Pharm Res (2013) 30:1968–1978
DOI 10.1007/s11095-013-1040-5

RESEARCH PAPER

Bioreducible Polypeptide Containing Cell-Penetrating Sequence for Efficient Gene Delivery

Si Chen • Kai Han • Juan Yang • Qi Lei • Ren-Xi Zhuo • Xian-Zheng Zhang

Received: 2 August 2012 / Accepted: 27 March 2013 / Published online: 19 April 2013

ABSTRACT

Purpose To design excellent polypeptide-based gene vectors and determine the gene delivery efficiency.

Methods Polypeptides (designated as xPolyK$_6$, xPolyK$_6$-R$_8$1 and xPolyK$_6$-R$_8$2), comprising the DNA condensing and buffering peptide HK$_6$H as well as cell penetrating peptide (CPP) R$_8$ were obtained by the oxidative polymerization of CHK$_6$HC and CR$_8$C at different molar ratios in 4 mL phosphate-buffered saline (PBS) containing 30% (*v/v*) DMSO at room temperature for 96 h. The cytotoxicity of vectors was studied by MTT assay. Moreover, particle size, zeta potential and morphology along with the *in vitro* transfection efficiency and cellular uptake of vector/plasmid DNA (pDNA) complexes were characterized at various *w/w* ratios to determine their potential in gene therapy.

Results All the vectors presented excellent ability of binding and condensing pDNA, additionally with low cytotoxicity. Simultaneously, transfection efficiency of the vectors appeared apparent dependence on the vector composition. The distinct correlation between the content of CR$_8$C with the transfection efficiency demonstrated the effective improvement in transfection efficacy by the oxidative polymerization. Particularly, xPolyK$_6$-R$_8$2 possessed the highest transfection efficiency at a *w/w* ratio of 50. Furthermore, xPolyK$_6$-R$_8$2 also presented the best cellular uptake capability demonstrated by confocal microscopy and flow cytometry.

Conclusions Bioreducible polypeptides incorporating with proper amount of CPP are promising as effective non-viral gene vectors in gene therapy.

KEY WORDS cell-penetrating peptide · disulfide bond · gene delivery · polypeptide

S. Chen • K. Han • J. Yang • Q. Lei • R.-X. Zhuo • X.-Z. Zhang (✉)
Key Laboratory of Biomedical Polymers (The Ministry of Education)
Department of Chemistry, Wuhan University, Wuhan 430072, China
e-mail: xz-zhang@whu.edu.cn

INTRODUCTION

Currently, gene therapy, as an extremely attractive method to cure serious diseases such as cancers and diabetes, has achieved great progress, and the success in gene therapy largely depends on the design and fabrication of admirable gene vectors (1). Despite the high gene transfection of viral vectors (2), the further development was restricted for their biosafety concern. During the past decades, non-viral gene vectors have obtained extensive interest because of their advantages over viral gene delivery carriers, such as biological safety, no integration of exogenous genes into host chromosomes, modifiable multiple functionality for gene delivery, and so on (3–5). However, their low transfection efficiency was still a major issue due to various barriers in gene delivery (6,7), including DNA packaging, cellular uptake, endosomal escape (8) and DNA release (9).

The pDNA condensing ability of gene vector affected the efficiency of gene delivery significantly and the formation of compact vector/pDNA complexes is helpful to promote the stability of DNA against different harsh enzyme environment. Various cationic polymers have been investigated as delivery vectors with satisfactory transfection efficiency (10,11). However, these polymers usually performed severe cytotoxicity due to their high density of positive charge in cytoplasm. To overcome this problem, many strategies aiming at lowering the cytotoxicity have been proposed (12). Introduction of bio-stimuli responsive behaviors to vectors, for example, pH responsiveness under weak acidic environment of endosome (13) as well as enzyme responsiveness in cytoplasm (14), have endowed non-viral vectors with rapid biodegradability and low toxicity. Specifically, bioreducible non-viral vectors have garnered widespread attention. Disulfide-linked bioreducible polymers show excellent stability in extracellular aqueous environment and well degradability

in cytoplasm at a high concentration of glutathione, leading unpacking of DNA (15).

Furthermore, cellular uptake is another major barrier in the gene delivery. Previous reports showed that the incorporation of guanidine groups could improve cellular uptake, which might be attributed to the well interaction between guanidine groups and phosphate groups on the cell surface (16,17). Meanwhile, CPPs, a kind of arginine-rich cationic peptides, have been demonstrated to possess good ability to delivery various biological macromolecules into cells both *in vivo* and *in vitro*. Among them, R_8, confirmed to be capable of spaning cell membrane, was well developed (18,19).

In this work, a series of bioreducible polypeptides comprised of CHK_6HC and CR_8C in various ratios via reducible disulfide bonds were designed and studied. CHK_6HC, an excellent candidate of $CWK_{17}C$ was reported to be able to condense DNA with similar particle size and transfection efficiency, even provide buffering capacity to enhance *in vitro* gene transfection (20,21). R_8 is a well investigated CPP, which also has advanced DNA condensing capability. After cross-linked by disulfide bonds, these polypeptides were capable of condensing and delivering pDNA into cells effectively and then degraded to release pDNA in cytoplasm under the stimulus of glutathione as illustrated in Scheme 1. In addition, the properties of the polypeptides were characterized in terms of cytotoxicity, transfection *in vitro*, and cellular uptake with confocal microscopy and flow cytometry etc.

MATERIALS AND METHODS

Materials

O-benzotriazole-N,N,N',N'-tetramethyluroniumhexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt), N-Fluorenyl-9-methoxycarbonyl (Fmoc) protected L-amino acids (Fmoc-Cys(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-His(Boc)-OH, Fmoc-Lys(Boc)-OH), thioanisole, piperidine, ethanedithiol (EDT), 2-chlorotrityl chloride resin (100–200 mesh, loading: 1.20 mmol/g) were purchased from GL Biochem. Ltd. (Shanghai, China) and used as received. Diisopropylethylamine (DIEA) was acquired from GL Biochem. Ltd. (Shanghai, China) and used after distillation. Trifluoroacetic acid (TFA), N,N-dimethylformamide (DMF), methanol and dichloromethane (DCM) were provided by Shanghai Chemical Co. (China) and distilled prior to use. Dimethylsulfoxide (DMSO), phenol, anhydrous ether and 1,4-dithiothreitol (DTT) were obtained from Shanghai Chemical Reagent Co. (Shanghai, China) and used as received. All other reagents and solvents were analytical grade and used directly.

QIAfilter™ plasmid purification Giga Kit was purchased from Qiagen (Hilden, Germany). GelRed™ was purchased from Biotium (CA, USA). Dulbecco's Modified Eagle's Medium (DMEM), penicillin-streptomycin, fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Dulbecco's phosphate buffered saline (PBS) were purchased from Invitrogen Corp. Micro BCA protein assay kit was purchased from Pierce. PEI was purchased from Poly-Plus-transfection. Molecular probes (Hoechst 33258, YOYO-1 iodide).

