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# Chitosan-*graft*-polyethylenimine with improved properties as a potential gene vector

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ABSTRACT

A novel chitosan-*graft*-polyethylenimine (CHI-*g*-PEI) copolymer with biocleavable disulfide linkages between chitosan chains and PEI grafts was synthesized, characterized, and examined as a potential nonviral gene vector. The chemical structure of the obtained product was characterized by $^{1}$H NMR, FTIR and Raman spectroscopy, respectively. Agarose gel retardation assay, dynamic light scattering, and scanning electron microscopy experiments revealed that CHI-*g*-PEI had a good ability of condensing plasmid DNA into spherical nanoparticles in the size range of 200–300 nm. In the imitative physiological environment the polymer/pDNA complexes are relatively stable, meanwhile, an efficient release of pDNA was detected in the presence of 25 mM DTT, mimicking the intracellular reductive environment. These results show that the bioreducible CHI-*g*-PEI copolymer, thus obtained, can be used as a promising nonviral gene carrier due to its excellent properties.

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## 1. Introduction

Recent years, nonviral cationic vectors have been applied to protect the DNA from degradation based on the condensation of negatively charged DNA into compact particles essentially by electrostatic interactions (Mintzer & Simanek, 2009; Nguyen, Green, Chan, Langer, & Anderson, 2009). To improve the safety of gene transfer vectors, the materials used for preparing cationic polymers must be biocompatible and biodegradable (Hussain et al., 2009). With high stability and favorable biocompatibility (Ravi Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004), chitosan was first applied in the efforts of gene delivery (Mumper, Wang, Claspell, & Rolland, 1995) and widely acknowledged as one of the most attractive cationic vectors (Liu & Yao, 2002). However, the transfection efficiency of chitosan polyplexes are poor and among all those efforts to solve this problem, direct grafting chitosan with polyethylenimine was considered to be one of the most prominent methods (Jiang et al., 2007; Lu, Xu, Zhuo, Cheng, & Zhuo, 2008; Wong et al., 2006). With a substantial increased charge density (Kunath et al., 2003; Neu, Fischer, & Kissel, 2005), the modified chitosan polymer creates a hydrophilic exterior that reduces interactions of the cationic vector with plasma proteins and erythrocytes.

The transfection efficiency may depend on several factors, such as the chemical composition of the synthetic polymer and nanoparticle size of the complex (Jeong, Kim, & Park, 2009). The conjugation mechanism and grafting ratios of PEI are two influential factors contributing to the properties of CHI-*g*-PEI obtained (Kircheis, Wightman, & Wagner, 2001). Cho et al. synthesized CHI-*g*-PEI through the reaction between periodate-oxidized chitosan and PEI (Jiang et al., 2007). Another strategy was carried out by grafting 800 Da PEI to *N*-maleated chitosans using Michael addition (Lu et al., 2008). Their results showed that these polymers remarkably enhanced transfection efficiency compared to chitosan. Despite the improved DNA condensing ability, these compounds still had a slow degradation rate, which can directly affect DNA release. It has been suggested that one of the primary causes of low efficiency in gene delivery is the inefficient release of pDNA from endosomes into the cytoplasm. The inability to efficiently release pDNA inside the cell is one of the principal limitations of 25 kDa bPEI applied in gene transfer, which would limit the accessibility of transcription machinery in the nucleus and therefore hinder gene expression (Godbey, Wu, & Mikos, 1999).

The disulfide linkage has been well-established to keep relatively stable state in the extracellular milieu and break rapidly in the intracellular compartment with degradation times ranging from minutes to hours, due to the presence of comparatively high concentrations of glutathione (Gilbert, 1995; Kuppusamy et al., 2002; Raina & Missiakas, 1997). This phenomenon has been exploited for disulfide-containing polymers used as a reduction-triggered gene delivery (Meng, Hennink, & Zhong, 2009). Peng et al. adopted two simple approaches to obtain disulfide cross-linked PEI, i.e., via oxidation of thiolated 800 Da PEI with DMSO to form the disulfide cross-links (Peng, Zhong,

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& Zhuo, 2008) and via Michael addition reaction between cystamine bisacrylamide and 800 Da PEI (Sun et al., 2008). The transfection experiments *in vitro* showed that SS-PEI exhibited comparable transfection efficiency and reduced cytotoxicity as compared to 25 kDa PEI. A synthesized thiolated chitosan derivative that could form reducible disulfide linkages exhibited a significantly higher gene transfer potential. This effect was mainly attributed to the enhancement of cellular uptake compared to chitosan (Lee et al., 2007).

