NJC

PAPER

View Article Online
View Journal

Cite this: DOI: 10.1039/c9nj02396h

Glutathione modified low molecular weight PEI for highly improved gene transfection ability and biocompatibility†

Yu-Rong Zhan, Qing-Ying Yu, Ji Zhang, * Yan-Hong Liu, Ya-Ping Xiao, Ju-Hui Zhang, Xi He and Xiao-Qi Yu *

Received 9th May 2019,
Accepted 4th July 2019

DOI: 10.1039/c9nj02396h

rsc.li/njc

The efficient delivery of therapeutic genes remains a major challenge in realizing a feasible gene-based treatment. Herein, a versatile oligopeptide, glutathione, was introduced to construct novel non-viral cationic gene vectors. Reduced/oxidized forms of glutathione (GSH/GSSG) and relevant amino acids (Glu, Cys, and Gly) were used to modify low molecular weight PEI through surface modification or crosslinking. These polymers could bind well and condense DNA into spherical nanoparticles, which were stable in the presence of serum. The disulfide bonds within the crosslinked polymer **GSSG-PEI** may facilitate polymer degradation and DNA release under a reductive environment. *In vitro* transfection experiments reveal that the modification could largely improve the gene transfection efficiency of low molecular weight PEI, especially in the presence of serum. In HeLa cells, **GSSG-PEI** could even give up to 150 times higher efficiency than PEI 25 kDa. TEM and serum concentration effect assay also demonstrate the good serum tolerance of the polymers. Flow cytometry results show that **GSSG-PEI** might induce cellular uptake with higher efficiency than PEI 25 kDa, especially in the presence of serum. Results reveal that GSSG is a good candidate for the crosslinking of small cationic molecules to form polymeric gene vectors with improved transfection efficiency and biocompatibility.

1. Introduction

With the development of molecular biology and gene transfer techniques, therapeutic interventions at the genetic level have gained more attention and achievement in recent decades, and gene therapy is regarded as a potential clinical treatment.[1–5] To achieve efficient gene delivery, it is necessary to overcome several extracellular and intracellular barriers including gene loading, systemic long circulation, cellular uptake, endosomal escape and cargo release. Thus, a safe and efficient vector is essential in gene therapy.[6] Although some viral vectors exhibited high efficiency, it is difficult to control their immunogenicity and tropism.[7,8] In contrast, non-viral vectors are more advantageous in biosafety and preparation, making them the research focus of gene vectors.[9,10]

Among the numerous non-viral vectors, cationic polymer is the most studied type for its stability, high nucleic acid loading, and easy modification. Polyethylenimine (PEI) is one of the promising non-viral cationic polymeric vectors due to its high transfection efficiency resulting from its effective endosomal escape through the "proton-sponge" mechanism.[11] However, high molecular weight PEI shows not only high transfection efficiency but also high cytotoxicity, while low molecular weight PEI shows low cytotoxicity but poor transfection efficiency.[12,13] Therefore, development of PEI derivatives with high efficiency together with low toxicity *via* appropriate modification is of special significance. Some strategies have been applied for the modification of PEI: (1) crosslinking low molecular weight PEI with biodegradable bonds, such as ester,[14,15] disulfide,[16,17] ketal,[18] imine[19] and hydrazine,[20] which are cleavable under an intracellular acid and reductive environment, facilitating the release of gene cargo and the decrease of cytotoxicity; (2) attaching biocompatible components, including amino acids,[21–23] PEG,[24,25] and polysaccharides,[26,27] to decrease the cytotoxicity of PEI and extend systemic circulation; (3) grafting targeting ligands, such as RGD peptides, folic acid, and hyaluronic acid, to promote the specificity of cellular uptake;[13,18,28–31] (4) introducing stimuli-responsive motifs to achieve effective controlled release;[28–32] (5) adding short hydrophobic substituents to endow the vector with amphiphilicity, which could promote interaction with the cell membrane and polyplex stability.[33]

Glutathione (GSH), L-γ-glutamyl-L-cysteinyl-glycine, as a versatile oligopeptide widely existing in cells, plays significant

Key Laboratory of Green Chemistry and Technology (Ministry of Education), College of Chemistry, Sichuan University, Chengdu 610064, P. R. China. E-mail: jzhang@scu.edu.cn, xqyu@scu.edu.cn

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c9nj02396h

roles in free radical scavenging, detoxification, liver protection and anti-cancer.[34,35] In cells, glutathione is simultaneously present in another form, glutathione disulfide (GSSG), which is formed upon oxidation and is also referred to as "oxidized glutathione".[36] According to previous reports, GSH has been applied as a capping agent to impart biocompatibility and monodispersity to cell-imaging quantum dots and bimodal imaging probes.[37,38] Besides, the GSSG-modified nanoparticle (GSSG@Fe_3O_4) was found to show high biocompatibility and effective cellular uptake.[39] It was also reported that the addition of GSH could reduce the cytotoxicity of PEI-coated nanoparticles.[40] At present, GSH is mainly used as a biocompatible shield on the surface of nanoparticles. Therefore, we herein introduced GSH/GSSG moieties into low molecular weight PEI though covalent surface modification (GSH) or crosslinking (GSSG) to create novel gene vectors. The unique nature of GSH was expected to endow vectors with excellent biocompatibility and transfection efficiency. Furthermore, the disulfide bond in GSSG is a classical reduction-responsive motif whose cleavage under an intracellular reducing environment contributes to cargo release during gene delivery.