Synthesis of Peptides

CHK_6HC (CHKKKKKKHC) and CR_8C (CRRRRRRRRC) were synthesized manually by using standard solid phase methodologies based on classical Fmoc chemistry (22). All the peptide sequences were loaded on a 2-chlorotrityl chloride resin (1.20 mmol/g), using DIEA/HBTU/HOBt in DMF to promote coupling of the Fmoc-protected amino acid, and 20% piperidine/DMF (*v*/*v*) solution was used to remove the Fmoc protected groups. The Kaiser reagent was utilized to test the coupling efficiency. Cleavage of the targeted peptide and deprotection of the side chain protected residues were performed using a 30 mL cocktail of TFA-phenol-thioanisole-EDT-DI water (83 : 6.3 : 4.3 : 2.1 : 4.3 in *v*/*v*) for 1.5 h at room temperature (23). The collected solution was concentrated to a viscous solution by rotary evaporation and then dropped into cold ether to precipitate the product. The precipitate was centrifuged and dried under vacuum for 24 h, after that, the precipitate was dissolved in distilled water and then freeze-dried. The purity of the products was examined by high-pressure liquid chromatography (HPLC) with a C_{18} column and using a linear gradient of acetonitrile and DI water containing 0.1% TFA. The molecular weights of CHK_6HC and CR_8C were measured by electrospray ionization mass spectrometry (ESI-MS).

Synthesis and Characterization of Disulfide Linked Polypeptides

A series of polypeptides were obtained by oxidative polymerization of CHK_6HC and CR_8C at various molar ratios in 4 mL phosphate-buffered saline (PBS) containing 30% DMSO at room temperature for 96 h (24–26). The $xPolyK_6$ was synthesized by oxidative polymerization of CHK_6HC peptide under the same condition. Noted here, $xPolyK_6$ means the chemical linked linear bioreducible polypeptide. All polypeptides were purified by dialysis using a regenerated cellulose acetate membrane (MWCO: 3,500 Da). The molecular weights and polydispersity (M_w/M_n) of the polypeptides ($xPolyK_6$, $xPolyK_6$-$R_8$1 and $xPolyK_6$-$R_8$2,1 and 2 was used to distinguish different R_8 content vectors) were determined by size-exclusion chromatography and multiangle laser light scattering (SEC-MALLS) analysis were employed to monitor the molecular weight distribution of $xPolyK_6$, $xPolyK_6$-$R_8$1and $xPolyK_6$-$R_8$2. A dual

detector system consisting of a MALLS device (DAWNEOS, Wyatt Technology) and an interferometric refractometer (Optilab DSP, Wyatt Technology) was adopted 0.1 M HAc/NaAc (pH 4.2) buffer solution were used as the eluent at a flow rate of 0.3 mL/min. The MALLS detector was operated at a laser wavelength of 690.0 nm. The structure of these polypeptides was analyzed by ^{1}H NMR by a Varian Unity 300 MHz spectrometer using D_2O as the solvent.

Cell Culture and Amplification of Plasmid DNA

Human cervix adenocarcinoma (HeLa) cells and African green monkey SV40-transformed kidney fibroblast (COS7) cells were used in this study, and they were incubated in DMEM which was supplemented with 10% fetal bovine serum and 1% antibiotics (penicillin-streptomycin, 10,000 U mL^{-1}) in a humidified atmosphere of 5% CO_2 at 37°C. Luciferase reporter gene plasmid (pGL-3) was transformed in *E. coli* JM109 (the positive clones were screened by Amp resistance) as below: *E. coli* JM109 was amplified in LB media at 37°C overnight at 250 rpm and collected by centrifugation, and then plasmid was extracted and purified by an EndoFree QiAfilter™ Plasmid Giga Kit (5), after that, the purified plasmid was dissolved in TE buffer solution at a final concentration of 200 ng/μL and stored at −20°C.

Preparation of Polypeptide/pGL-3 DNA Complexes

The complexes of peptides/pGL-3 DNA at various weight ratios (*w*/*w*) ranging from 0.5 to 60 were prepared by adding dropwise 1 μg of pGL-3 (200 ng/μL in 40 mM Tris–HCl buffer solution) into an appropriate volume of polypeptide solutions (in 150 mM NaCl solution), and then the complexes were diluted to a total volume of 100 μL with 150 mM NaCl and vortexed for 5 s. The complexes were incubated at 37°C for 30 min to allow formation of the complexes. All complexes were used immediately after their preparation.

Acid–Base Titration Assay

The buffering capacity was measured by acid–base titration (27). Briefly, 10 mL solution of each polymer (0.2 mg/mL) was adjusted to pH 10 with 0.1 M NaOH, and then 0.02 M HCl was gradually added until the solution pH reached 3.5. After the each addition of 0.02 M HCl, the pH was measured by a microprocessor pH meter. 150 mM NaCl was titrated as a control.

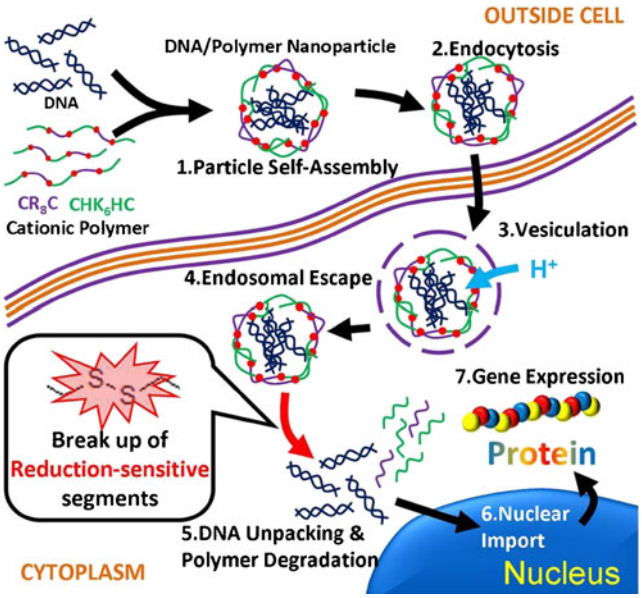
Agarose Gel Retardation Assay

xPolyK$_6$/pGL-3, xPolyK$_6$-R$_8$1/pGL-3 and xPolyK$_6$-R$_8$2/pGL-3 complexes at various *w*/*w* ratios ranging from 0.5 to 12 were prepared by adding dropwise 0.1 μg of pGL-3 DNA (200 ng/μL in 40 mM Tris–HCl buffer solution) into polypeptide solutions, and then the complexes were diluted to 8 μL and incubated at 37°C for 30 min. Subsequently, the samples were loaded onto the 0.7% (*w*/*v*) agarose gel containing GelRed™ and with Tris-acetate (TAE) running buffer at 80 V for 60 min. DNA was visualized under a UV lamp in the Vilber Lourmat imaging system (France).

To estimate the ability of the complexes to release DNA, a reductive environment of cytoplasm was simulated with DTT. The *w*/*w* ratio of vector/DNA was 8. After the complexes were incubated at 37°C for 30 min, DTT was added into the complex solutions at 20 mM and incubated at 37°C for another 15 min, other treatment were the same as that without DTT.