The purpose of this study is to synthesize a novel chitosan-*graft*-polyethylenimine copolymer with biocleavable disulfide linkages between chitosan chains and PEI grafts, which are sensitive to intracellular glutathione concentrations. The physiochemical characteristics and morphology of the CHI-*g*-PEI/DNA complexes were evaluated and examined as a potential reduction-triggered gene delivery system. The prepared CHI-*g*-PEI/DNA complexes are relatively stable before cellular uptake. While, in the reduction environment of the cytoplasm, the cleavage of the disulfide linkage would facilitate DNA release effectively, resulting in an increased gene expression level.

## 2. Materials and methods

### 2.1. Materials

Chitosans ($M_w$ = 10 kDa, 80 kDa, deacetylation degree = 85.3%) were purchased from Haidebei Marine Bioengineering Co. Ltd., Jinan, China. 3,3′-Dithiodipropionic acid was purchased from Shanghai Jingchun Chemical Reagent Co., China. Branched polyethylenimine (PEI) with molecular weight of 800 Da, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were purchased from Sigma–Aldrich. *N*-Hydroxysuccinimide was purchased from Sinopharm Chemical Reagent Co. Ltd., Shanghai, China. 1,4-Dithiothreitol (DTT) were purchased from Shanghai Chemical Reagent Co., China. GelRed™ was purchased from Biotium (CA, USA). All reagents were analytical grade and used as received.

### 2.2. Instrumentations

The chemical structure of the modified chitosan was characterized by $^1$H NMR, FTIR and Raman spectra. $^1$H nuclear magnetic resonance (NMR) spectra were recorded on a Varian Unity 300 MHz spectrometer using $D_2O$ as a solvent. Fourier transformed infrared (FTIR) spectra were recorded on a Lambda Bio40 UV–vis spectrometer (Perkin-Elmer). Raman spectra were recorded on a Thermo Nicolet Model Nexus 670 FT-RAMAN spectrometer.

### 2.3. Synthesis of chitosan-graft-PEI

0.526 g of 3,3′-dithiodipropionic acid (2.5 mmol) was dissolved in 15 mL of aqueous DMF. Then a solution of 0.479 g EDC (2.5 mmol) in 5 mL distilled water was added, the reaction was stirred at 40 °C for 30 min. Subsequently, 0.2 g of chitosan powder dispersed in 10 mL distilled water was slowly added dropwise into the above solution with continuous stirring. The reaction was performed at 40 °C for 24 h until the solution became clear. The solution was dialyzed against distilled water (MWCO: 3500 Da) for 3 days, followed by freeze drying.

In the second step, 800 Da PEI (0.246 g, 0.308 mmol), EDC (0.059 g, 0.308 mmol) and NHS (0.035 g, 0.308 mmol) were dissolved in distilled water (20 mL) and then adjusted to pH 6.5 with 0.1 M hydrochloric acid. The mixture was placed in a flask equipped with a magnetic stirrer. The activated CHI-SS-COOH aqueous solution was added dropwise to the flask, and the mixture was heated at 40 °C for 24 h. Subsequently, the final solution was purified by dialysis (MWCO: 3500 Da) at room temperature and lyophilized for 2 days. Chitosans with the molecular weights of 10 and 80 kDa were used and the obtained products were designated as $CHI_{10k}$-*g*-PEI and $CHI_{80k}$-*g*-PEI, respectively.

### 2.4. Determination of buffering capacity of polymers

The buffer capability of 800 Da PEI, 10 kDa CHI and CHI-*g*-PEI was determined by acid–base titration assay over the pH values ranging from 10 to 3 as described previously by Benns (Benns, Choi, Mahato, Park, & Kim, 2000; Benns, Mahato, & Kim, 2002). Briefly, each sample was dissolved in 30 mL of 150 mM NaCl solution with a concentration of 0.2 mg/mL. The sample solution was first alkalized by 0.1 M NaOH to a pH of 10, and then different volumes of 0.1 M HCl were added dropwise to the solution. The different pH values were measured using a microprocessor pH meter.

