDV-PPP2/pZNF580 complexes,

Fig. 8 Cell uptake of (A) REDV-PPP1/pZNF580 complexes, (B) REDV-PPP1/pZNF580 complexes, (C) REDV-PPP2/pZNF580 complexes and (D) REDV-PPP2/pZNF580 complex treated group with (E) PEI25/pZNF580 complex treated as the positive control and (F) without any treatment as the negative control. (1) Cy5 fluorescence intensity and corresponding counts, (2) mean fluorescence intensity (the column chart) and percentages of cell uptake (the line chart) measured by flow cytometry (mean $\pm$ SD, $n = 3$, * statistically different from (F) the group with $P < 0.05$).

and REDV-PPP2/pZNF580 complexes was evaluated on HUVECs by MTT assay with PEI10/pZNF580 complexes and PEI25/pZNF580 complexes as controls. The concentrations of pZNF580 ranged from $1 \mu\text{g mL}^{-1}$ to $10 \mu\text{g mL}^{-1}$ in the evaluation test. The cytotoxicity increased by increasing the concentration of pZNF580 at the same N/P molar ratio as shown in Fig. 9. At the same concentration, the NP/pZNF580 complexes showed significantly higher cell viability than the two control groups. Especially when the pZNF580 concentration increased to $5 \mu\text{g mL}^{-1}$, the NP/pZNF580 complexes could be maintained above 65% relative viability for HUVECs while the control groups exhibited beneath 25%. In particular, for the PEI to PEG molar ratio of 1 : 1 (REDV-PPP1/pZNF580 complexes and REDV-PPP1/pZNF580 complexes), the relative cell viabilities were over 60% even at the pZNF580 concentration of $10 \mu\text{g mL}^{-1}$. These results indicated that the peptide functionalized NPs could strongly condense pZNF580 in the shell with the hydrophilic PEG and the peptide corona effectively shielded the positively charged shell thus significantly decreasing the cytotoxicity of the NP/pZNF580 complexes and showing excellent cytocompatibility.

3.9. EC migration assays

Cell migration plays an important role in the wound healing process. What is more, EC migration is essential to angiogenesis and the formation of a confluent EC monolayer. Here, the wound healing assay and transwell migration assay were performed to assess the migration capability of transfected HUVECs. The cells were firstly transfected by different NP/pZNF580 complexes with PEI25/pZNF580 complexes as the positive control and only pZNF580 as the negative control. For the wound healing assay, the monolayer of HUVECs was artificially scratched as shown in

Fig. 9 Relative cell viability of HUVECs in the presence of REDV-PPP1/pZNF580 complexes (A), REDV-PPP1/pZNF580 complexes (B), REDV-PPP2/pZNF580 complexes (C), and REDV-PPP2/pZNF580 complexes (D) with various pZNF580 concentrations ($1, 3, 5, 8$ and $10 \mu\text{g mL}^{-1}$) based on the MTT assay, PEI10/pZNF580 complexes (E) and PEI25/pZNF580 complexes (F) were used as controls (mean $\pm$ SD, $n = 6$, # statistically different from (E) the group with $P < 0.05$, * statistically different from (F) the group with $P < 0.05$).

Fig. 10 Recovered process of HUVECs after 0, 6 and 12 h (1) and the relative recovered area after 12 h calculated using the Image-J software (2). (A) REDV-PPP1/pZNF580 complex treated group, (B) REDV-PPP1/pZNF580 complex treated group, (C) REDV-PPP2/pZNF580 complex treated group and (D) REDV-PPP2/pZNF580 complex treated group with (E) PEI25/pZNF580 complexes treated as the positive control and (F) only pZNF580 treated as the negative control (mean $\pm$ SD, $n = 3$, * statistically different from (F) the group (only pZNF580) with $P < 0.05$).

Fig. 10(1) 0 h. The cells migrated to recover the surface area and the process was monitored by capturing the images by extending the time. The relative migration area at 12 h was calculated and is shown in Fig. 10(2). REDV peptide functionalized NP/pZNF580 complex groups showed a significantly promoting effect on the migration of HUVECs. After 12 h, the relative migration area of cells transfected by REDV-PPP1/pZNF580 complexes and REDV-PPP2/pZNF580 complexes reached $79.1 \pm 1.6\%$ and $65.2 \pm 1.3\%$, respectively. Therefore, REDV peptide functionalized NP/pZNF580 complexes were beneficial for EC migration. In contrast, the REDV peptide linked NP/pZNF580 complexes exhibited relatively lower recovered area than the positive control group, because the REDV peptide cannot enhance EC adhesion.