Particle Size and Zeta Potential Measurements

The complexes samples of xPolyK$_6$/pGL-3, xPolyK$_6$-R$_8$1/pGL-3 and xPolyK$_6$-R$_8$2/pGL-3 were prepared at *w*/*w* ratios ranging from 10 to 60 by adding 1 μg of pGL-3 to appropriate volumes of polypeptides solutions and incubated at 37°C for 30 min. After that, the samples were diluted to 1 mL volume with 150 mM NaCl solution. Complexes with the treatment of DTT were described as below: after 100 μL complexes incubated at 37°C for 30 min, DTT was added into the complex solutions at 20 mM and incubated at 37°C for another 15 min. The samples were diluted to 1 mL volume with 150 mM NaCl



Scheme 1 Illustration of polypeptide/pDNA complex mediated gene delivery. For CPPs mediated clathrin-mediated endocytosis, the complexes were mainly located in endosome, after endosome escape and the followed immersing in cytoplasm, complexes degraded with the trigger of high level of glutathione, subsequently, pDNA was unpacking and delivered to nucleus for gene expression.

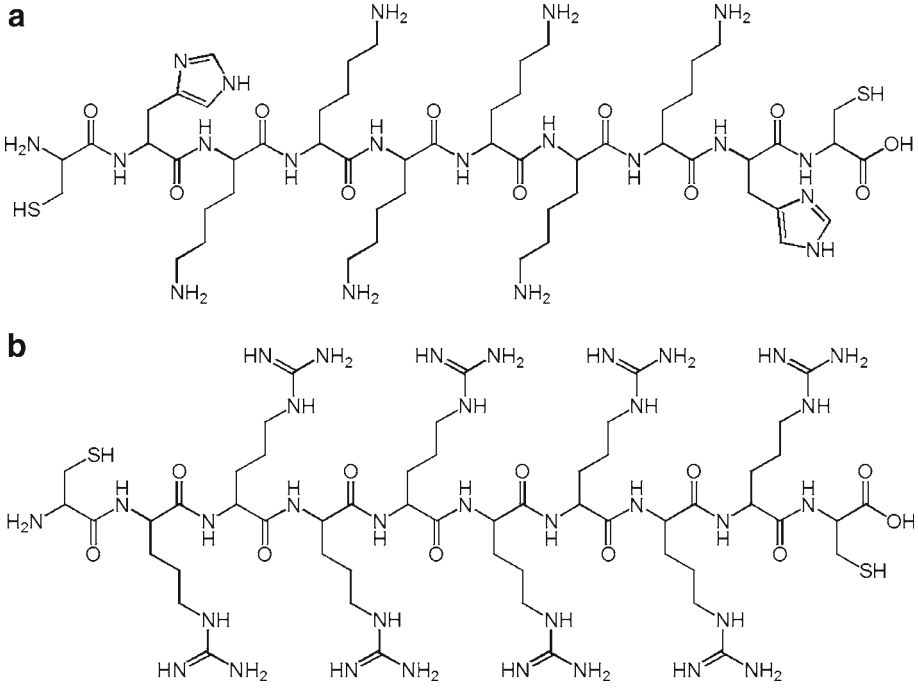


Fig. 1 Chemical structures of CHK_6HC (**a**) and CR_8C (**b**).

solution, and then the particle size and zeta potential of the complexes were measured by a Nano-ZS ZEN3600 (Malvern Instruments) at 25°C (28).

Transmission Electron Microscopy (TEM)

The morphologies of vector/DNA complexes at *w*/*w* ratio of 30 were observed by TEM using a JEM-2100 microscope operating at an acceleration voltage of 100 kV. The TEM samples were prepared by dropping the vector/DNA complex solution onto a copper grid with a layer of formvar film, and then the samples were stained by 0.2% (*w*/*v*) phosphotungstic acid solution before measurement.

Cytotoxicity Assay *In Vitro*

The toxicity of the polypeptides ($xPolyK_6$, $xPolyK_6$-$R_8$1 and $xPolyK_6$-$R_8$2) and PEI was performed in HeLa and COS7 cells by MTT assay. The cells were seeded respectively in a 96-well plate at a density of 6,000 cells/well and incubated in 100 μL DMEM containing 10% FBS for 1 day, and then the peptides at different concentration were added. After incubated for 2 day, the medium was replaced with 200 μL of fresh medium, then 20 μL MTT (5 mg/ml) solutions was added to each well and further incubated for 4 h. Finally, the medium was removed and 200 μL DMSO was added. The absorbance at 570 nm was measured using a microplate reader (Bio-Rad, Model 550, USA). The relative cell viability was calculated as: cell viability (%) = (OD_{570} (samples)/OD_{570}(control)) × 100, where OD_{570}(samples) was obtained in the presence of peptides (29).

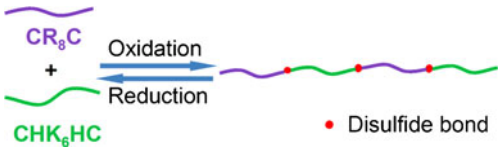


Fig. 2 Schematic illustration of the formation of reductive polycation.

In Vitro Transfection and Cellular Uptake Study

The transfection efficiency of the complexes ($xPolyK_6$/pGL-3, $xPolyK_6$-$R_8$1/pGL-3 and $xPolyK_6$-$R_8$2/pGL-3) was evaluated at various *w*/*w* ratios ranging from 5 to 60, 25 kDa PEI was used as positive control. To compare the transfection abilities between chemical linked bioreducible polymer and physical mixed peptide monomer, the luciferase expression of the complexes by mixing certain weight of CHK_6KC, CR_8C and pGL-3 (designated as K_6/pGL-3, K_6/$R_8$1/pGL-3 and K_6/$R_8$2/pGL-3) was also measured. The contents of CHK_6HC and CR_8C peptides in the K_6/R_8/pGL-3 complex

Table 1 Characterization of Reductive Polypeptides

Polypeptides	Molar Ratio CHK_6HC : CR_8C	M_w (Da)	Polydispersity
$xPolyK_6$	1:0	6950	1.46
$xPolyK_6$-$R_8$1	4:1	6507	1.42
$xPolyK_6$-$R_8$2	3:2	6852	1.48

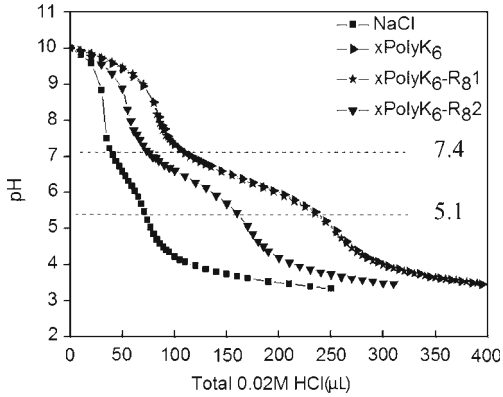


Fig. 3 Acid–base titration of polymer to evaluation the buffering capability, 150 mM NaCl was employed as a control.

were equal to that in the xPolyK$_6$-R$_8$/pGL-3 complex at the same *w*/*w* ratio.

The cells were seeded in the 24-well plate at a density of 6×10^4 cells/well and cultured with 1 mL DMEM containing 10% FBS at 37°C until they reached about 80% confluence. Then the cells were cultured with complexes supplemented with serum-free DMEM for 4 h at 37°C. After this period, the medium was replaced with fresh DMEM containing 10% FBS and the cells were incubated for an additional 2 day. For luciferase assay, the medium was removed, the cells were washed with 0.2 mL PBS and lysed using 200 μL reporter lysis buffer (Pierce). The relative light units (RLUs) were measured with a chemiluminometer (Lumat LB9507, EG&G Berthold, Germany). The amount of protein in the cell lysate was determined using a BCA protein assay kit (Pierce) and the luciferase activity was expressed as RLU per milligram protein (30).