### 2.5. Preparation of CHI-g-PEI/DNA complexes

The CHI-*g*-PEI/DNA complexes were prepared at various weight ratios of CHI-*g*-PEI to DNA by adding the copolymer solution to equal volumes of DNA solution. The plasmid DNA solution was stocked in 40 mM Tris–HCl buffer solution (104 ng/μL) and CHI-*g*-PEI was dissolved in 150 mM NaCl solution at corresponding concentrations. The mixture was gently vortexed for 3–5 s and incubated at 37 °C for 30 min to complete the complex formation. All CHI-*g*-PEI/DNA complexes were freshly prepared and filtered with a 0.22 μm membrane filter before use.

### 2.6. Agarose gel retardation assay

CHI-*g*-PEI/DNA complexes with different weight ratios ranging from 0 to 7 were prepared by adding appropriate volume of CHI-*g*-PEI (in 150 mM NaCl solution) to 1 μL of plasmid DNA (104 ng/μL in 40 mM Tris–HCl buffer solution) with or without 25 mM DTT. The chitosan and CHI-SS-COOH aqueous solution were used as control in this experiment. The complexes were diluted by 150 mM NaCl solution to 6 μL and then incubated at 37 °C for 30 min. Then the complexes were electrophoresed on the 0.7% (W/V) agarose gel containing GelRed™ with Tris–acetate (TAE) running buffer at 80 V for 80 min. DNA was visualized with an UV lamp using a GelDoc system (Imago).

### 2.7. Size and ζ-potential measurements

The particle size and surface charge of CHI-*g*-PEI/DNA complexes were determined by Nano-ZS ZEN3600 (MALVERN Instrument) at room temperature. The complexes were prepared as described in Section 2.5 above and diluted with 150 mM NaCl solution for particle size measurement or distilled water for ζ-potential measurement respectively. The final volume of each sample was 1 mL, containing DNA concentration of 1 μg/mL. All samples were measured in triplicate.

### 2.8. Scanning electron microscopy

The polymer/DNA complexes were prepared according to the conditions described above for CHI-*g*-PEI/DNA complexes. A total of 100 μL of complex suspension was deposited onto a glass slide. After drying at room temperature, the samples were fixed on an aluminum stub and coated with gold for 7 min. The morphology of the sample was observed by a scanning electron microscope (SEM, FEI-QUANTA 200, Holland).

## 3. Results and discussion

### 3.1. Synthesis and characterization of chitosan-graft-PEI

In this study, a CHI-*g*-PEI copolymer with a disulfide linkage between chitosan and PEI was prepared as shown in Scheme 1. Initially, an excess of 3,3′-dithiodipropionic acid was reacted with the amino groups of chitosan in an aqueous DMF solution, yielding the intermediate CHI-SS-COOH. Then, the target product CHI-*g*-PEI was obtained by the reaction of the carboxylic acid groups of the CHI-SS-COOH activated by NHS ester with the amine groups of PEI.

