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Preparation of arginine modified PEI-conjugated chitosan copolymer for DNA delivery

Xi Zhang, Yajing Duan, Dongfang Wang, Fengling Bian *

Key Laboratory of Nonferrous Metal Chemistry and Resources Utilization of Gansu Province, College of Chemistry and Chemical Engineering, Lanzhou University, Lanzhou 730000, China

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ABSTRACT

Polyethylenimine-conjugated chitosan (CS-PEI) and arginine modified polyethylenimine-conjugated chitosan (CS-PEI-Arg) were prepared, and the copolymers were characterized by FTIR, ^{1}H NMR, and XRD. The properties of these copolymers like plasmid DNA (pDNA) binding capacity, complexes' size and zeta potential, cytotoxicity and transfection efficiency were also evaluated. The results show that CS-PEI-Arg derivatives can bind pDNA thoroughly, and form complexes with sizes about 170 nm. Cytotoxicity assay in HepG2 and 293 T cells show that CS-PEI-Arg has lower cytotoxicity compared with CS-PEI, which is similar to CS and far below that of PEI. In vitro luciferase assay show that CS-PEI-Arg has better transfection efficiency than CS-PEI, which is superior to that of PEI. The best transfection efficiency of CS-PEI-Arg ($N/P = 50$) is 2.3-fold, 4.2-fold of those of CS-PEI ($N/P = 20$) and PEI's ($N/P = 10$) efficiency respectively. These results display that CS-PEI-Arg is a promising candidate as an efficient gene vector.

1. Introduction

Chitosan has been a superior candidate for gene vectors due to its advantages like biocompatibility, biodegradability and low toxicity (Jayakumar et al., 2010; Liu & Yao, 2002; Mao, Sun, & Kissel, 2010; Muzzarelli, 2010). However, chitosan/DNA complexes have poor proton buffering capacity, which affect its escape from endosomes and further hinder intracellular gene expression. According to the literature (Tang & Szoka, 1997), the escaping ability of chitosan/DNA complexes is related to its buffering capacity in pH range of 5–7. Thus various molecules including polyethylenimine (PEI) were employed in order to improve chitosan's buffering capacity and enhance gene expression.

PEI is an efficient non-viral vector for gene delivery, which has high charge density, good DNA binding capacity and excellent ability to promote endosome escape through proton sponge effect (Godbey, Barry, Saggau, Wu, & Mikos, 2000; Godbey, Wu, & Mikos, 1990). Wong et al. (2006) reported the synthesis of PEI-g-chitosan by cationic polymerization of aziridine in the presence of chitosan. Jiang et al. (2007) synthesized chitosan-graft-polyethylenimine (CHI-g-PEI) copolymer by an imine reaction between periodate-oxidized chitosan and polyethylenimine. The above chitosan derivatives modified by low molecular weight PEI have good DNA binding capacity, lower cytotoxicity and higher

* Corresponding author. Tel.: +86 931 8912582; fax: +86 931 8912582.
E-mail address: bianfl@lzu.edu.cn (F. Bian).

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gene transfection than PEI (25 kDa). Therefore, it's a useful method to modify chitosan with PEI in order to prepare gene vectors with efficient gene transfection and low cytotoxicity.

Low molecular weight PEI modification has many advantages including low toxicity and eminent gene transfection. However, compared with PEI (25 kDa) which has excellent performance in gene delivery, PEI (800 Da) has lower DNA condensing capability due to its lower molecular weight and thus has inferior gene expression level (Baker et al., 1997; Kunath et al., 2003). It is reported that arginine residue is an effective component of cell penetrating peptides (Futaki et al., 2001; Richard et al., 2003) and arginine modification can raise the gene transfection level of chitosan and maintain low cytotoxicity (Gao et al., 2008; Ho et al., 2011).

Therefore, the present work aims at employing PEI to increase the solubility and proton buffering capacity of chitosan and further improve cell internalization and transfection performance by combining with arginine modification. Based on theses purposes, CS-PEI and CS-PEI-Arg copolymers were synthesized. CS-PEI/DNA and CS-PEI-Arg/DNA complexes were prepared and characterized in terms of DNA binding efficiency, particle size, zeta potential, cell viability and in vitro transfection efficiency.

2. Materials and methods

2.1. Materials

Chitosan (Mv = 32 kDa, deacetylation degree = 77.5%) was prepared according to our previous work (Zhang, Yao, Zhang, Fang, & Bian, 2014). Maleic anhydride was purchased from Tianjin Chemical Reagent Co., China, which was recrystallized by methylene dichloride before use. Branched polyethylenimines (PEI, Mw = 800 Da and Mw = 25 kDa), *N*-hydroxysuccinimide (NHS) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma–Aldrich. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl) was purchased from Energy Chemical and L-arginine chloride was obtained from Shanghai Chemical Reagent Co., China. Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were obtained from Invitrogen. All other chemicals were of analytical grade and were used as received.