Confocal Microscopy and Flow Cytometry

HeLa cells were seeded in 24-well plates at a density of 5×10^4 cells/well and cultured with 1 mL DMEM containing 10% FBS for 1 day. xPolyK$_6$/pGL-3, xPolyK$_6$-R$_8$1/pGL-3 and xPolyK$_6$-R$_8$2/pGL-3 complexes were prepared at *w*/*w* ratio of 30, respectively. 1 μg pGL-3 was incubated with YOYO-1 for 15 min at 37°C followed by adding the appropriate polypeptide solution. The polypeptide/pGL-3/YOYO-1 complex was diluted to 100 μL with PBS at 37°C for 30 min. Then the complex sample was supplemented to 1 mL by serum-free DMEM and added to the plates. After 4 h of incubation, the medium was discarded and the cells were washed three times with PBS, the nuclei were stained with 20 μL (2 mg/mL) of Hoechst 33258 for 20 min at 37°C. Subsequently, the cells were washed with PBS three times and incubated with 200 μL DMEM. The cells were visualized on a confocal laser scanning microscope (C1-Si, Nikon, Japan) (31).

Cellular uptake of the complex was quantitatively estimated by flow cytometry. pGL-3 plasmid was labeled with YOYO-1. The transfected cells were washed with PBS 2 times and trypsinized with 0.25% trypsin for 1 min at 37°C. Subsequently, the cells were collected and resuspended in 500 μL PBS, then filtrated and examined by flow cytometry (BD FACSAria™ III, USA). The instrument was calibrated with non-transfected cells (negative control) to identify viable cells, and the transfected cells were determined from a fluorescence scan performed with 1×10^4 cells using the FL1-H channel.

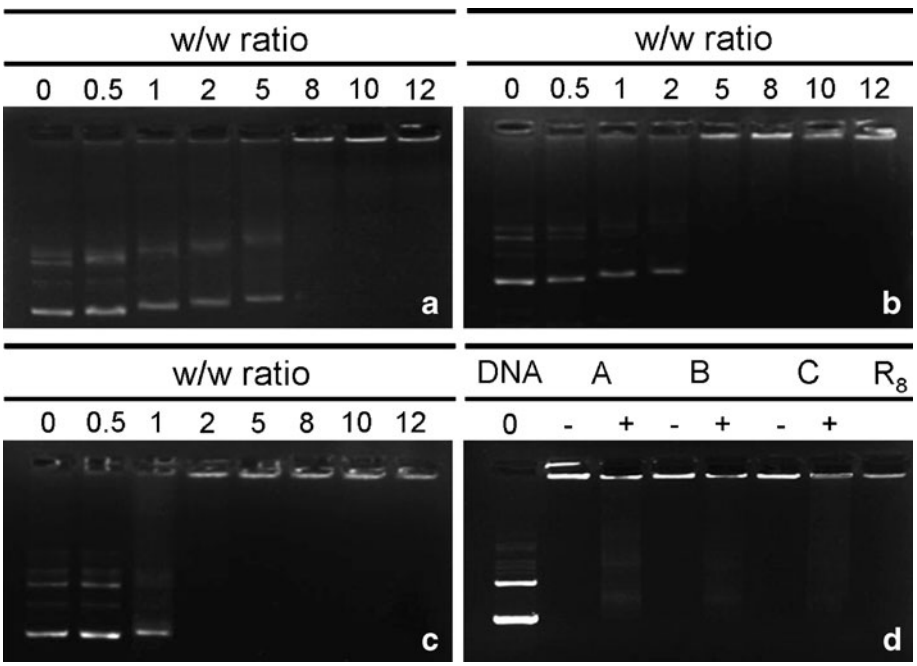


Fig. 4 Agarose gel electrophoresis retardation assay of (**a**) xPolyK$_6$/pGL-3 complexes; (**b**) xPolyK$_6$-R$_8$1/pGL-3 complexes; (**c**) xPolyK$_6$-R$_8$2/pGL-3 complexes at the indicated (*w*/*w*) ratios; (**d**) xPolyK$_6$/pGL-3 (A), xPolyK$_6$-R$_8$1/pGL-3 (B) and xPolyK$_6$-R$_8$2/pGL-3 (C) complexes at *w*/*w* ratio of 8 were incubated without (−) or with (+) 20 mM DTT. The R$_8$ lane was CR$_8$C/pGL-3 complex at *w*/*w* ratio of 8.

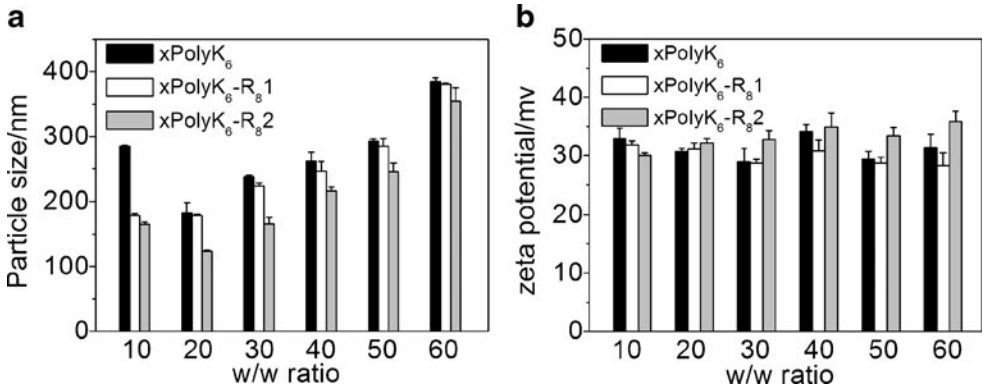


Fig. 5 Particle sizes of polypeptides/pGL-3 complexes. (**a**) At *w/w* ratios ranging from 10 to 60 and zeta potential of polypeptides/pGL-3 complexes; (**b**) at *w/w* ratios ranging from 10 to 60. Data are shown as the mean ± SD ($n = 3$).

RESULTS AND DISCUSSION

Synthesis of Peptides

CHK_6HC and CR_8C were synthesized manually on the 2-chlorotrityl chloride resin by employing a standard Fmoc solid-phase peptide synthesis (SPPS) technique, and the chemical structures were illustrated in Fig. 1. The products were assayed for purity by analytical high performance liquid chromatography and the corresponding purities were determined at least 90%. The molecular weight was measured by ESI-MS. CHK_6HC: molecular weight, calculated 1268.6, found 1269.49 $(M+H)^+$ and 635.48 $(M+2H)^+$. CR_8C: molecular weight, calculated 1472.84, found 1473.8 $(M+H)^+$.

Synthesis and Characterization of Polypeptides

Polypeptides ($xPolyK_6$, $xPolyK_6$-$R_8$1 and $xPolyK_6$-$R_8$2) were synthesized by oxidation of the terminal cysteinyl thiol groups of CHK_6HC and CR_8C. Schematic illustration of the formation of reductive polypeptides was shown in Fig. 2. The molecular weights of the polypeptides were easily adjusted by controlling the polymerization time. The molecular weights (Mws) of $xPolyK_6$, $xPolyK_6$-$R_8$1 and $xPolyK_6$-$R_8$2 were measured by SEC-MALLS to be 6950, 6507 and 6852, respectively. The molar ratios of CHK_6HC : CR_8C in $xPolyK_6$, $xPolyK_6$-$R_8$1 and $xPolyK_6$-$R_8$2 were 1: 0, 4 : 1 and 3 : 2, respectively, as shown in Table I.