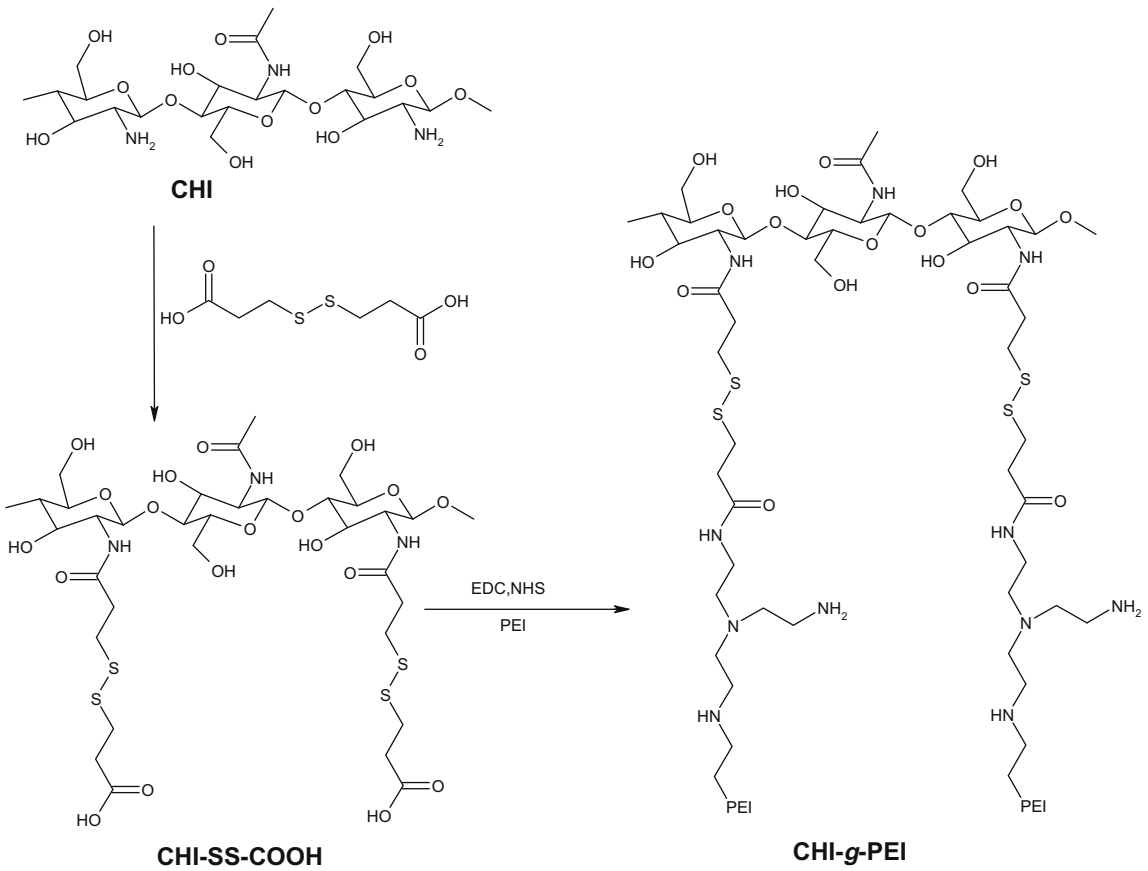
It's worth noting that the process for preparing CHI-*g*-PEI copolymer was very simple and controllable. 3,3′-Dithiodipropionic acid was used as the linker between chitosan and branched 800 Da PEI (Williams, Hekmat, & Withers, 2006). Chitosan is a weak base with a $pK_a$ value of about 6.2–7.0 (due to the D-glucosamine residue), thus shows a very poor aqueous solubility at neutral and alkaline pH values. After adding the DMF solution of 3,3′-dithiodipropionic acid, the amino groups were positively charged in acidic medium, resulting in a good water solubility. The condensation reaction of carboxylic groups and pendant amino groups of chitosan was performed by using water-soluble carbodiimide.

Although the procedure proved to be highly reproducible, it should be noted that crosslinking may occur since chitosan and the branched PEI are both multivalent polymers. In order to avoid difficulties in the purification of the final product, twofold of 3,3′-dithiodipropionic acid had to be added and the nonreacted material should be removed by dialysis. To avoid the possibility of glycosidic bond hydrolysis, the reaction time was necessary to be controlled. In the following grafting, similarly, the excess amount of PEI was employed and the solution of CHI-SS-COOH were added dropwise in order to prevent the possible crosslinking reaction. The activated NHS ester could react with PEI very quickly due to the high amine density of the branched PEI. The obtained products showed excellent aqueous solubility as applied to the following experimental parts.

### 3.2. Characterization of CHI-g-PEI

Structure changes of chitosan derivative were confirmed by $^1$H NMR, FTIR, and Raman spectra. The $^1$H NMR spectra of the chitosan derivatives are shown in Fig. 1. The proton peaks of $-CH_2CH_2S-$ appeared at 2.8–2.95 and 2.5–2.6 ppm in the spectrum of CHI-SS-COOH (Fig. 1b), indicating that 3,3′-dithiodipropionic acid was grafted to the chitosan chain. By comparing the intensity of peak at 3.4–3.8 ppm (multiplet, assigned to the proton peaks of glucosamine unit, H-3, H-4, H-5, H-6, H-6′), the grafting degrees per glucosamine unit were calculated as 38.08% in $CHI_{10k}$-SS-COOH, and 32.11% in $CHI_{80k}$-SS-COOH. The proton peaks of PEI ($-NHCH_2CH_2-$) appeared at 2.5–3.0 ppm which overlapped with those of the $-CH_2CH_2S-$ (Fig. 1c). The PEI grafting degree per glucosamine unit was determined by comparing the $^1$H NMR signal integrals from $-CH_2$ protons of PEI, which should subtract the absorption areas of $-CH_2CH_2S-$, with integrals of the chitosan backbone proton signals. The grafting degrees were 16.48 and 18.49% for $CHI_{10k}$-*g*-PEI and $CHI_{80k}$-*g*-PEI, respectively, which is presumed that the length of polymer may influence the grafting degree of PEI. These results indicated that the $CHI_{80k}$-*g*-PEI possess a higher cationic charge density due to an increased number of amine groups per copolymer molecule.