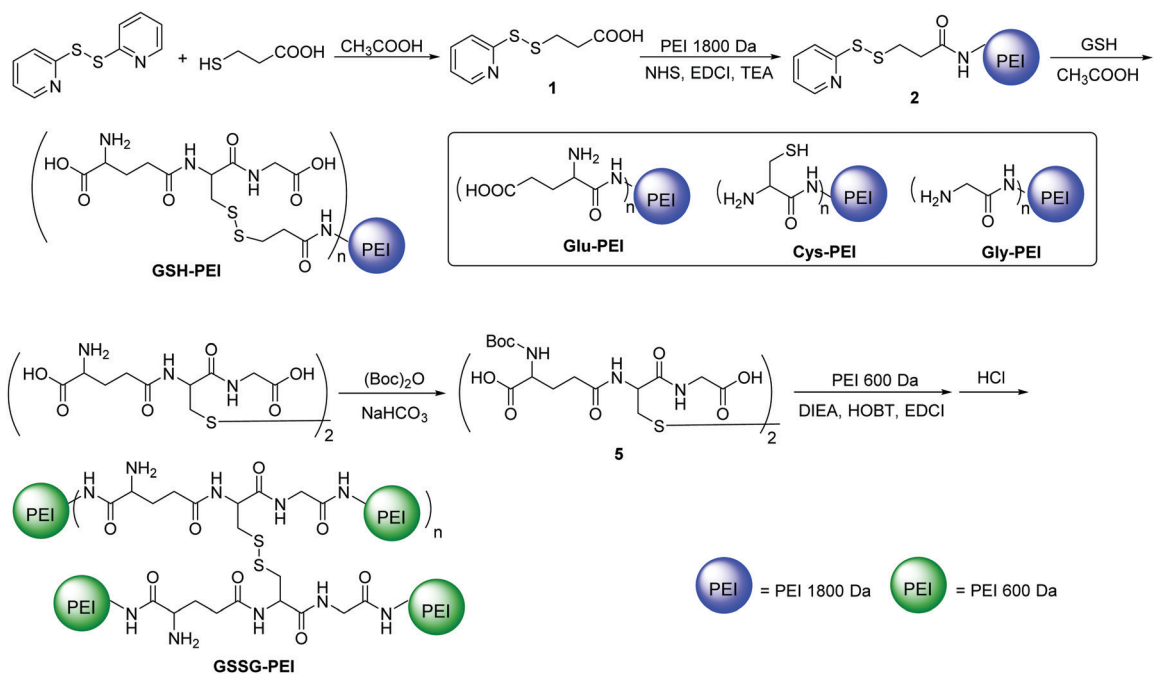
2. Results and discussion

2.1. Preparation of target polymers

The target polymers were prepared according to the route shown in Scheme 1 and Scheme S1 (ESI†). PEI with a molecular weight of 1800 Da was chosen for the modification. GSH was grafted on PEI through several steps starting from dithiodipyridine, which first reacted with mercaptopropionic acid to give compound **1**. The carboxyl of **1** was subsequently condensed with the amino group of PEI to yield material **2**. After the mercapto-exchange reaction with GSH, target polymer **GSH-PEI** could be obtained. For comparison, PEI modified with relevant amino acids including glutamic acid, cysteine, and glycine was also prepared (named as **Glu-PEI**, **Cys-PEI**, and **Gly-PEI** respectively). Different feed ratios (mole ratio of amino acid or GSH to amino groups in PEI 1800 Da) were used to obtain a series of modified PEI derivatives with different substitution degrees (SDs), which could be calculated according to the ratio of characteristic peak integrals in the ^{1}H NMR spectra. The data shown in Table S1 (ESI†) indicate that the actual SDs were lower than the feed ratio because of the steric hindrance and different reactivity of the various amino groups. According to previous results,[22] the modified PEI with a SD of ~10% was chosen for subsequent study. Besides, the oxidized form of GSH (GSSG) was also used to connect PEI to form crosslinked polymers (**GSSG-PEI**). Since several PEI units may exist in one polymer molecule, a lower molecular weight (600 Da) was used for the crosslinking. Similarly, the actual mole ratio of PEI 600 Da to GSSG in the polymer was estimated to be ~3 according to the characteristic peak integrals in the ^{1}H NMR spectrum, slightly lower than the feed mole ratio of 4. GPC was used to measure the molecular weight (M_w) and polydispersity indexes (PDIs) of polymers, which are shown in Table S2 (ESI†). The M_ws of single amino acid modified PEI were found to be around 5 kDa, while **GSH-PEI** and **GSSG-PEI** exhibited a higher M_w (7–7.5 kDa). The molecular weight of all polymers was distinctly lower than that of PEI 25 kDa, which would be beneficial for the biocompatibility.

2.2. Formation and characterization of polymer/DNA complexes

It is essential for gene vectors to have the ability to bind and condense DNA into nanoparticles with suitable size and surface



Scheme 1 Synthetic route of target polymers.

potential. Agarose gel retardation assay was carried out to assess the capacity of polymers to bind DNA. As shown in Fig. 1A, all the polymers could hinder the migration of DNA entirely at polymer/DNA weight ratios (w/w) of 0.8, which was just slightly higher than PEI 25 kDa (0.4), suggesting their good cationic capabilities to bind and condense DNA.

The stability of polymer/DNA complexes (polyplexes) was subsequently investigated, and heparin was used to bind the cationic polymers and induce the DNA release. The results shown in Fig. 1B indicate that DNA release could be observed at a relatively higher heparin/DNA weight ratio of 8–16, reflecting the good stability of the polyplexes, which is important for DNA protection. It could be found that PEI 25 kDa bound to DNA so tightly that heparin could not release DNA from its polyplex even at high dosage. Such tight binding may hinder the DNA release in the transfection process, leading to a negative effect on transfection efficiency.

The disulfide bond in **GSSG-PEI** was expected to be cleaved under the reductive environment in cells to facilitate the release of DNA. Thus, DTT was used to verify the reduction responsiveness of **GSSG-PEI**. As shown in Fig. 2, upon incubation with DTT, DNA could be released by heparin more easily. In other words, DTT could promote the release of DNA. However, since PEI 600 Da could also interact and bind with DNA, total release could not be achieved.

The physical properties of the polyplexes, such as particle size and zeta potential, were evaluated by using the dynamic light scattering (DLS) method. As shown in Fig. 3A, with the increase of w/w, the particle size of polyplexes gradually decreased and tended to be stable at 200 to 400 nm, which is a size suitable for cellular uptake.[41] Meanwhile, the zeta potential of the polyplexes underwent charge reversal from negative to positive with a w/w increase and ultimately stabilized at around +30 mV (Fig. 3B), which is indispensable for polyplexes to facilitate their interaction with the cell membrane and subsequent cellular uptake. In addition, the morphology of the polyplex was observed directly *via* TEM. Taking **GSSG-PEI** as an example, the polyplex particles under serum-free or serum-containing conditions are shown in Fig. 3C. The polyplex existed in the form of approximately spherical particles that were seldom affected by serum, indicating their good stability and serum tolerance.

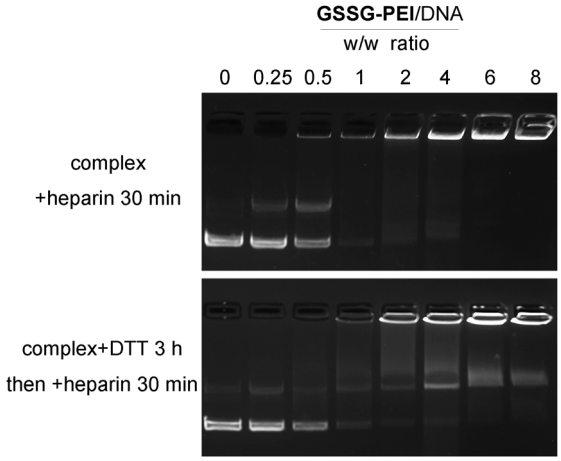


Fig. 2 DTT assisted DNA release from the polyplexes at various weight ratios (DTT: 20 mM, heparin: 0.4 mg mL^{-1}).