Buffering Capability

Buffering capability is a vital parameter for the complexes to escape from endosomes and avoid trafficking and degrading in lysosomes, and it is expected that the polypeptides possessed certain buffering capacity (pH ranging from 7.4 to 5.1) due to existence of primary amine, secondary amine or imidazole group. Acid–base titration assay was employed to determine the buffering capability. As shown in Fig. 3, all polypeptides presented good buffering capability compared with NaCl solution. Meanwhile, $xPolyK_6$ and $xPolyK_6$-$R_8$1 performed better buffering capacity than $xPolyK_6$-$R_8$2 which was owing to the lower content of histidine in the later polypeptide.

Agarose Gel Electrophoresis Assay

The ability of the polypeptides to form stable complexes with pGL-3 DNA was important for gene delivery. The binding capability of the polypeptides was evaluated by agarose gel electrophoresis assay and the results were shown in Fig. 4. $xPolyK_6$, $xPolyK_6$-$R_8$1 and $xPolyK_6$-$R_8$2 could completely stop the mobility of pGL-3 at *w/w* ratios of 8, 5

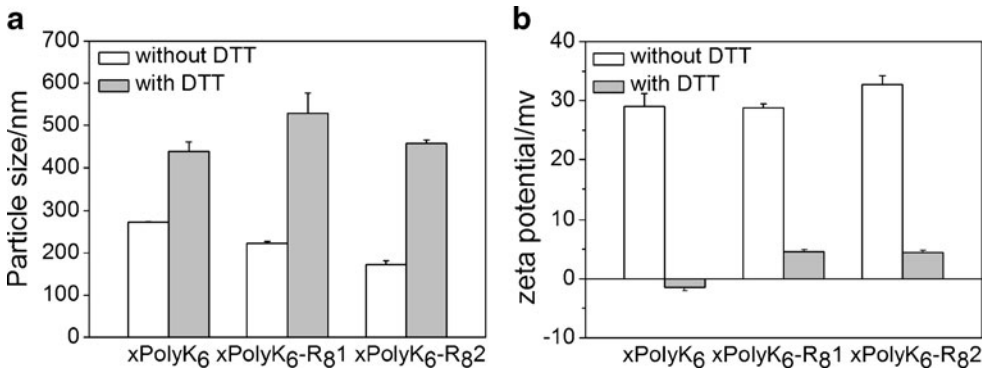


Fig. 6 Particle sizes of polypeptides/pGL-3 complexes treated by DTT (**a**) at *w/w* ratio of 30 and zeta potential of polypeptides/pGL-3 complexes treated by DTT; (**b**) at *w/w* ratio of 30. Data are shown as the mean ± SD ($n = 3$).

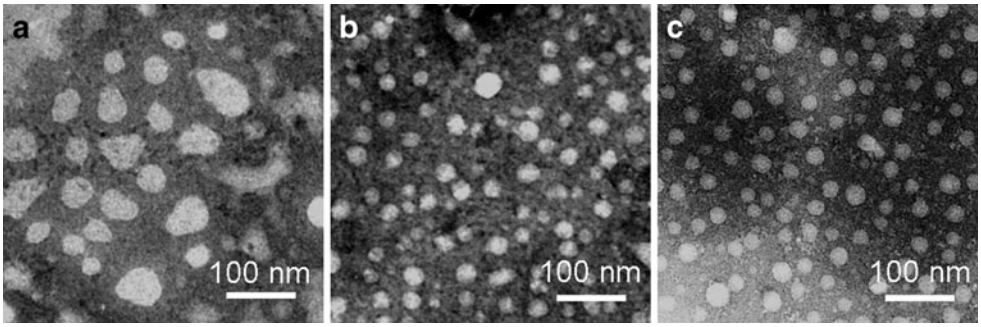


Fig. 7 TEM images of polypeptides/pGL-3 complexes at *w/w* ratio of 30. (**a**) xPolyK$_6$; (**b**) xPolyK$_6$-R$_8$1; (**c**) xPolyK$_6$-R$_8$2.

and 2, respectively. Obviously, with the increase of CR$_8$C content, the ability of the polypeptides to bind pDNA increased. Nevertheless, all the polypeptides were able to bind and condense pDNA effectively at appropriate *w/w* ratios.

In cytoplasm, the reductive cleavage of disulfide bonds could promote the DNA release and decrease the toxicity of vectors. The reduction sensitivity of the polypeptide/pGL-3 complex at *w/w* ratio of 8 was also evaluated by incubation of complexes under reducing conditions of 20 mM DTT. The results in Fig. 4d showed that the polypeptides that were able to bind and condense pDNA effectively without DTT, could not bind pDNA under reducing conditions of 20 mM DTT at *w/w* ratio of 8, indicating the successful cleavage of disulfide bonds of the polypeptides.

Particle Size and Zeta Potential

The particle size and zeta potential of the polypeptide/pGL-3 complexes could influence the intracellular trafficking of particles and subsequent transfection efficiency. The hydrodynamic size and zeta potential of the polypeptides/pGL-3 complexes at various *w/w* ratios ranging from 10 to 60 without DTT were investigated. As shown in Fig. 5, the particle sizes of all polypeptides/pGL-3 exhibited a similar trend. In detail, upon increasing of *w/w* ratio, the particle size initially decreased and then increased. At the *w/w* ratio of 20, all the particles have the minimum size of 182.4 nm, 178.5 nm and 123.6 nm for xPolyK$_6$/pGL-3, xPolyK$_6$-R$_8$1/pGL-3 and xPolyK$_6$-R$_8$2/pGL-3 complex, respectively. Meanwhile, their mean particle sizes were reduced gradually at the same *w/w* ratio, due to the sequentially increased content of CR$_8$C in the complexes. The results suggested that appropriate content of CR$_8$C in the polypeptides could decrease the particle size of the complexes, and the polypeptides had an optimum *w/w* ratio to bind and condense pDNA. When the *w/w* ratio exceeded the optimum *w/w* ratio, the redundant polypeptides were bundled to the polypeptide/pGL-3, leading to increase of the particle size. As shown in Fig. 5, the zeta potential of binary complexes was around 30 mv at all *w/w* ratios.

The hydrodynamic size and zeta potential of the polypeptides/pGL-3 complexes under reducing conditions of 20 mM DTT were also studied. As shown in Fig. 6, after the addition of DTT, the particle size of xPolyK$_6$/pGL-3, xPolyK$_6$-R$_8$1/pGL-3 and xPolyK$_6$-R$_8$2/pGL-3 complexes increased significantly from 200 to 500 nm and the zeta potential was declined sharply from 30 to 0 mV, due to the fact that DTT could effectively cleavage the disulfide bond, leading to the dissociation of the complexes. Above results suggested that the polypeptides cross-linked with disulfide bonds could be degraded by reductive reagents.