As shown in Fig. 2, the absorption of $\nu_{(C-O-C)}$ at 1155 cm$^{-1}$, $\nu_{(C-OH)}$ at 1030 cm$^{-1}$ and $\nu_{(C-O)}$ of glucosamine at 899 cm$^{-1}$ in the FTIR spectra were not shifted. The spectra of CHI-SS-COOH showed absorption




**Scheme 1.** Synthesis of CHI-*graft*-PEI.

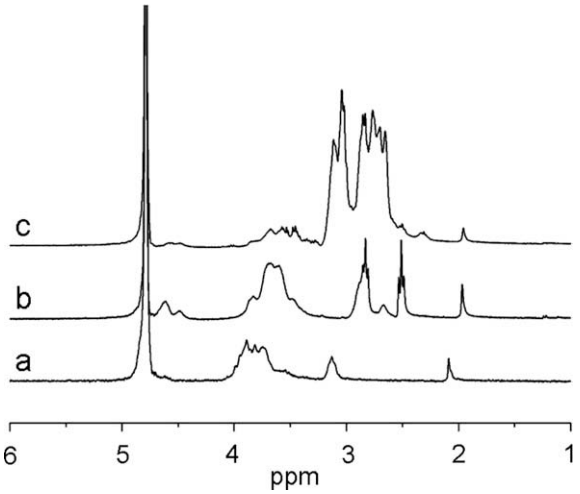



**Fig. 1.** The $^1$H NMR spectra of (a) CHI, (b) CHI-SS-COOH, and (c) CHI-*g*-PEI in $D_2O$.

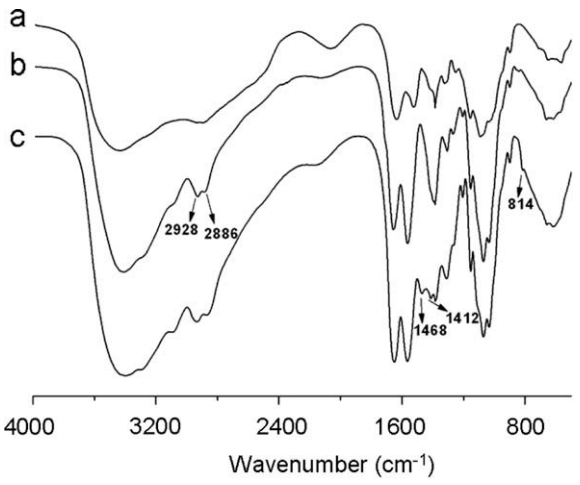



**Fig. 2.** FTIR spectra of (a) CHI, (b) CHI-SS-COOH, and (c) CHI-*g*-PEI.

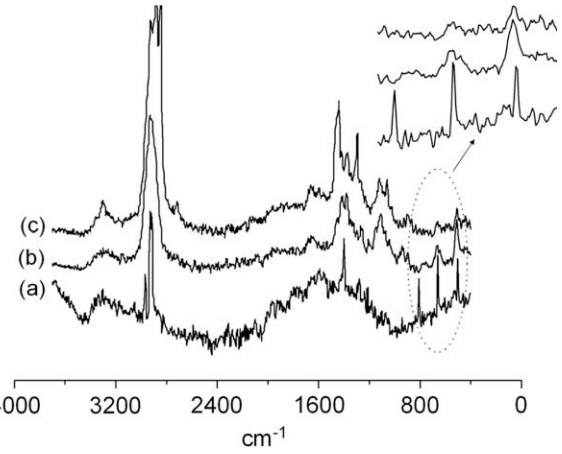



**Fig. 3.** Raman spectra of (a) 3,3′-dithiodipropionic acid, (b) CHI-SS-COOH , and (c) CHI-*g*-PEI.

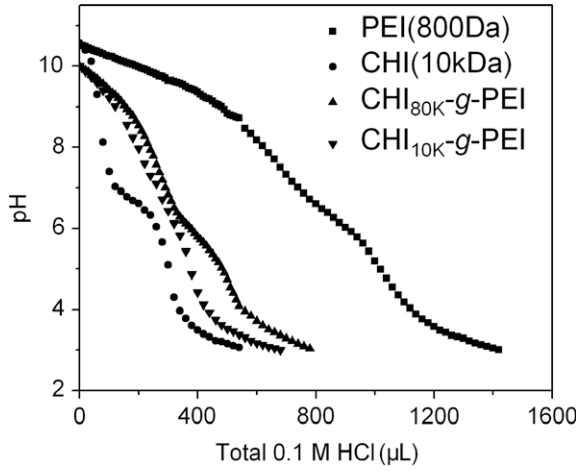



**Fig. 4.** Buffering capacity of PEI (800 Da), CHI (10 kDa), $CHI_{10k}$-*g*-PEI and $CHI_{80k}$-*g*-PEI in 150 mM NaCl solutions.

for $-CH_2CH_2-$ at 2928 and 2886 $cm^{-1}$. After grafting PEI to CHI, new peaks appeared at 1468, 1412, and 814 $cm^{-1}$ attributed to the absorption of $-CH_2CH_2NH-$ moiety. These results suggested that chitosan was conjugated with polyethyleneimine successfully.

In Fig. 3, the absorption of $\nu_{(-S-S-)}$ at 501 $cm^{-1}$ and $\nu_{(C-S)}$ at 659 $cm^{-1}$ in the Raman spectra were not shifted obviously. These results confirmed the existence of "–S–S–" bond in the structure of products.

### 3.3. Buffer capability

The buffer capability of gene vectors is extremely important for the escaping of complexes from the endosomes and facile release of DNA from the complex. As for chitosan complexes, optimal transfection effect can be obtained in the range of pH 6.8–7.0. Above pH 7.5, the complex showed a very poor ability of condensing pDNA, thus preventing cellular uptake