2.3. *In vitro* gene transfection

To evaluate the gene transfection efficiency of these polymers, experiments on luciferase reporter gene pGL-3 plasmid expression were carried out in two cell lines, and the results are shown in Fig. 4. In the absence of serum in HeLa cells (Fig. 4A), all the polymers performed better than PEI 1800 Da, demonstrating the advantage of modification. Nevertheless, compared to PEI 25 kDa, only **GSSG-PEI** showed higher efficiency, suggesting that crosslinking might be a better strategy than surface modification for low molecular weight PEI. In 7702 cells, all the polymers could give higher transfection efficiency than PEI 25 kDa (Fig. 4C). Unfortunately, although the crosslinked polymer **GSSG-PEI** showed good results, the GSH modified material **GSH-PEI** did not exhibit superior performance compared to the single amino acid decorated PEIs. On the other hand, in the presence of serum, all the studied polymers exhibited higher transfection efficiency relative to PEI 25 kDa. For **GSSG-PEI** in HeLa cells, up to 150 times higher transfection efficiency than PEI 25 kDa could be achieved (Fig. 4B). Without the presence of serum, only 2.7 times higher efficiency was obtained. Other amino acid/GSH modified PEIs also showed such a trend. Besides, the enhanced green fluorescent protein expression experiment results (Fig. S1, ESI†) also visually reveal that all the modified polymers

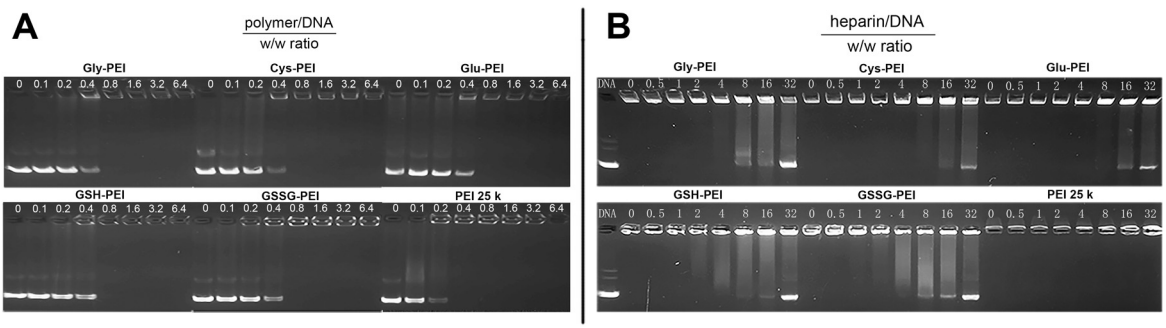


Fig. 1 (A) DNA retardation by polymers at different weight ratios. (B) DNA release assay by the addition of heparin.

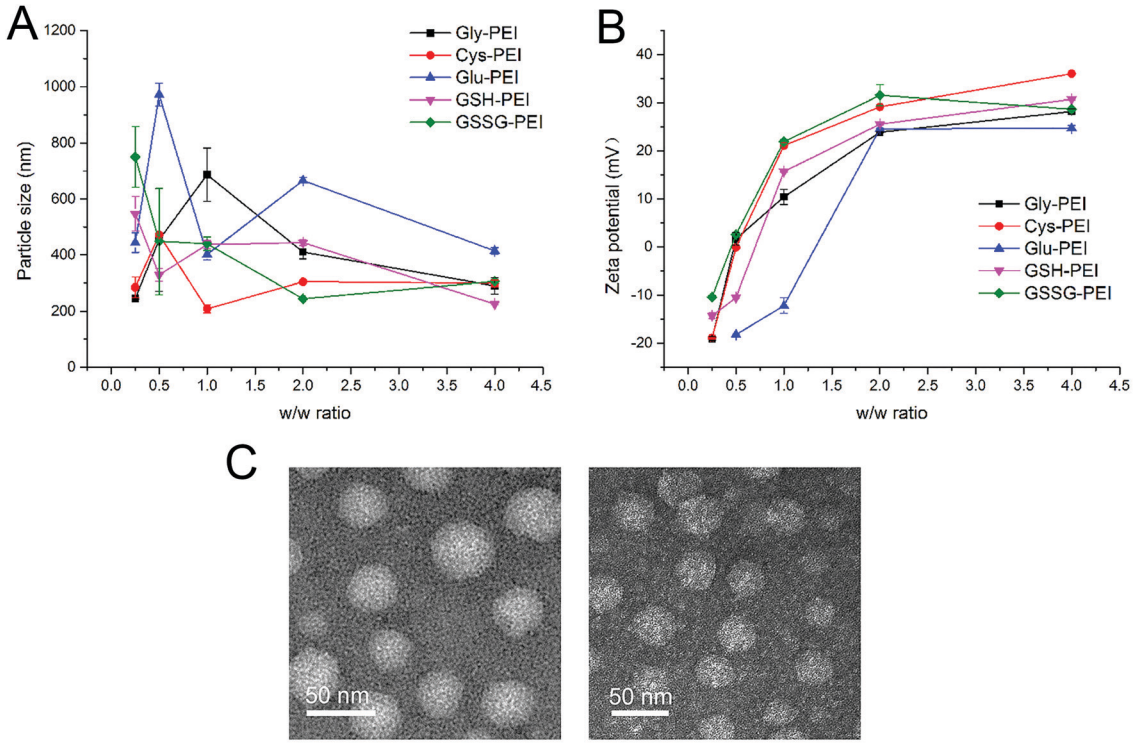


Fig. 3 Particle size (A) and zeta potential (B) of polyplexes at various weight ratios. Data represent mean ± SD (n = 3). (C) TEM images of **GSSG-PEI**/DNA complex (w/w = 16) without serum (left) or with serum (right), scale bar: 50 nm.

could induce more green fluorescence than PEI 1800 Da both in tumor and normal cells, and **GSSG-PEI** showed better transfection behavior than PEI 25 kDa, especially in the presence of serum. These results suggest that the modification PEI could not only enhance the transfection efficiency of the cationic materials, but also improve the serum tolerance of the vectors. The higher transfection efficiency of **GSSG-PEI** might also come from its reduction responsiveness, which may facilitate DNA release in cells. Furthermore, the effect of serum concentration on the transfection efficiency of **GSSG-PEI** was also investigated. The results in Fig. 5 show that the increase of serum concentration only caused a slight decrease of efficiency. On the contrary, the PEI 25 kDa-mediated transfection efficiency gave rise to a dramatic inhibition, further verifying the excellent serum tolerance of **GSSG-PEI**.