Morphology Observation

The morphology of polypeptide/pGL-3 complexes was observed by TEM. As shown in Fig. 7, most of the complexes performed a uniform spherical shape with the size less than

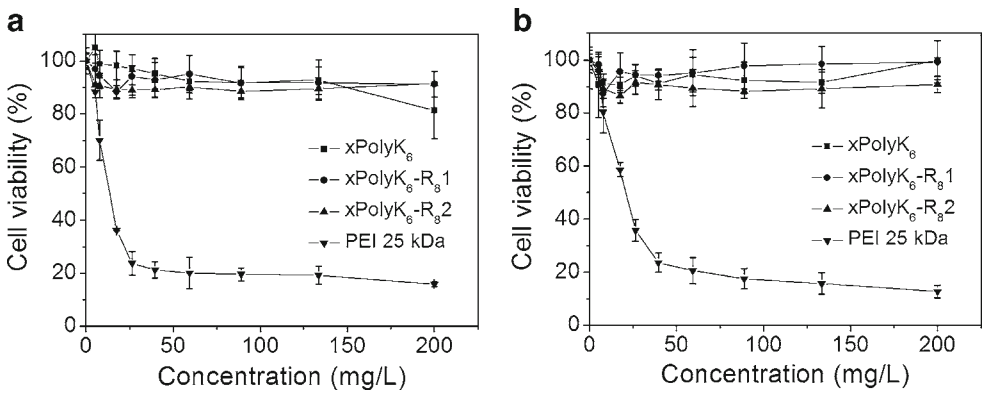


Fig. 8 Cell viabilities of polypeptides and PEI. (**a**) HeLa cells and (**b**) COS7 cells.

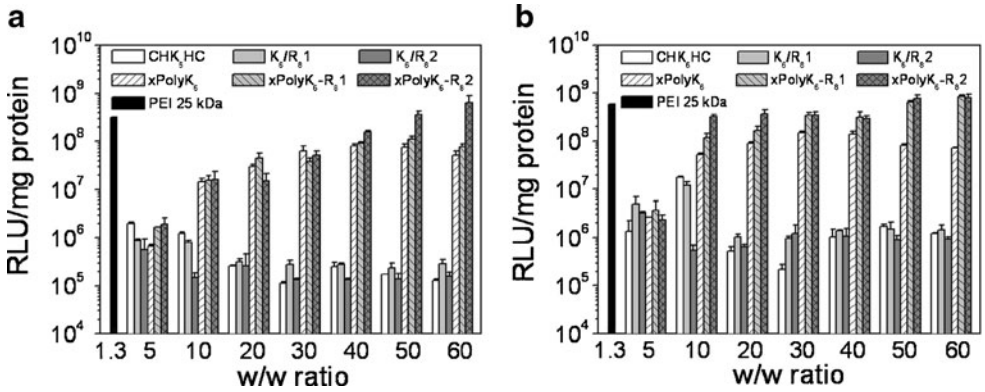


Fig. 9 Luciferase expression mediated by the mingle-peptide/pGL-3, polypeptide/pGL-3 and PEI/pGL-3. (**a**) HeLa cells and (**b**) COS7 cells.

100 nm, which was smaller than the particle size measured by the Nano-ZS ZEN3600 apparatus, since the former one was observed in dry condition and the latter one was measured in damp condition. The morphology of polypeptide/pGL-3 complexes indicated that the peptides could condense DNA compactly to form a regular sphere.

Cytotoxicity Assay *In Vitro*

The cytotoxicity of vectors is crucial for their applications *in vivo*, which depends on the properties of vectors to a great extent, including structural features, charge density, and molecular weight (32). It should be noted that a high positive charge density would always cause relative high cytotoxicity. In this study, the cytotoxicity of the bioreducible polypeptides was determined in HeLa and COS7 cells by MTT assay and the 25 kDa PEI was used as the control. As shown in Fig. 8, no significant toxicity was observed upon incubation in both cell lines for 48 h, while 25 kDa PEI exhibited a serious cytotoxicity when the concentration was over 25 mg/L. The low toxicity of polypeptides was attributed to the redox sensitivity of the polypeptides in cytoplasm. The existence of relative high

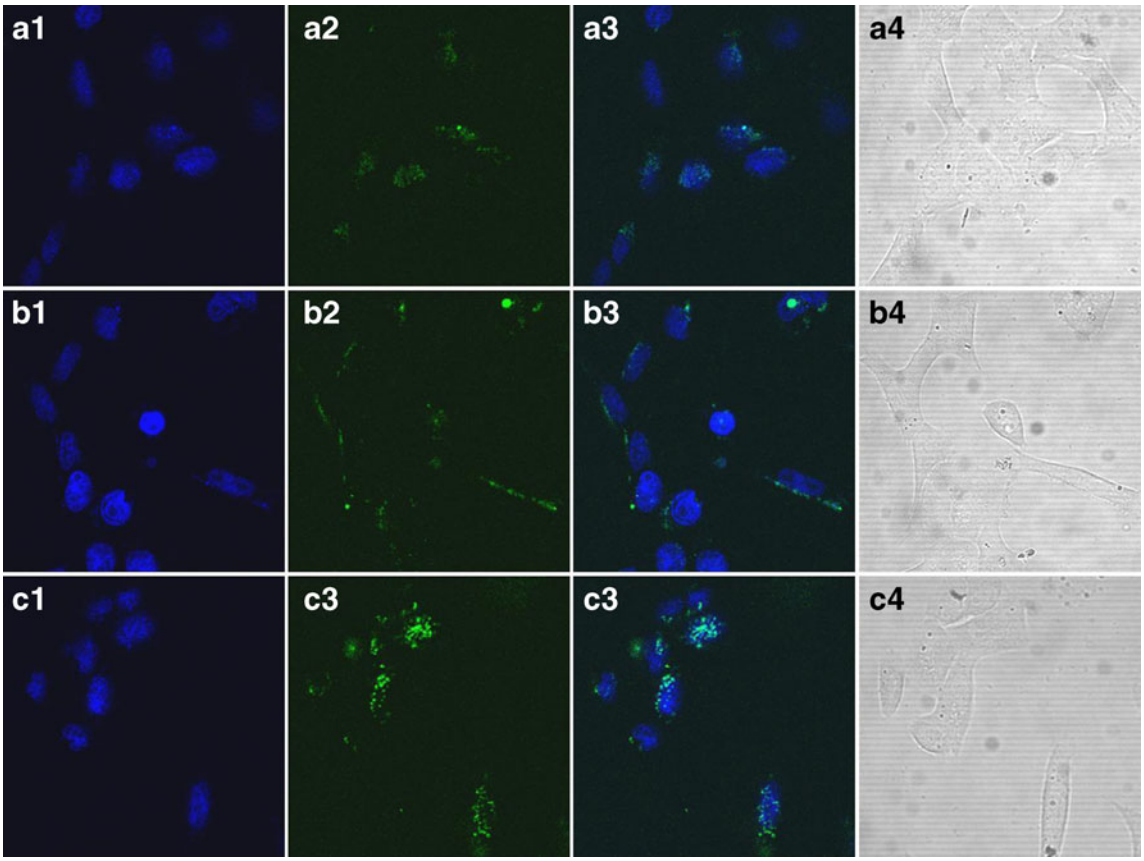


Fig. 10 Confocal microscopy images of the cellular uptake of polypeptides/pGL-3 complexes in HeLa cells. (**a**) xPolyK$_6$/pGL-3 complex at *w/w* ratio of 30; (**b**) xPolyK$_6$-R$_8$1/pGL-3 complex at *w/w* ratio of 30; **c** xPolyK$_6$-R$_8$2/pGL-3 complex at *w/w* ratio of 30.