and transfection efficiency. Below pH 6.5, cellular uptake is significant but transfection efficiency is low, possibly due to hindering complex release in the endosome (Sato, Ishii, & Okahata, 2001). The high density of amines, resulting in a significant buffering capacity, allows PEI polyplexes to avoid degradation in endolysosomes (Suh, Paik, & Hwang, 1994). To improve the buffer capacity of chitosan-based polyplexes, conjugating chitosan with polyethyleneimine proved to be an effective modification.

In this study, the buffer capabilities of 10 kDa CHI, 800 Da PEI, and CHI-*g*-PEI were contrasted by acid–base titration. As shown in Fig. 4, 800 Da PEI had a strong buffer capacity, and the buffer capacity of chitosan was very weak. When chitosan grafted with PEI, the buffer capacity was improved as compared with chitosan, which was attributed to the increased density of amine groups. In addition, $CHI_{80k}$-*g*-PEI had a higher cationic charge density than $CHI_{10k}$-*g*-PEI resulting in an increasing buffer capacity.

### 3.4. Agarose gel retardation assay

The influence of variation in the side groups of the polymers and the favorable effect of the presence of the disulfide linkages on the degree of binding between CHI-*g*-PEI and DNA at different weight ratios is illustrated.

As shown in Fig. 5, chitosan possessed a low condensation capacity led to low transfection efficiency. The CHI-SS-COOH requires a higher weight ratio for condensation of DNA compared to the chitosan due to the decreased charge density. The migrations of DNA were completely retarded at *w*/*w* ratios higher than 3 for $CHI_{10k}$-*g*-PEI and 2 for $CHI_{80k}$-*g*-PEI. The high molecular weight polymer showed an obvious increase in condensation