Flow cytometry was performed to measure the cell internalization of Cy5-labeled DNA carried by the polymers using the fluorescence activated cell sorting (FACS) technique. After 4 h of incubation with the polyplexes in HeLa cells, the percentage of positive cells for Cy5-labeled DNA and mean fluorescence intensity (MFI) could be calculated. Among the two quantities, the MFI may reflect the amount of internalized Cy5-labeled DNA more accurately. As shown in Fig. 6A, **GSSG-PEI** exhibited slightly lower cellular uptake percentages than PEI 25 kDa both in the absence and presence of serum. However, it could induce higher fluorescence intensity (Fig. 6B), suggesting that **GSSG-PEI** may induce cellular uptake with higher efficiency. This might be due to the enhanced biocompatibility *via* the GSSG modification, which made the polyplexes enter cells more easily, especially in the presence of serum. Similar to the transfection results, it could be found that serum has a less negative effect on the modified PEIs than that on PEI 25 kDa, especially for the MFI. The dramatic decrease of transfection efficiency of PEI 25 kDa could be attributed to the largely reduced amount of internalized DNA. Therefore, protecting DNA from extracellular degradation by nuclease or early release by serum proteins and other components is significant for the design of PEI derived vectors. Besides, confocal laser scanning microscopy (CLSM) was also used to visualize the effective internalization of Cy5-labeled DNA delivered by the polymer vectors in the presence of serum (Fig. S2, ESI†).

Cytotoxicity is also an important factor to evaluate non-viral gene vectors. The cell viability of the polyplexes prepared at various weight ratios was measured by MTS assay in HeLa cells, and the results are shown in Fig. 7. The modification led to no obvious change in the cell viability, except for the polymers with higher molecular weight at high weight ratios. All of the polymers exhibited higher cell viability than PEI 25 kDa, indicating that molecular weight is still the main factor in the cytotoxicity of cationic polymers. Combined with the transfection results, it is proved that the rational modification of low molecular weight PEI with amino acids or small peptides is a promising strategy to enhance the gene transfection efficiency while reducing the toxicity of the vectors.

3. Experimental section

3.1. Materials and methods

All reagents and chemicals were purchased from commercial providers and used without further purification unless specially

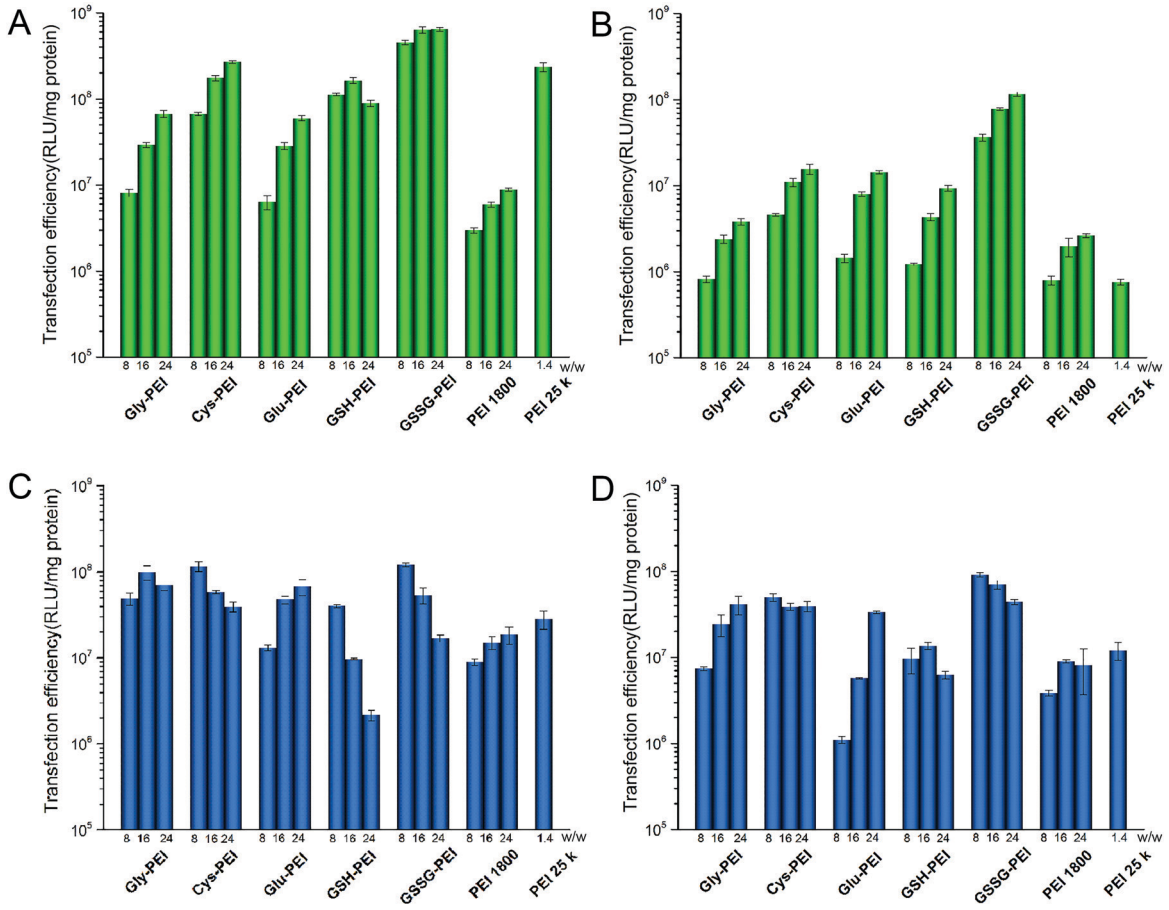


Fig. 4 Luciferase gene expression mediated by the polyplexes at different weight ratios in HeLa (A: without serum; B: with 10% serum) and 7702 cells (C: without serum; D: with 10% serum). PEI 1.8 and 25 kDa as control.

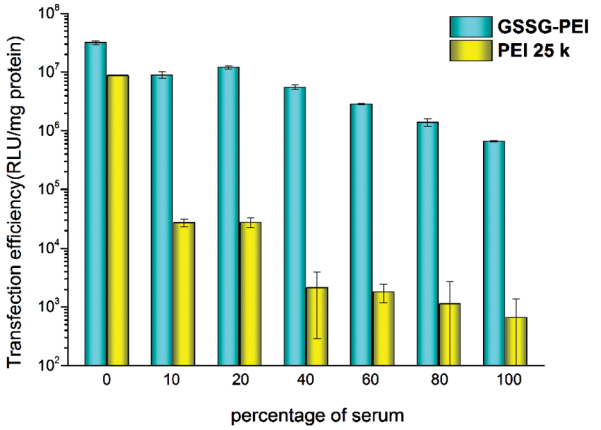


Fig. 5 Effect of serum concentration on the transfection efficiency of **GSSG-PEI** (w/w = 16) and PEI 25 kDa (w/w = 1.4) in HeLa cells.