The results of transwell migration assay are shown in Fig. 11. For the REDV peptide functionalized groups, the numbers of migrated ECs were much larger than the dysfunctional REDV-PPP/pZNF580 complex groups, which were in agreement with the wound healing assay.

The migration results of ECs transfected by different NP/pZNF580 complexes verified the targeting recognition of the REDV peptide to ECs and demonstrated that the REDV peptide functionalized NP/pZNF580 complexes could significantly promote the migration of ECs.

Fig. 11 Transwell migration assay of HUVECs after 6 h (1) and the average migrating cell number (2). (A) REDV-PPP1/pZNF580 complex treated group, (B) REDV-PPP1/pZNF580 complex treated group, (C) REDV-PPP2/pZNF580 complex treated group and (D) REDV-PPP2/pZNF580 complex treated group with (E) PEI25/pZNF580 complexes treated as the positive control and (F) only pZNF580 treated as the negative control (mean $\pm$ SD, $n = 3$, * statistically different from (F) the group with $P < 0.05$).

4. Discussion

Gene therapy has recently attracted much attention for rapid endothelialization in cardiovascular diseases.⁵² The transfected ECs with enhanced migration and proliferation abilities could quickly form a robust and confluent EC monolayer on the inner surface of the artificial vascular thus effectively preventing thrombosis and restenosis. However, there are still many challenges for safe and effective delivery of DNA. Especially for ECs, the intrinsic resistance to foreign genes and high sensitivity to the toxic effects make ECs one of the most difficult transfected cell types. Therefore, the design of gene carriers for EC-specific transfection is particularly important. Liposomes, PEI, polycations based on PEI and dendrimers, as well as cationic polysaccharides have been extensively studied in gene delivery. Among them, NPs containing core-shell structures have been demonstrated to have advantages due to the enhanced stability and the improved transfection efficiency. For rapid endothelialization *in vivo*, previous studies have demonstrated the targeting adhesion of ECs on REDV peptide functionalized scaffolds. So, for the selective accumulation of pDNA in blood vessel ECs, it is of great interest to link a precise amount of REDV peptide onto NPs using a convenient method and to investigate the specific adhesion function of REDV peptide in the enhancement of EC-specific transfection.

In the present study, we prepared NPs with a precise immobilization amount *via* a NHS-PEG-MAL spacer. The hydrophobic

core of NPs composed of biodegradable PLMD blocks could stabilize the NP structure and simultaneously enable the complexes to unpack and release pZNF580 at the desired rate. PEG blocks were grafted onto PEI-PLMD-PEI copolymers to form a highly hydrophilic shell which could decrease the cytotoxicity and improve the stability as well as the long circulation of NPs. The REDV peptide provided these NPs with specific EC adhesion ability to improve the cell uptake and transfection. What is more, the REDV peptide could maintain its biofunction benefiting from the flexible PEG spacer. The dysfunctional REDV peptide was used to substitute the REDV peptide for verifying the targeting adhesion and the enhanced transfection efficiency of REDV peptide functionalized NP/pZNF580 complexes on HUVECs. The transfection efficiencies of NP/pZNF580 complexes were evaluated using different methods. From the fluorescence images (Fig. 5), Western blot assay (Fig. 6) and quantitative real-time RT-PCR (Fig. 7), we could find that REDV peptide functionalized NP/pZNF580 complexes showed higher transfection efficiencies ($27.4 \pm 1.2\%$ for REDV-PPP1/pZNF580 complexes and $20.9 \pm 1.4\%$ for REDV-PPP2/pZNF580 complexes) than the dysfunctional REDV peptide linked NP/pZNF580 groups ($10.0 \pm 1.2\%$ for REDV-PPP1/pZNF580 complexes and $12.9 \pm 0.4\%$ for REDV-PPP2/pZNF580 complexes), even comparable with the positive control of PEI25/pZNF580 complexes ($25.6 \pm 2.9\%$), indicating that the REDV peptide functionalized NPs could enhance the transfection of HUVECs by specific interactions between the REDV peptide and the HUVECs. Compared with REDV-PPP1/pZNF580 (15.5 ± 0.7 mV, $N/P = 15$), REDV-PPP2/pZNF580 complexes (19.8 ± 0.4 mV, at $N/P = 15$) had higher zeta potential but approximately the same hydrodynamic diameter. However, in this study, the REDV-PPP2/pZNF580 complex group showed lower transfection than the REDV-PPP1/pZNF580 complex group.⁵³ Although the zeta potential of complexes usually facilitates cellular uptake, our results have demonstrated that the REDV amount in NP/pZNF580 complexes also benefits to cellular uptake.