2.2. Synthesis of maleated chitosan (CS-MA)

Maleated chitosan was prepared by a modified method of the literature (Lu et al., 2009). 1 g of chitosan (CS) and 1.5 g of maleic anhydride (MA) were dispersed in 100 mL of DMSO and the reaction mixture was continuously stirred at 60 °C for 8 h. The product was precipitated by 250 mL of acetone and then collected and washed by acetone and ether for three times. The final product was obtained by vacuum drying.

2.3. Synthesis of polyethylenimine-conjugated chitosan (CS-PEI)

0.2 g of CS-MA was dissolved in 20 mL of 0.25% NaOH solution. 5 mL of PEI solution (0.1 g/mL) was added and the mixture was stirred for 24 h at 60 °C. After that, the pH value of the reaction solution was adjusted to 7 by HCl (pH = 1.0). The product was dialyzed (MWCO = 3500) against distilled water for 3 days and lyophilized.

2.4. Synthesis of arginine modified polyethylenimine-conjugated chitosan (CS-PEI-Arg)

CS-PEI-Arg was prepared according to the literature (Morris & Sharma, 2010). Briefly, 0.13 g of Arg·HCl was dissolved in 10 mL of HCl solution (pH = 5.0), and then 0.35 g of NHS and 0.58 g of EDC·HCl were added into the solution. After stirring for 1 h, 5 mL of HCl solution (pH = 5.0) dissolving 0.1 g of CS-PEI was mixed with the above solution and stirred for another 24 h at the room temperature. The product was dialyzed (MWCO = 3500) against distilled water for 3 days and lyophilized.

2.5. Polymer characterization

The Fourier transform infrared (FTIR) spectra were carried out with a NEXUS670 FTIR spectroscopy (Nicolet, USA). The samples were mixed with KBr and KBr pallets were prepared for measurements.

The ^{1}H nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Bruker AV-400 NMR spectrometer at 400 MHz using DMSO-d_6 or D_2O as the solvents.

The X-ray diffraction spectra of the samples were performed by a X-ray scattering diffractometer (Shimadzu XRD-6000, Japan) with Cu Kα radiation (λ = 1.5444) in the range of 5–50° (2θ) at a voltage of 40 kV and a current of 40 mA.

The molecular weight of CS-PEI-Arg was measured by gel permeation chromatography (Agilent Technologies PL-GPC 50 Integrated GPC System) equipped with a Agilent PL aquagel-OH MIXED-H 8 μm Serial NO. 00067184-38 column using $KH_2PO_4/Na_2HPO_4{\cdot}12H_2O$ buffer (pH = 5.5) and 0.2 mol/L $NaNO_3$ as the eluent at a flow rate of 1 mL/min at 45 °C.

2.6. Buffer capacity

The buffer capacity of different polymers was determined by acid-base titration assay according to the literature (Benns, Mahato, & Kim, 2002). Briefly, 0.2 mg/mL of sample solutions were prepared in 30 mL of 150 mM NaCl solutions and then were titrated to pH = 10 by 0.1 M NaOH. After that, 0.1 M HCl solution with appropriate volume was added to the solution to obtained mixtures with different pH values. The data were measured using a pH meter.

2.7. Plasmid DNA preparation

The reporter plasmid encoding luciferase (pGL3-Control, Promega) was propagated in *Escherichia coli* DH5a and purified by the EndoFree Plasmid Kit (Qiagen, German). The purity of plasmid DNA (pDNA) was checked by electrophoresis on a 1% agarose gel, and the concentration of DNA was determined by measuring the UV absorbance of 260 nm. The purified pDNA was resuspended in Tris–EDTA buffer (pH = 8.0) and stored at −20 °C.

2.8. Preparation of CS-PEI/DNA and CS-PEI-Arg/DNA complexes

CS-PEI and CS-PEI-Arg samples were dissolved in PBS buffer (pH = 7.4) with a concentration of 1 mg/mL and then the solutions were filtered using 0.22 μm filters. The pDNA stock solutions (0.1 mg/mL) were prepared in Tris–HCl buffer (pH = 7.6). Complexes were prepared by adding polymer solutions to equal volumes of pDNA solution (containing 1 μg pDNA) at various *N*/*P* ratios and vortexed for 30 s. The resulting complexes were incubated for 30 min at room temperature before use.

2.9. Agarose gel retardation assay

CS-PEI and CS-PEI-Arg copolymers were examined for their abilities to bind pDNA through gel electrophoresis experiments. The CS-PEI/DNA and CS-PEI-Arg/DNA complexes with different *N*/*P* ratios ranging from 0.5 to 5.0 were prepared according to the procedure described above. Then 10 μL of complexes suspension was electrophoresed on 1.0% (w/v) agarose gel containing ethidium bromide (0.5 μg/mL) with 1 × TAE running buffer (40 mM Tris-acetate,