mentioned. Anhydrous dichloromethane (DCM) was distilled after being dried with calcium hydride (CaH_2). All aqueous solutions were prepared using deionized water. Column chromatography was performed using 200–300 mesh silica gel. Branched polyethylenimine (PEI 25 kDa, M_w = 2.5 × 10^4) was supplied by Sigma-Aldrich (St. Louis, MO, USA) and low molecular weight PEI (PEI 1800 Da, M_w = 1800; PEI 600 Da, M_w = 600) was purchased from Aladdin (Shanghai, China). *N*-Boc-glycine, cysteine, γ-benzyl-glutamate, reduced glutathione and 2,2′-dithiodipyridine were purchased from Energy Chemistry (Shanghai, China). Oxidized glutathione was purchased from J&K Scientific Ltd. The plasmids used in this work were pEGFP-N1 (Clontech, Palo Alto, CA, USA) coding for Enhanced Green Fluorescent Protein (EGFP) DNA and pGL-3 (Promega, Madison, WI, USA) coding for luciferase DNA. HeLa and 7702 cell lines were purchased from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. Cy5™ was supplied by Molecular Probe (Mirus, Madison, WI, USA). DMEM, 1640 medium and fetal bovine serum (FBS) were supplied by Invitrogen Corp (Carlsbad, CA, USA). The MicroBCA protein assay kit was obtained from Pierce (Rockford, IL, USA). The luciferase assay kit and MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfo-phenyl)-2*H* tetrazolium, inner salt) were obtained from Promega (Madison, WI, USA).

The ^{1}H NMR and ^{13}C NMR spectra were measured on a Bruker AM400 NMR spectrometer (Bruker Corporation, Billerica, MA, USA). HRMS spectral data were recorded on a Bruker Daltonics Bio TOF mass spectrometer. The molecular weights

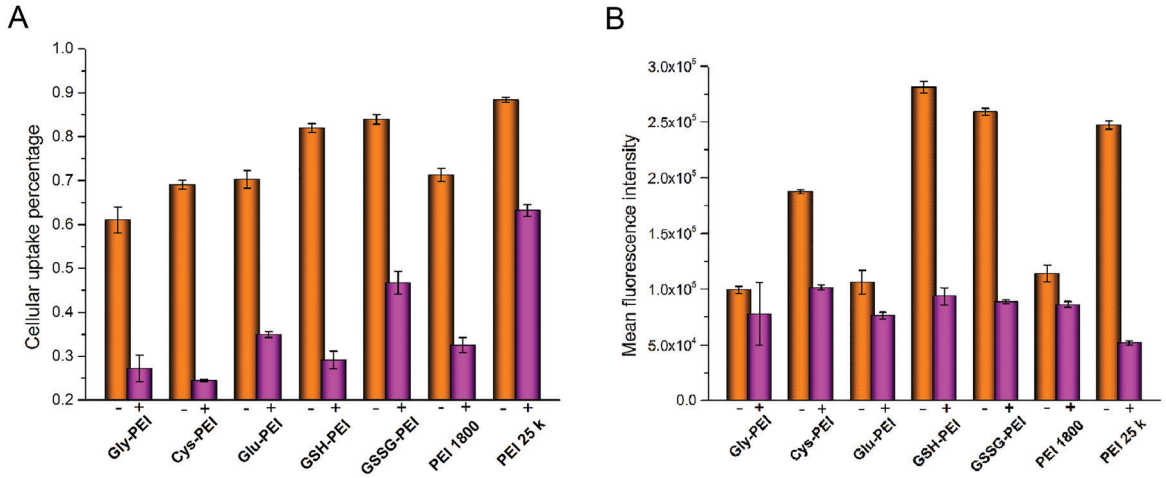


Fig. 6 Cellular uptake percentage (A) and mean fluorescence intensity (B) of Cy5-labeled DNA induced by the polymers without (−) or with serum (+) in HeLa cells. w/w (polymer/DNA) = 1.4 for PEI 25 kDa, and 16 for other materials.

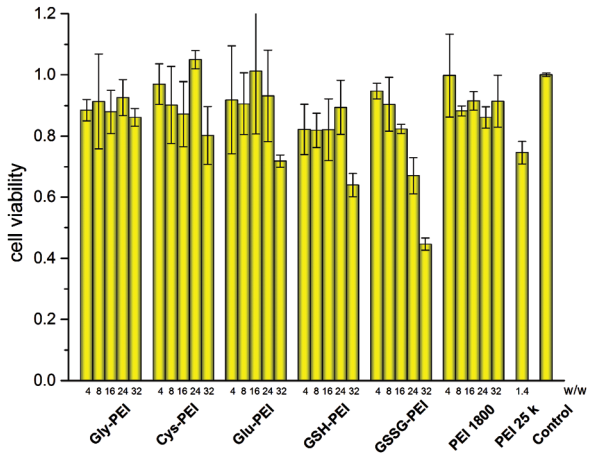


Fig. 7 Cytotoxicity of complexes at various weight ratios in HeLa cells.

of the polymers were determined *via* gel permeation chromatography (GPC), which consisted of a Waters 515 isocratic HPLC pump, a Linear 7.8 × 300 mm column (Waters Corp, Milford, MA, USA), an 18-angle laser scattering instrument (Wyatt Technology Corporation, USA), and an OPTILAB DSP interferometric refractometer (Wyatt Technology Corporation, USA). 0.5 M HAc/NaAc buffer solution was used as the mobile phase at a flow rate of 1 mL min^{-1}. Molecular weights were calculated against poly(ethylene glycol) standards, with average molecular weights ranging from 601 to 106 000.

3.2. Preparation of target polymers

Preparation of GSH-PEI. Compound **1** was prepared according to a previous report.[42] Compound **1** (2 mmol), *N*-hydroxysuccinimide (NHS, 2.4 mmol), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDCI, 2.4 mmol), and triethylamine (TEA, 2.4 mmol) were dissolved in anhydrous DCM (30 mL) and stirred for 3 h at 0 °C. Then, the desired amount of PEI 1800 Da (0.86, 0.43 and 0.28 g, mole ratios of compound **1** to amino groups in PEI 1800 Da were 10%, 20% and 30%, respectively) dissolved in anhydrous DCM (20 mL) was added to the reaction system dropwise. Here, different feed ratios were chosen to obtain a series of polymers with different substitution degrees. After stirring for 24 h at room temperature, the excess solvent was removed under reduced pressure and the residue was dialyzed (MWCO 1000 Da) against DMF and deionized water for 3 days. Compound **2** was then obtained as a pale yellow solid after lyophilization.

Compound **2** was dissolved in 20 mL of solvent (methanol/H_2O = 1/1) with acetic acid (100 μL), and GSH was added to the reaction mixture. After stirring overnight, methanol was removed under reduced pressure and the residue was dialyzed (MWCO 1000 Da) against deionized water for 3 days. The product **GSH-PEI** was obtained as a white solid after lyophilization. Total yield of the two steps: 23% (calculated by comparing the mass of the product to starting PEI, and the same below).

Compound **2**: ^{1}H NMR (D_2O, 400 MHz) δ 8.34, 7.84–7.78, 7.27–7.22 (d, J = 4 Hz, m, m, Ar-*H*), 3.03–2.63 (m, PEI-*H*, -S-CH_2CH_2-COOH).