Furthermore, we used a flow cytometer to analyze the cellular uptake. The mean fluorescence intensities of the active REDV-PPP/pZNF580 groups were more than twice those of the dysfunctional REDV control ones, indicating that the REDV peptide could specifically enhance EC transfection. The REDV-PPP1/pZNF580 group had much higher mean fluorescence intensities in the cellular uptake (Fig. 8), which is consistent with transfection efficiencies shown in Fig. 5. The enhanced transfection efficiency was attributed to the increased cell uptake of REDV-PPP1/pZNF580 complexes into ECs and relied on the amount of the peptide in NPs.

Another challenge for gene therapy is the expression of transgene and the activity of the expressed protein. As the overexpression of the ZNF580 protein is beneficial for the enhanced migration of ECs, the wound healing assay and transwell migration assay were used to evaluate the migration of transfected HUVECs by the NP/pZNF580 complexes. As shown in Fig. 10 and 11, the highest migration rate was achieved for REDV peptide functionalized NP/pZNF580 complexes while the dysfunctional REDV peptide linked NP/pZNF580 complexes

showed relatively low migration. This result demonstrated that the expressed ZNF580 protein maintained its function for improving the migration of ECs which was accordant with a previous report.⁵⁴

Based on the experiment and discussion above, it can be concluded that the improved transfection and migration of HUVECs were attributed to the REDV peptide functionalized NPs *via* increasing the cell uptake and relied on the amount of the active peptide on NPs.

5. Conclusion

In this study, we have prepared EC-specific NPs as gene carriers for the delivery of pZNF580 into HUVECs. The transfection efficiency was evaluated using fluorescence imaging, ZNF580 protein and ZNF580 RNA in the cell, protein and transcriptional aspects. The results demonstrated that HUVECs were efficiently transfected by REDV peptide functionalized NP/pZNF580 complexes. And the high transfection efficiency was attributed to the REDV peptide which could actively target ECs and enhance cell uptake. The significantly promoted cell migration in turn proved the successful transfection and the retained bioactivity of the expressed ZNF580 protein. The convenient construction of targeting REDV peptide functionalized gene carriers with low cytotoxicity and high transfection efficiency should be a potentially promising strategy in gene delivery, especially for the endothelialization of artificial vascular implants.

Acknowledgements

This project was supported by the National Natural Science Foundation of China (Grant No. 31370969) and the International Cooperation from Ministry of Science and Technology of China (Grant No. 2013DFG52040).