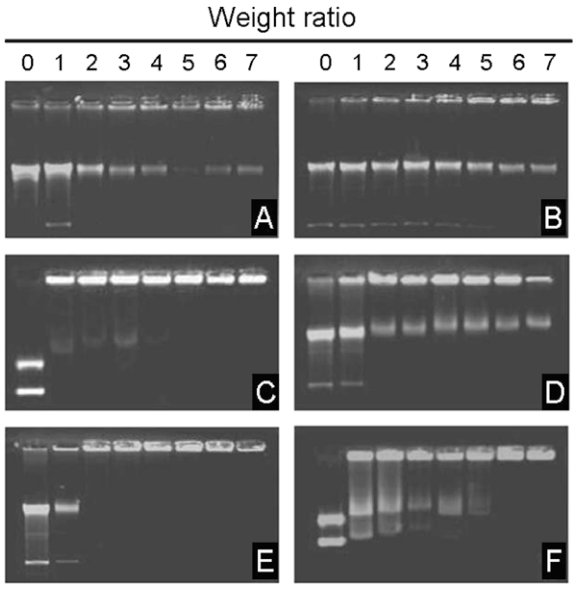



**Fig. 5.** Agarose gel retardation assay of the complexes at various weight ratios. (A) $CHI_{80k}$/DNA complexes, (B) $CHI_{80k}$-SS-COOH/DNA complexes, (C) $CHI_{10k}$-*g*-PEI/DNA complexes, (D) $CHI_{10k}$-*g*-PEI/DNA complexes with 25 mM DTT. (E) $CHI_{80k}$-*g*-PEI/DNA complexes, (F) $CHI_{80k}$-*g*-PEI/DNA complexes with 25 mM DTT.

capacity due to the chain entanglement effect (Kiang, Wen, Lim, & Leong, 2004). Superadd the higher cationic charge density, $CHI_{80k}$-*g*-PEI can entrap DNA to form stable complex more effectively than $CHI_{10k}$-*g*-PEI.

However, when the complexes were incubated with 25 mM DTT, the migrations of DNA were observed obviously at *w*/*w* ratios ranging from 4 to 7 for $CHI_{10k}$-*g*-PEI, and 2 to 5 for $CHI_{80k}$-*g*-PEI (Fig. 5(D, F)). It was inferred that, in the reduction environment of the cytoplasm, CHI-*g*-PEI/DNA complex would effectively release pDNA, resulting in an increase in DNA uptake and gene expression inside the nucleus. These results indicate that it is essential to balance between disulfide crosslinking and the density of the cationic charge in the thiolated polyplex to obtain the optimal conditions in forming complexes (Miyata et al., 2004).

### 3.5. Particle size and ζ-potential measurements

Particle size and surface charge of the complex are crucial requirements for successful gene transfection. It has been clearly demonstrated that particle size is one of the most important factors determining the behavior of the complexes *in vitro and in vivo* (Liu & Reineke, 2005). As shown in Fig. 6(A), the particle sizes of $CHI_{10k}$-*g*-PEI/DNA and $CHI_{80k}$-*g*-PEI/DNA complexes at *w*/*w* ratios of 10–50 were in the scope of 250–350 nm. The cationic charge of complex surfaces prevented aggregation due to the electrostatic repulsion between complexes, therefore, the particle size gradually decreased with the increasing *w*/*w* ratio. The excess of polycation was essential to generate a hydrophilic cationic coating around the complex for sufficient solubilization. $CHI_{80k}$-*g*-PEI revealed higher condensing ability to form smaller complexes, which belonged to the high molecular weight (Kim et al., 2001).