GSH-PEI: ^{1}H NMR (D_2O, 400 MHz) δ 3.71–3.68 (t, J = 12 Hz, COOH-CH_2-NHCO-, -CH_2CH$_2$-C*H*(NH_2)-COOH), 3.44–2.45 (m, PEI-*H*, -CH_2-SH, -NHCO-CH_2CH$_2$-CH(NH_2)-COOH), 2.12–2.06 (m, -NHCO-CH$_2$CH_2-CH(NH_2)-COOH).

Preparation of Glu/Cys/Gly-PEI. γ-Benzyl-glutamate (16 mmol) and TEA (32 mmol) were dissolved in 100 mL of solvent (THF/H_2O = 2/1), di-*tert*-butyl dicarbonate ((Boc)$_2$O, 19.2 mmol) in 50 mL of THF was added to the mixture dropwise in an ice bath and the system was stirred overnight at room temperature. THF was removed under reduced pressure and the aqueous solution of the crude product was adjusted to pH 2 with HCl hydrous solution (2N). The resulting solution was extracted with EA three times and the combined extracts were washed with brine. After drying over anhydrous Na_2SO_4, the organic phase was concentrated and purified *via* column chromatography (silica gel, PE/EA = 1/1). Compound **3** was obtained as a pale yellow viscous liquid. In the same way, compound **4** could be obtained as a light red viscous liquid from cysteine.

Compound **3** (Scheme S1, ESI†): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.35 (s, 5H, Ph-*H*), 5.12 (s, 2H, Ph-CH_2-), 4.37–4.25 (m, 1H, Boc-NH-C*H*-COOH), 2.57–2.44 (m, 2H, -CH-CH$_2$-CH_2-COOPh), 2.25–2.05 (m, 2H, -CH-CH_2-CH$_2$-COOPh), 1.43 (s, 9H, Boc-*H*). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 176.09, 172.72, 155.68, 135.85, 128.67, 128.30, 128.25, 80.27, 66.62, 52.76, 30.53, 28.41, 27.38. HRMS (DCM): *m*/*z*: calcd: 360.1423, found: 360.1421 $[M + Na]^+$.

Compound **4** (Scheme S1, ESI†): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.64 (s, 1H, Boc-NH-C*H*-COOH-), 3.07–2.96 (m, 2H, -CH_2-SH), 1.46 (s, 9H, Boc-*H*). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 174.69, 155.48, 80.80, 54.40, 28.27, 27.04. HRMS (DCM): *m*/*z*: calcd: 244.0619, found: 244.0614 $[M + Na]^+$.

N-Boc-amino acid (2 mmol), EDCI (2.4 mmol), 1-hydroxybenzotriazole (HOBT, 2.4 mmol) and ethyldiisopropylamine (DIEA, 2.4 mmol) were dissolved in anhydrous DCM (30 mL) and stirred for 3 h at 0 °C. Then, the desired amount of PEI 1800 Da (0.86, 0.43, 0.28 and 0.17 g, mole ratios of *N*-Boc-amino acid to amino groups in PEI 1800 Da were 10%, 20%, 30% and 50%, respectively) dissolved in anhydrous DCM (20 mL) was added to the reaction system dropwise. After stirring for 48 h at room temperature, HCl hydrous solution (1N) was added to remove the protecting group. The excess solvent was removed under reduced pressure and the residue was dialyzed (MWCO 1000 Da) against deionized water for 3 days. The product amino acid–PEI was obtained as a pale yellow solid after lyophilization.

Glu-PEI: yield 26%. ^{1}H NMR (D_2O, 400 MHz) δ 3.91–3.85 (m, -NH-C*H*-COOH), 3.15–2.74 (m, PEI-*H*).

Cys-PEI: yield 9%. ^{1}H NMR (D_2O, 400 MHz) δ 3.58 (s, NH_2-C*H*-CONH-), 3.23–2.76 (m, PEI-*H*, -CH_2-SH).

Gly-PEI: yield 19%. ^{1}H NMR (D_2O, 400 MHz) δ 3.77 (d, J = 8 Hz, NH_2-CH_2-CONH-), 3.20–2.73 (m, PEI-*H*).

Preparation of the crosslinked polymer GSSG-PEI. Compound **5** could be obtained according to a previous report.[43] Compound **5** (0.5 mmol), DIEA (2.4 mmol), HOBT (2.4 mmol), and EDCI (2.4 mmol) were dissolved in anhydrous DCM (20 mL) and stirred for 3 h at 0 °C. Then, PEI 600 Da (1.2 g, mole ratio of PEI 600 Da to compound **5** was 4) dissolved in anhydrous DCM (30 mL) was added to the reaction system dropwise. After stirring for 48 h at room temperature, HCl hydrous solution (1N) was added to remove the protecting Boc group. The excess solvent was removed under reduced pressure and the residue was dialyzed (MWCO 1000 Da) against deionized water for 3 days. The product **GSSG-PEI** was obtained as a pale yellow solid after lyophilization with a yield of 34%.

GSSG-PEI: ^{1}H NMR (D_2O, 400 MHz) δ 4.57–4.53 (m, -C*H*-CH$_2$-S-), 3.92 (s, -CH$_2$CH$_2$-C*H*(NH_2)-CONH-), 3.74–3.73 (m, -NHCO-CH$_2$-N*H*CO-), 3.39–2.73 (m, PEI-*H*, -CH-CH_2-S-), 2.50 (s, -CH_2CH$_2$-CH(NH_2)-CONH-), 2.14–2.13 (m, -CH$_2$CH_2-CH(NH_2)-CONH-).

3.3. Gel retardation assay

Polymer/DNA complexes at different w/w ratios ranging from 0.25 to 16 were prepared by adding an appropriate volume of polymer solution to 5 μL of pUC-19 (0.025 mg mL^{-1}). The resulting complex solutions were diluted to 10 μL and incubated at 37 °C for 30 min. After that, the complexes were electrophoresed on 1% (w/v) agarose gel containing GelRed and Tris-acetate (TAE) running buffer at 130 V for 30 min. DNA was visualized under an ultraviolet lamp using a Bio-Rad Universal Hood II (Berkeley, CA, USA).

The ability of polymers to bind DNA could be studied by the heparin sodium replacement assay. At first, the desired amount of polymers and 0.125 μg of pUC-19 were incubated to obtain the complexes (w/w = 2). Then, a series of heparin sodium solutions with different concentrations were added to the complexes and the mixture was incubated at 37 °C for another 30 min. Similarly, gel retardation assay was carried out as above.

Besides, DTT was used to investigate the reduction responsiveness of **GSSG-PEI**. Two groups of experiments were undertaken; in one group, DTT solution (20 nM) was added to the polymer/DNA complexes, while in the other group, water was added as a control. Then, the mixtures were incubated for 3 h at 37 °C. Heparin sodium (0.4 mg mL^{-1}) was added to replace DNA.