References

- X. Ren, Y. Feng, J. Guo, H. Wang, Q. Li, J. Yang, X. Hao, J. Lv, N. Ma and W. Li, *Chem. Soc. Rev.*, 2015, **44**, 5680–5742.
- J. Yang, J. Lv, B. Gao, L. Zhang, D. Yang, C. Shi, J. Guo, W. Li and Y. Feng, *Front. Chem. Sci. Eng.*, 2014, **8**, 188–196.
- W. Zhou, Y. Feng, J. Yang, J. Fan, J. Lv, L. Zhang, J. Guo, X. Ren and W. Zhang, *J. Mater. Sci.: Mater. Med.*, 2015, **26**, 56.
- C. Huang, S. Wang, L. Qiu, Q. Ke, W. Zhai and X. Mo, *ACS Appl. Mater. Interfaces*, 2013, **5**, 2220–2226.
- Y. Feng, H. Zhao, M. Behl, A. Lendlein, J. Guo and D. Yang, *J. Mater. Sci.: Mater. Med.*, 2013, **24**, 61–70.
- K. Jin, X. Ye, S. Li, B. Li, C. Zhang, C. Gao and J. Ye, *Graefes. Arch. Clin. Exp. Ophthalmol.*, 2016, **254**, 83–89.
- M. Q. Tan, Y. K. Feng, H. Y. Wang, L. Zhang, M. Khan, J. T. Guo, Q. L. Chen and J. S. Liu, *Macromol. Res.*, 2013, **21**, 541–549.
- H. Y. Wang, Y. K. Feng, Z. C. Fang, R. F. Xiao, W. J. Yuan and M. Khan, *Macromol. Res.*, 2013, **21**, 860–869.
- W. Yuan, Y. Feng, H. Wang, D. Yang, B. An, W. Zhang, M. Khan and J. Guo, *Mater. Sci. Eng., C*, 2013, **33**, 3644–3651.
- Y. Yao, J. Wang, Y. Cui, R. Xu, Z. Wang, J. Zhang, K. Wang, Y. Li, Q. Zhao and D. Kong, *Acta Biomater.*, 2014, **10**, 2739–2749.
- M. Khan, J. Yang, C. C. Shi, Y. K. Feng, W. C. Zhang, K. Gibney and G. N. Tew, *Macromol. Mater. Eng.*, 2015, **300**, 802–809.
- J. Yang, J. Lv, M. Behl, A. Lendlein, D. Yang, L. Zhang, C. Shi, J. Guo and Y. Feng, *Macromol. Biosci.*, 2013, **13**, 1681–1688.
- C. Shi, W. Yuan, M. Khan, Q. Li, Y. Feng, F. Yao and W. Zhang, *Mater. Sci. Eng., C*, 2015, **50**, 201–209.
- L. Yu, Y. Feng, Q. Li, X. Hao, W. Liu, W. Zhou, C. Shi, X. Ren and W. Zhang, *React. Funct. Polym.*, 2015, **91–92**, 19–27.
- M. Khan, J. Yang, C. Shi, Y. Feng, W. Zhang, K. Gibney and G. N. Tew, *RSC Adv.*, 2015, **5**, 11284–11292.
- D. V. Bax, A. Kondyurin, A. Waterhouse, D. R. McKenzie, A. S. Weiss and M. M. Bilek, *Biomaterials*, 2014, **35**, 6797–6809.
- H. Zhang, X. Jia, F. Han, J. Zhao, Y. Zhao, Y. Fan and X. Yuan, *Biomaterials*, 2013, **34**, 2202–2212.
- Q. Lin, X. Ding, F. Qiu, X. Song, G. Fu and J. Ji, *Biomaterials*, 2010, **31**, 4017–4025.
- A. J. Melchiorri, N. Hibino, T. Yi, Y. U. Lee, T. Sugiura, S. Tara, T. Shinoka, C. Breuer and J. P. Fisher, *Biomacromolecules*, 2015, **16**, 437–446.
- H. Yin, R. L. Kanasty, A. A. Eltoukhy, A. J. Vegas, J. R. Dorkin and D. G. Anderson, *Nat. Rev. Genet.*, 2014, **15**, 541–555.
- Y. Wei, Y. Ji, L. Xiao, Q. Lin and J. Ji, *Colloids Surf., B*, 2011, **84**, 369–378.
- Y. Wei, Y. Ji, L. L. Xiao, Q. K. Lin, J. P. Xu, K. F. Ren and J. Ji, *Biomaterials*, 2013, **34**, 2588–2599.
- J. Yang, M. Khan, L. Zhang, X. K. Ren, J. T. Guo, Y. K. Feng, S. P. Wei and W. C. Zhang, *J. Mater. Chem. B*, 2015, **3**, 7682–7687.
- M. I. Castellanos, A. S. Zenses, A. Grau, J. C. Rodriguez-Cabello, F. J. Gil, J. M. Manero and M. Pegueroles, *Colloids Surf., B*, 2015, **127**, 22–32.
- W. Wang, L. Guo, Y. Yu, Z. Chen, R. Zhou and Z. Yuan, *J. Biomed. Mater. Res., Part A*, 2015, **103**, 1703–1712.
- W. Chen, F. Meng, R. Cheng, C. Deng, J. Feijen and Z. Zhong, *J. Controlled Release*, 2014, **190**, 398–414.
- D. T. Clarke and N. A. McMillan, *Nat. Nanotechnol.*, 2014, **9**, 568–569.
- T. Abel, E. El Filali, J. Waern, I. C. Schneider, Q. Yuan, R. C. Munch, M. Hick, G. Warnecke, N. Madrahimov, R. E. Kontermann, J. Schuttrumpf, U. C. Muller, J. Seppen, M. Ott and C. J. Buchholz, *Blood*, 2013, **122**, 2030–2038.
- J. Zhang, Y. Duan, D. Wei, L. Wang, H. Wang, Z. Gu and D. Kong, *J. Biomed. Mater. Res., Part A*, 2011, **96**, 212–220.
- M. N. Chavez, T. L. Schenck, U. Hopfner, C. Centeno-Cerdas, I. Somlai-Schweiger, C. Schwarz, H. G. Machens, M. Heikenwalder, M. R. Bono, M. L. Allende, J. Nickelsen and J. T. Egana, *Biomaterials*, 2015, **75**, 25–36.
- N. Monteiro, D. Ribeiro, A. Martins, S. Faria, N. A. Fonseca, J. N. Moreira, R. L. Reis and N. M. Neves, *ACS Nano*, 2014, **8**, 8082–8094.
- J. Devalliere, W. G. Chang, J. W. Andrejcsk, P. Abrahimi, C. J. Cheng, D. Jane-wit, W. M. Saltzman and J. S. Pober, *FASEB J.*, 2014, **28**, 908–922.