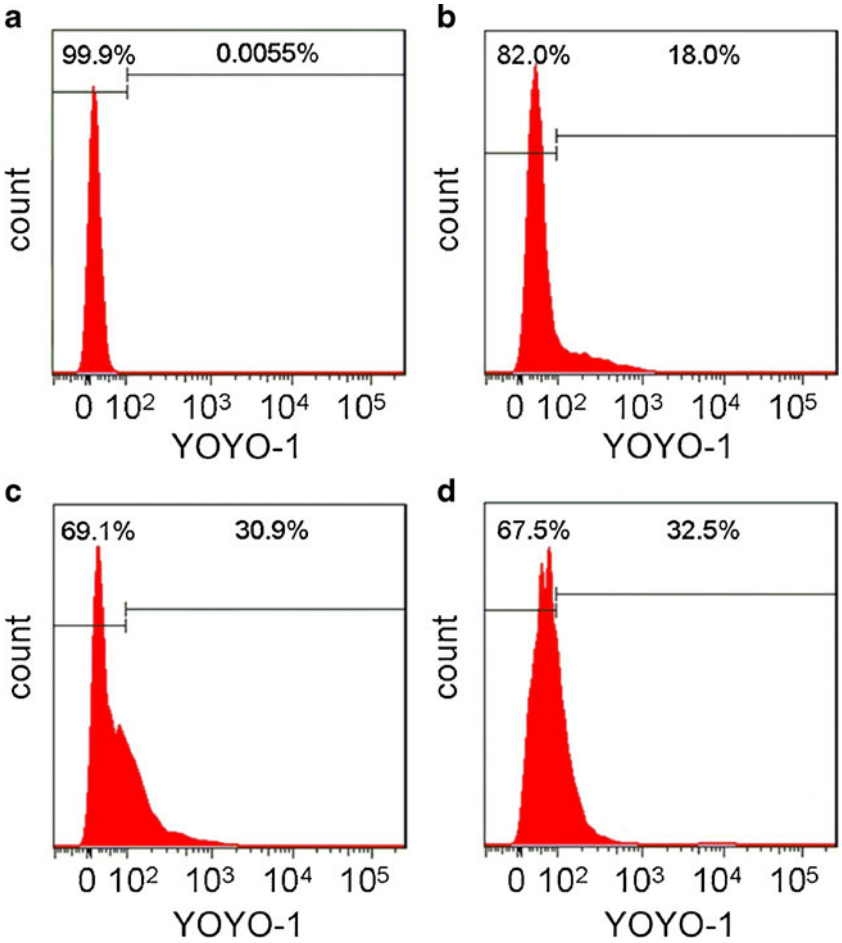


Fig. 11 Flow cytometry images of complexes at *w/w* ratio of 30 in HeLa cells. (**a**) Blank cells; (**b**) xPolyK$_6$/pGL-3 complex; (**c**) xPolyK$_6$-R$_8$1/pGL-3 complex; (**d**) xPolyK$_6$-R$_8$2/pGL-3 complex.

concentration of glutathione in cytoplasm disrupted the disulfide bond in the polypeptide, leading to the decrease of positive charge on the surface of the complexes as well as the cytotoxicity.

In Vitro Transfection and Cellular Uptake Study

The transfection efficiency of polypeptide/pGL-3 complexes was evaluated at various *w/w* ratios ranging from 5 to 60. PEI/pGL-3 complex with optimized *w/w* ratio of 1.3 was used as the positive control.

The results of luciferase transfection were shown in Fig. 9. For all the bioreducible polypeptides, the transfection efficiency showed similar trends in both HeLa and COS7 cells, which increased initially and then reached stable value, indicating that the more compact of the complexes the better transfection efficiency. Furthermore, xPolyK$_6$-R$_8$2/pGL-3 exhibited the highest transfection efficiency, which was even comparable with PEI 25 kDa. The remarkable transfection efficiency of the xPolyK$_6$-R$_8$2/pGL-3 complex demonstrated that the addition of appropriate amounts of CR$_8$C peptide, as well as the introduction of cleavable disulfide bonds could enhance the transfection efficiently. Obviously, the introduction of the CR$_8$C peptide could be favorable for the transfection efficiency of xPolyK$_6$.

The transfection efficiency of physical mixture of CK$_6$C and CR$_8$C (designated as CK$_6$C/CR$_8$C) at various *w/w* ratios was also investigated. As shown in Fig. 9, both in HeLa and COS7 cells, all the complexes exhibited the similar level of gene transfection at different *w/w* ratios which was significantly lower than that of chemical linked bioreducible polypeptides/pGL-3 complexes, since the physical mixture of peptide could not form compact complexes. The results also suggested that the strategy to incorporate CR$_8$C peptide by disulfide bonds was an effective approach to promote gene transfection.

Confocal Microscopy and Flow Cytometry Assays

The cellular uptake of polypeptide/DNA complexes was explored by confocal microscopy and flow cytometry. The polypeptide/pGL-3 complexes were evaluated at *w/w* ratio of 30. Nuclei stained by Hoechst 33258 were observed in Fig. 10–1, pGL-3 labeled with YOYO-1 was visible in Fig. 10–2, the merged images were presented in Fig. 10–3

and bright-field cells were exhibited in Fig. 10–4. As shown in Fig. 10, the green fluorescence of pGL-3 could be found in the cytoplasm and nucleus after transfection for 4 h, which implied that the pGL-3 was delivered to the nucleus by polypeptides. Stronger green fluorescence was observed in the nucleus and cytoplasm after the cellular uptake by the xPolyK$_6$-R$_8$2/pGL-3 complex compared with that by xPolyK$_6$/pGL-3 or xPolyK$_6$-R$_8$1/pGL-3 complex, indicating that xPolyK$_6$-R$_8$2 exhibited enhanced gene delivery to cells. Meanwhile, the fluorescence intensity of pGL-3 delivered by xPolyK$_6$-R$_8$1 was much stronger than that transferred by xPolyK$_6$. It was inferred that the introduction of CR$_8$C could enhance cellular uptake, and the cellular uptake ability was associated with the content of CR$_8$C peptide.

To further confirm the cellular uptake behaviors of polypeptides, pGL-3 transferred HeLa cells was studied quantitatively by flow cytometry. The pGL-3, as a reporter gene, was labeled by YOYO-1. The polypeptide/pGL-3 complexes were evaluated at *w*/*w* ratio of 30. As shown in Fig. 11, the proportion of transfected cells by xPolyK$_6$, xPolyK$_6$-R$_8$1 and xPolyK$_6$-R$_8$2 complex were 18.0%, 30.9% and 32.5%, respectively. Clearly, the phagocytized cell number by xPolyK$_6$-R$_8$2 was higher than that by the other complexes, which demonstrated that xPolyK$_6$-R$_8$2 was the most efficient vector among the polypeptides and these results were consistent with the transfection activity.