3.4. Morphology study of polymer/DNA complexes

The size and zeta potential of complexes at various weight ratios were evaluated by dynamic light scattering (DLS) at 25 °C by using a Nano-ZS ZEN 3600 (Malvern Instruments, Malvern, Worcestershire, UK). The complexes were prepared by adding 1 μg of pUC-19 to the appropriate volume of polymer solution. After incubating for 30 min at 37 °C, the solution was diluted to 1 mL with deionized water before measurement. All data were averaged and a variance was obtained after 3 parallel experiments.

The morphology of the complexes was also observed *via* TEM (Tecnai G2 F20 S-TWIN, Hillsboro, OR, USA) with an acceleration voltage of 100 kV. 2 μg of pUC-19 was added to the appropriate volume of polymer (weight ratio of **GSSG-PEI** relative to DNA, w/w = 16), and the mixture was incubated at 37 °C for 30 min. 15 min before measurements, the complex solution was diluted with deionized water (or PBS containing 10% FBS) to 200 μL. A drop of complex suspension was placed onto the copper grid. After 3 min, the excess solution was removed with filter paper. Then, a drop of 2% (w/v) phosphotungstic acid was placed on the above grid. The grid was dried at room temperature at atmospheric pressure for several minutes before observation.

3.5. Cell culture

HeLa cells were cultured in DMEM medium containing 10% FBS and 1% antibiotics (penicillin–streptomycin, 10 kU mL^{-1}), while 7702 cells were incubated in 1640 medium containing 10% FBS and 1% antibiotics (penicillin–streptomycin, 10 kU mL^{-1}) at 37 °C in a humidified atmosphere containing 5% CO_2.

3.6. Gene transfection assay *in vitro*

Gene transfection efficiency of complexes was investigated in HeLa and 7702 cells. For the expression of the EGFP gene, HeLa and 7702 cells were seeded in 48-well plates (5×10^4 cells per well) and incubated for 24 h at 37 °C in 5% CO_2. Before transfection, the medium in every well was exchanged for fresh serum-free or 10% serum-containing medium containing polymer/DNA (0.4 μg)

complexes at various weight ratios. After incubation for 4 h, the medium was replaced with 10% serum-containing medium. After another 20 h incubation, cells were observed using an inverted fluorescence microscope (Nikon TS100, Tokyo, Japan).

For luciferase expression, the cells were transfected with complexes containing pGl-3 plasmids. After a similar transfection process as described above, cells were washed with PBS and lysed with 60 μL 1× Lysis reporter buffer (Promega, Madison, WI, USA). The luciferase activity was measured by using a microplate reader (Imark, Bio-Rad, Berkeley, CA, USA). The protein content of the lysed cell was determined by BCA protein assay (Pierce, Rockford, IL, USA). Gene transfection efficiency was shown as the relative fluorescence intensity per mg protein (RLU per mg protein). All the experiments were carried out in triplicate.

3.7. Cellular uptake of plasmid DNA (flow cytometry)

The cellular uptake efficiency of polymer/fluorescein labeled DNA complexes was evaluated *via* flow cytometry (Accuri C6, BD, Franklin Lakes, NJ, USA). Here, polymers and DNA labeled using a Label IT Cy5 Labeling Kit were used to form complexes at a weight ratio of 16. After 4 h transfection, the cells were washed with 1× PBS and digested with trypsin to obtain a dispersive cell suspension. Cy5-labeled plasmid DNA uptake was measured in the FL4 channel using a red diode laser (633 nm). The mean fluorescence intensity was analyzed using a flow cytometer (Accuri C6, BD, USA).

3.8. Confocal laser scanning microscopy (CLSM) analysis

HeLa cells were seeded at a density of 2.5×10^5 cells per well in a 35 mm confocal dish, 24 h prior to transfection. Then, the medium was exchanged with fresh serum-containing medium. Complexes of polymers and Cy5-labeled pGL-3 at a weight ratio of 24 were added to each well. After incubation at 37 °C for 4 h, the medium was removed and cells were rinsed three times with PBS (pH = 7.4). Then, cells were fixed with 4% paraformaldehyde (dissolved with PBS solution) for 15 min and nuclear staining was done using Hoechst 33342. The CLSM observation was performed *via* confocal laser scanning microscopy (LSM 780, Zeiss, Jena, Germany) at excitation wavelengths of 361 nm for Hoechst 33342 (blue) and 633 nm for Cy5 (red), respectively.

3.9. Cell viability assay

The cytotoxicity of the complexes towards HeLa and 7702 cells was studied by using the MTS Kit. After 24 h incubation, the medium containing complexes was removed and MTS in PBS was added for another 1 h incubation. Then, the absorbance of each sample was measured using an enzyme-linked immunosorbent assay (ELISA) plate reader (Model 680, Bio-Rad, Berkeley, CA, USA) at a wavelength of 490 nm. The cell viability (%) was obtained according to the manufacturer's instructions. The untreated cell controls were taken as 100% cell viability. All the experiments were carried out in triplicate.

4. Conclusion

In summary, low molecular weight PEI was modified by reduced/oxidized glutathione and three relevant amino acids to obtain a series of target polymers. Gel retardation and heparin replacement assays proved that all of these polymers could bind and condense DNA into stable polyplexes. Besides, the crosslinked polymer **GSSG-PEI** showed a reduction responsive property, which was beneficial to the DNA release in an intracellular reductive environment. The modification could largely improve the gene transfection efficiency of low molecular weight PEI, and their transfection efficiencies were even higher than PEI 25 kDa. In particular, in the presence of serum, **GSSG-PEI** had 150 times higher efficiency than PEI 25 kDa. TEM, serum concentration effect assay and flow cytometry assay also demonstrated the good serum tolerance of the target polymer. **GSSG-PEI** might induce cellular uptake with higher efficiency than PEI 25 kDa, especially in the presence of serum. These results revealed that GSSG is a good candidate for the crosslinking of small cationic molecules to form polymeric gene vectors with improved transfection efficiency and biocompatibility.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (No: 21672155 and 21877082). We also acknowledge the Comprehensive Training Platform of Specialized Laboratory, College of Chemistry, Sichuan University for sample analysis.

Notes and references

1 U. Lachelt and E. Wagner, *Chem. Rev.*, 2015, **115**, 11043–11078.
2 R. Voelker, *JAMA, J. Am. Med. Assoc.*, 2018, **319**, 434.
3 C. H. Evans, S. C. Ghivizzani and P. D. Robbins, *Nat. Rev. Rheumatol.*, 2011, **7**, 244–249.
4 J. C. T. van Deutekom and G. J. B. van Ommen, *Nat. Rev. Genet.*, 2003, **4**, 774–783.
5 A. D. Miller, *Nature*, 1992, **357**, 455–460.
6 S. M. Dizaj, S. Jafari and A. Y. Khosroushahi, *Nanoscale Res. Lett.*, 2014, **9**, 252–260.
7 R. Waehler, S. J. Russell and D. T. Curiel, *Nat. Rev. Genet.*, 2007, **8**, 573–587.
8 M. A. Kay, *Nat. Rev. Genet.*, 2011, **12**, 316–328.
9 M. A. Mintzer and E. E. Simanek, *Chem. Rev.*, 2009, **109**, 259–302.
10 M. S. Al-Dosari and X. Gao, *AAPS J.*, 2009, **11**, 671–681.
11 R. V. Benjaminsen, M. A. Mattebjerg, J. R. Henriksen, S. M. Moghimi and T. L. Andresen, *Mol. Ther.*, 2013, **21**, 149–157.