- 33 Y. Hu, W. Yuan, N. N. Zhao, J. Ma, W. T. Yang and F. J. Xu, *Biomaterials*, 2013, **34**, 5411–5422.
- 34 J. J. Nie, X. B. Dou, H. Hu, B. Yu, D. F. Chen, R. X. Wang and F. J. Xu, *ACS Appl. Mater. Interfaces*, 2015, **7**, 553–562.
- 35 F. J. Xu and W. T. Yang, *Prog. Polym. Sci.*, 2011, **36**, 1099–1131.
- 36 H. Y. Sun, S. P. Wei, R. C. Xu, P. X. Xu and W. C. Zhang, *Biochem. Biophys. Res. Commun.*, 2010, **395**, 361–366.
- 37 Y. Y. Yang, H. Hu, X. Wang, F. Yang, H. Shen, F. J. Xu and D. C. Wu, *ACS Appl. Mater. Interfaces*, 2015, **7**, 12238–12248.
- 38 J. Li, C. Zheng, S. Cansiz, C. Wu, J. Xu, C. Cui, Y. Liu, W. Hou, Y. Wang, L. Zhang, I. T. Teng, H. H. Yang and W. Tan, *J. Am. Chem. Soc.*, 2015, **137**, 1412–1415.
- 39 Q. F. Zhang, Q. Y. Yu, Y. Geng, J. Zhang, W. X. Wu, G. Wang, Z. Gu and X. Q. Yu, *ACS Appl. Mater. Interfaces*, 2014, **6**, 15733–15742.
- 40 H. L. Cao, Y. X. Dong, A. Aied, T. Y. Zhao, X. Chen, W. X. Wang and A. Pandit, *Chem. Commun.*, 2014, **50**, 15565–15568.
- 41 T. Y. Zhao, H. Zhang, B. Newland, A. Aied, D. Z. Zhou and W. X. Wang, *Angew. Chem., Int. Ed.*, 2014, **53**, 6095–6100.
- 42 H. Q. Song, X. B. Dou, R. Q. Li, B. R. Yu, N. N. Zhao and F. J. Xu, *Acta Biomater.*, 2015, **12**, 156–165.
- 43 Y. Zhu, G. Liang, B. Sun, T. Tian, F. Hu and Z. Xiao, *Int. J. Nanomed.*, 2016, **11**, 399–411.
- 44 J. He, Y. Su, T. Huang, B. Jiang, F. Wu and Z. Gu, *Colloids Surf., B*, 2014, **116**, 303–308.
- 45 J. Lv, X. F. Hao, J. Yang, Y. K. Feng, M. Behl and A. Lendlein, *Macromol. Chem. Phys.*, 2014, **215**, 2463–2472.
- 46 X. Zhang, S. R. Pan, M. Feng, Z. J. Li, W. Zhang and X. Luo, *Prog. Biochem. Biophys.*, 2007, **34**, 1065–1071.
- 47 Q. Li, C. Shi, W. Zhang, M. Behl, A. Lendlein and Y. Feng, *Adv. Healthcare Mater.*, 2015, **4**, 1225–1235.
- 48 C. Shi, F. Yao, Q. Li, M. Khan, X. Ren, Y. Feng, J. Huang and W. Zhang, *Biomaterials*, 2014, **35**, 7133–7145.
- 49 H. Wang, Y. Feng, J. Yang, J. Guo and W. Zhang, *J. Mater. Chem. B*, 2015, **3**, 3379–3391.
- 50 X. F. Hao, Q. Li, J. Lv, L. Yu, X. K. Ren, L. Zhang, Y. K. Feng and W. C. Zhang, *ACS Appl. Mater. Interfaces*, 2015, **7**, 12128–12140.
- 51 M. W. Pfaffl, *Nucleic Acids Res.*, 2001, **29**, e45.
- 52 B. C. Dash, D. Thomas, M. Monaghan, O. Carroll, X. Chen, K. Woodhouse, T. O'Brien and A. Pandit, *Biomaterials*, 2015, **65**, 126–139.
- 53 X. Zheng, T. B. Zhang, X. Y. Song, L. Zhang, C. Q. Zhang, S. B. Jin, J. F. Xing and X. J. Liang, *J. Mater. Chem. B*, 2015, **3**, 4027–4035.
- 54 Y. Luo, Y. Zhao, X. Li, J. Zhao and W. Zhang, *Mol. Cell. Biochem.*, 2014, **393**, 199–207.