CONCLUSIONS

In this study, a series of polypeptides with different CR$_8$C contents were synthesized for gene delivery by the oxidative polymerization of CHK$_6$HC and CR$_8$C peptides. The reduction-responsive polypeptides showed low cytotoxicity and xPolyK$_6$-R$_8$2 exhibited the highest transfection efficiency among these gene vectors, which was also comparable to 25 kDa PEI. Results suggested that increasing the amount of R$_8$ could promote the transfection efficiency significantly. Besides, xPolyK$_6$-R$_8$2 performed the best ability of cellular uptake based on the results of confocal microscopy and flow cytometry, which was in correspondence with the results of gene transfection. These bioreducible polypeptides incorporating with proper amount of CPPs are promising as effective non-viral gene vectors in gene therapy.

ACKNOWLEDGMENTS AND DISCLOSURES

This work was financially supported by National Natural Science Fundodation of China (51125014, 51233003) and the Ministry of Science and Technology of China (2011CB606202).

REFERENCES

1. Pack DW, Hoffman AS, Pun S, Stayton PS. Design and development of polymers for gene delivery. Nat Rev Drug Discov. 2005;4:581–93.
2. Maheshri N, Koerber JT, Kaspar BK, Schaffer DV. Directed evolution of adeno-associated virus yields enhanced gene delivery vectors. Nat Biotechnol. 2006;24:198–204.
3. Luo D, Saltzman WM. Synthetic DNA delivery systems. Nat Biotechnol. 2000;18:33–7.
4. Park TG, Jeong JH, Kim SW. Current status of polymeric gene delivery systems. Adv Drug Deliv Rev. 2006;58:467–86.
5. Jeong JH, Kim SW, Park TG. Molecular design of functional polymers for gene therapy. Prog Polym Sci. 2007;32:1239–74.
6. Neu M, Fischer D, Kissel TJ. Recent advances in rational gene transfer vector design based on poly(ethylene imine) and its derivatives. Gene Med. 2005;7:992–1009.
7. Wong SY, Pelet JM, Putnam D. Polymer systems for gene delivery —past, present, and future. Prog Polym Sci. 2007;32:799–837.
8. Sun XL, Liu CX, Liu DH, Li P, Zhang N. Novel biomimetic vectors with endosomal-escape agent enhancing gene transfection efficiency. Int J Pharm. 2012;425:62–72.
9. Wiethoff CM, Middaugh CR. Barriers to nonviral gene delivery. J Pharm Sci. 2003;92:203–17.
10. Newland B, Zheng Y, Jin Y, Abu-Rub M, Cao HL, Wang WXA. Single cyclized molecule *versus* single branched molecule: a simple and efficient 3D "Knot" polymerstructure for nonviral gene delivery. J Am Chem Soc. 2012;134:4782–9.
11. Shan YB, Luo T, Peng C, Sheng RL, Cao A, Cao XY, *et al.* Gene delivery using dendrimer-entrapped gold nanoparticles as nonviral vectors. Biomaterials. 2012;33:3025–35.
12. Zhou JB, Liu J, Cheng CJ, Patel TR, Weller CE, Piepmeier JM, *et al.* Biodegradable poly(amine-co-ester) terpolymers for targeted gene delivery. Nat Mater. 2012;11:82–90.
13. Shim MS, Kwon YJ. Stimuli-responsive polymers and nanomaterials for gene delivery and imaging applications. Adv Drug Deliv Rev. 2012. doi:10.1016/j.addr.2012.01.018.
14. Hu JM, Zhang GQ, Liu SY. Enzyme-responsive polymeric assemblies, nanoparticles and hydrogels. Chem Soc Rev. 2012. doi:10.1039/C2CS35103J.
15. Nam HY, Nam K, Lee M, Kim SW, Bull DA. Dendrimer type bio-reducible polymer for efficient gene delivery. J Control Release. 2012;160:592–600.
16. Ho YC, Liao ZX, Panda N, Tang DW, Yu SH, Mi FL, *et al.* Self-organized nanoparticles prepared by guanidine- and disulfide-modified chitosan as a gene delivery carrier. J Mater Chem. 2011;21:16918–27.
17. Kima T, Lee M, Kima SW. A guanidinylated bioreducible polymer with high nuclear localization ability for gene delivery systems. Biomaterials. 2010;31:1798–804.
18. Lehto T, Mager I, Sork H, Copolovici DM, Oskolkov N, Suhorutsenko J. A peptide-based vector for efficient gene transfer *in vitro* and *in vivo*. Mol Ther. 2011;19:1457–67.
19. Ma DX, Shi NQ, Qi XR. Distinct transduction modes of arginine-rich cell-penetrating peptides for cargo delivery into tumor cells. Int J Pharm. 2011;419:200–8.
20. McKenzie DL, Smiley E, Kwok KY, Rice KG. Low molecular weight disulfide cross-Linking peptides as nonviral gene delivery carriers. Bioconjug Chem. 2000;11:901–9.
21. Martin ME, Rice EG. Peptide-guided gene delivery. AAPS J. 2007;9:E18–29.
22. Chen CS, Ji TJ, Xu XD, Zhang XZ, Zhuo RX. Nanofibers Self-assembled from Structural Complementary Borono-decapeptides. Rapid Commun. 2010;31:1903–8.

23. Chen JX, Wang HY, Quan CY, Xu XD, Zhang XZ, Zhuo RX. Amphiphilic cationic lipopeptides with RGD sequences as gene vectors. Org Biomol Chem. 2010;8:3142–8.
24. Oupicky D, Parker AL, Seymour LW. Laterally stabilized complexes of DNA with linear reducible polycations: strategy for triggered intracellular activation of DNA delivery vectors. J Am Chem Soc. 2002;124:8–9.
25. Lo SL, Wang S. An endosomolytic Tat peptide produced by incorporation of histidine and cysteine residues as a nonviral vector for DNA transfection. Biomaterials. 2008;2:2408–14.
26. Manickam DS, Bisht HS, Wan L, Mao GZ, Oupicky D. Influence of TAT-peptide polymerization on properties and transfection activity of TAT/DNA polyplexes. J Control Release. 2005;102:293–306.
27. Han K, Yang J, Chen S, Chen JX, Liu CW, Li C, *et al.* Novel gene transfer vectors based on artificial recombinant multi-functional oligopeptides. Int J Pharm. 2012;436:555–63.
28. Yang J, Lei Q, Han K, Gong YH, Chen S, Cheng H, *et al.* Reduction-sensitive polypeptides incorporated with nuclear localization signal sequences for enhanced gene delivery. J Mater Chem. 2011. doi:10.1039/c2jm32223d.
29. Sun YX, Zhang XZ, Cheng H, Cheng SX, Zhuo RX. A low-toxic and efficient gene vector: Carboxymethyl dextran-graft-polyethylenimine. J Biomed Mater Res Part A. 2008;84:1102–10.
30. McKenzie DL, Kwok KY, Rice KG. A potent new class of reductively activated peptide gene delivery agents. J Biol Chem. 2000;275:9970–7.
31. Wang HY, Chen JX, Sun YX, Deng JZ, Li C, Zhang XZ, *et al.* Construction of cell penetrating peptide vectors with N-terminal steaylated nuclear localization signal for targeted delivery of DNA into the cell nuclei. J Control Release. 2011;155:26–33.
32. Godbey WT, Wu KK, Mikos AG. Characterization of a multifunctional PEG-based gene delivery systemcontaining nuclear localization signals and endosomalescape peptides. J Control Release. 1999;60:149–60.