12 Y. H. Wang, M. Zheng, F. H. Meng, J. Zhang, R. Peng and Z. Y. Zhong, *Biomacromolecules*, 2011, **12**, 1032–1040.
13 H. Y. Tian, L. Lin, J. Chen, X. S. Chen, T. G. Park and A. Maruyama, *J. Controlled Release*, 2011, **155**, 47–53.
14 Q. Liu, R. C. Su, W. J. Yi and Z. G. Zhao, *Molecules*, 2017, **22**, 566–577.
15 X. F. Hao, Q. Li, H. N. Wang, K. Muhammad, J. T. Guo, X. K. Ren, C. C. Shi, S. H. Xia, W. C. Zhang and Y. K. Feng, *ACS Biomater. Sci. Eng.*, 2018, **4**, 2870–2878.
16 G. Y. Zhang, X. L. Jiang, J. Liu, Q. Z. Yang and R. X. Zhuo, *J. Controlled Release*, 2013, **172**, E118–E119.
17 L. X. Xie, X. Ding, R. Budry and G. Z. Mao, *Int. J. Nanomed.*, 2018, **13**, 4943–4960.
18 H. He, Y. G. Bai, J. H. Wang, Q. R. Deng, L. P. Zhu, F. H. Meng, Z. Y. Zhong and L. C. Yin, *Biomacromolecules*, 2015, **16**, 1390–1400.
19 X. M. Li, X. S. Guo, Y. Cheng, X. T. Zhao, Z. W. Fang, Y. L. Luo, S. J. Xia, Y. Feng, J. J. Chen and W. E. Yuan, *Front. Oncol.*, 2018, **8**, 354–365.
20 G. Fang, F. Zeng, C. Yu and S. Wu, *Colloids Surf., B*, 2014, **122**, 472–481.
21 Q. Y. Yu, Y. R. Zhan, J. Zhang, C. R. Luan, B. Wang and X. Q. Yu, *Polymers*, 2017, **9**, 362–373.
22 Q.-F. Zhang, C.-R. Luan, D.-X. Yin, J. Zhang, Y.-H. Liu, Q. Peng, Y. Xu and X.-Q. Yu, *Polymers*, 2015, **7**, 2316–2331.
23 G. R. Dhanya, D. S. Caroline, M. R. Rekha and K. Sreenivasan, *Int. J. Biol. Macromol.*, 2018, **120**, 999–1008.
24 J. Z. Deng, N. N. Gao, Y. A. Wang, H. Q. Yi, S. T. Fang, Y. F. Ma and L. T. Cai, *Biomacromolecules*, 2012, **13**, 3795–3804.
25 R. Devulapally, T. Lee, A. Barghava-Shah, T. V. Sekar, K. Foygel, S. V. Bachawal, J. K. Willmann and R. Paulmurugan, *Nanomedicine*, 2018, **13**, 1051–1066.
26 Y. Zhang, L. Lin, L. Liu, F. Liu, A. Maruyama, H. Tian and X. Chen, *Carbohydr. Polym.*, 2018, **201**, 246–256.
27 X. L. Zhao, Z. Y. Li, H. B. Pan, W. G. Liu, M. M. Lv, F. Leung and W. W. Lu, *Acta Biomater.*, 2013, **9**, 6694–6703.
28 J. H. Wang, H. He, X. Xu, X. Wang, Y. B. Chen and L. C. Yin, *Biomaterials*, 2018, **171**, 72–82.
29 H. Kim, J. Kim, M. Lee, H. C. Choi and W. J. Kim, *Adv. Healthcare Mater.*, 2016, **5**, 1918–1930.
30 G. Q. Lin, W. J. Yi, Q. Liu, X. J. Yang and Z. G. Zhao, *Molecules*, 2018, **23**, 2061–2072.
31 W. X. Song, D. A. Gregory, H. Al-janabi, M. Muthana, Z. Q. Cai and X. B. Zhao, *Int. J. Pharmaceut.*, 2019, **555**, 322–336.
32 M. Bez, T. J. Kremen, W. Tawackoli, P. Avalos, D. Sheyn, G. Shapiro, J. C. Giaconi, S. Ben David, J. G. Snedeker, Z. Gazit, K. W. Ferrara, D. Gazit and G. Pelled, *Mol. Ther.*, 2018, **26**, 1746–1755.
33 C. C. Shi, F. L. Yao, J. W. Huang, G. L. Han, Q. Li, M. Khan, Y. K. Feng and W. C. Zhang, *J. Mater. Chem. B*, 2014, **2**, 1825–1837.
34 A. Meister and M. E. Anderson, *Annu. Rev. Biochem.*, 1983, **52**, 711–760.
35 B. G. Hill and A. Bhatnagar, *IUBMB Life*, 2007, **59**, 21–26.
36 H. Sies, *Free Radical Biol. Med.*, 1999, **27**, 916–921.
37 Y. Zheng, S. Gao and J. Y. Ying, *Adv. Mater.*, 2007, **19**, 376–380.
38 D. K. Jha, K. Saikia, S. Chakrabarti, K. Bhattacharya, K. S. Varadarajan, A. B. Patel, D. Goyary, P. Chattopadhyay and P. Deb, *Mater. Sci. Eng., C*, 2017, **72**, 415–424.
39 Y. J. Ma, Z. B. Yan, H. Xu and H. C. Gu, *Chin. Sci. Bull.*, 2012, **57**, 3525–3531.
40 K. Strojan, J. Lojk, V. B. Bregar, P. Veranic and M. Pavlin, *Toxicol. In Vitro*, 2017, **41**, 12–20.
41 H. Aldawsari, R. Edrada-Ebel, D. R. Blatchford, R. J. Tate, L. Tetley and C. Dufes, *Biomaterials*, 2011, **32**, 5889–5899.
42 C. Y. Ang, S. Y. Tan, S. Wu, Q. Qu, M. F. E. Wong, Z. Luo, P.-Z. Li, S. Tamil Selvan and Y. Zhao, *J. Mater. Chem. C*, 2016, **4**, 2761–2774.
43 K. Mitamura, M. Sogabe, H. Sakanashi, S. Watanabe, T. Sakai, Y. Yamaguchi, T. Wakamiya and S. Ikegawa, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.*, 2007, **855**, 88–97.