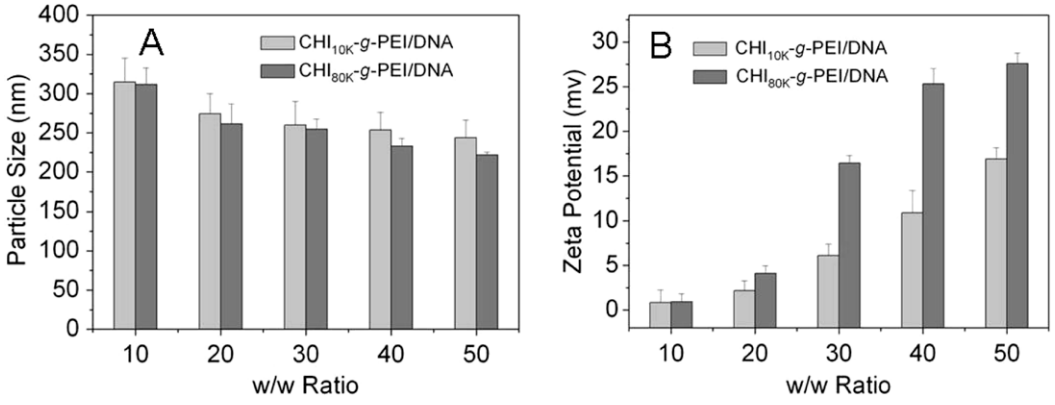



**Fig. 6.** Effect of the *w*/*w* ratio of the CHI-*g*-PEI/DNA complexes on the particle size (A) and ζ-potential (B).

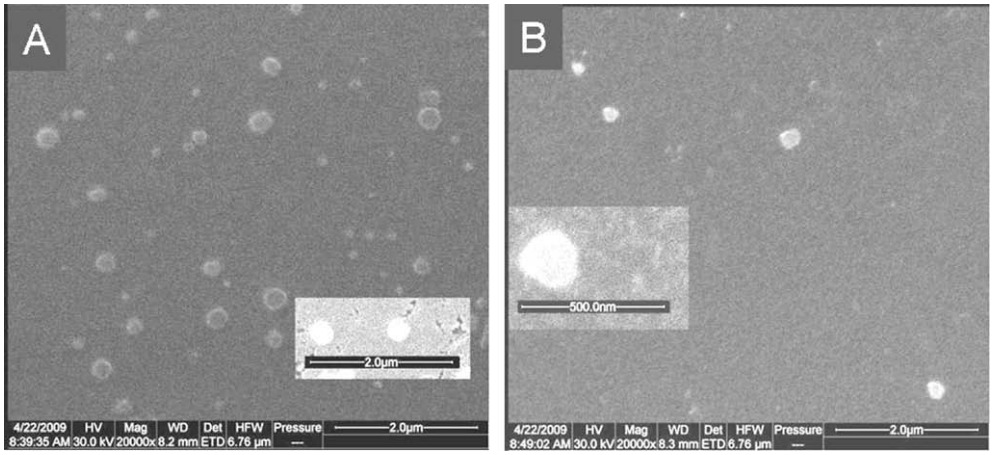



**Fig. 7.** SEM images of (A) $CHI_{10k}$-*g*-PEI/DNA complexes (*w*/*w* = 20/1), (B) $CHI_{80k}$-*g*-PEI/DNA complexes (*w*/*w* = 20/1).

Beside the solubilization enhancement, the cationic surface charge of complexes is required for electrostatic interactions with anionic cell-surface proteoglycans (Mislick & Baldeschwieler, 1996). The ζ-potential values of $CHI_{10k}$-*g*-PEI/DNA and $CHI_{80k}$-*g*-PEI/DNA complexes at *w*/*w* ratios ranging from 10 to 50 were tested as shown in Fig. 6(B). Positively charged complexes were obtained when the weight ratio was higher than 10, furthermore, the ζ-potential of the complexes rapidly increased from 0 mV to 15–25 mV with increasing *w*/*w* ratios. Compared with $CHI_{10k}$-*g*-PEI, $CHI_{80k}$-*g*-PEI with a higher charge density will release more counter ions upon binding with DNA, thus forming more stable complexes.

When the weight ratios were lower than 10:1, large-size complexes were obtained with a ζ-potential of the complexes close to 0 mV, indicating full retardation of DNA and aggregation of the complex at neutral surface charge.

### 3.6. Scanning electron microscopy

The sizes and the morphologies of $CHI_{10k}$-*g*-PEI/DNA and $CHI_{80k}$-*g*-PEI/DNA complexes are shown in the SEM images in Fig. 7. Most of complexes were spherical and compact, indicating that the CHI-*g*-PEI copolymer could interact with DNA through electrostatic attractions and condense them to form of nanoparticles. Besides, the hydrophilic exterior could reduce the interactions between the complexes. The size of the nanosphere was about 200 nm, which was in agreement with the particle size measured by Nano-ZS ZEN3600.

## 4. Conclusions

In this study, we have successfully prepared a new CHI-*g*-PEI copolymer through a biocleavable disulfide linkage and evaluated the physicochemical and biological characteristics in details. The resulting CHI-*g*-PEI/DNA complexes were shown to have stable sized particles, cationic surface charge and good DNA condensation capability. Favorable biocompatibility and improved safety could be expected for CHI-*g*-PEI due to the reduction-triggered degradation. Our results demonstrated that this novel CHI-*g*-PEI is a promising nonviral gene carrier and can be applied to the subsequent *in vitro and in vivo* measurements.

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