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Chitosan based oligoamine polymers: Synthesis, characterization, and gene delivery

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ABSTRACT

A series of chitosan-based oligoamine polymers was synthesized from *N*-maleated chitosan (NMC) via Michael addition with diethylenetriamine (DETA), triethylenetetramine (TETA), tetraethylenepentamine (TEPA) and linear polyethylenimine (M_n 423), respectively. The resulted polymers exhibited well binding ability to condense plasmid DNA to form complexes with size ranging from 200 to 600 nm when the polymer/DNA weight ratio was above 7. The polymer/DNA complexes observed by scanning electron microscopy (SEM) exhibited a compact and spherical morphology. The cytotoxicity assay showed that the synthesized polymers were less toxic than that of PEI(25 K). The gene transfection effect of resulted polymers was evaluated in 293T and HeLa cells, and the results showed that the gene transfection efficiency of these polymers was better than that of chitosan. Moreover, the transfection efficiency was dependent on the length of the oligoamine side chains and the molecular weight of the chitosan derivatives.

1. Introduction

The development of safe and efficient gene delivery vectors is a prerequisite for the success of gene therapy [1–3]. Non-viral gene vectors such as cationic liposomes and cationic polymers are being sought as alternatives for viral vectors since non-viral gene vectors have several advantages over viral counterparts, including low immune response, ease of production, the ability to transfer large DNA molecules and potential cell targeting property [3,4]. Among non-viral vectors, chitosan which is derived from natural sources has been considered as a safer alternative to other non-viral gene vector candidates since chitosan is known as a biocompatible, biodegradable, and low toxic material with high cationic potential [5]. Due to the D-glucosamine residue in chitosan having a p*K*a of around 6.2–7.0, the amines in chitosan could become positively charged at acidic pH below p*K*a. Therefore, these protonated amines enable chitosan to bind negatively charged DNA and condense it into particles. Besides, it was reported that chitosan has shown a promise to protect DNA from DNase I and II degradation and transfect DNA into different cell types [6]. To date, although many encouraging results have demonstrated chitosan as non-viral gene carriers [7–9], a main drawback is the low gene transfection efficiency for the chitosan-based gene delivery systems. It has been suggested that the low transfection efficiency was attributed to the strong interactions between chitosan and DNA, resulting in highly stable particles, thereby preventing dissociation within the cell and ultimately precluding translation of the DNA [10]. In addition, the poor water solubility of chitosan at physiological pH also has the negative influence on the transfection. To overcome these drawbacks, many chitosan derivatives have been developed in the last few years. For example, chemical modifications of chitosan using hydrophilic [11,12], hydrophobic [13,14] moieties were reported to enhance the water solubility of chitosan. To improve gene transfection efficiency, pH-sensitive [15], thermosensitive [16,17] and cell-specific [18–20] polymers or groups grafting with chitosan were also reported.

Polyethylenimine (PEI), a cationic polymer, has been demonstrated as an effective non-viral vector and widely used in gene transfection study [21–23]. It is widely accepted that the good transfection ability of PEI is due to its high buffer capacity over a broad pH, which is called "the proton sponge effect" [24,25]. Although PEI(25 K) has many advantages such as high condensation capability and strong buffer capacity [26,27], the high toxicity and lack of biodegradability of PEI(25 K) remain to be the drawbacks, especially preventing repeated administration in vivo gene delivery [28]. Further studies showed that the cytotoxicity of PEI is dependent on its molecular weight. A lower molecular weight PEI has a lower cytotoxicity but these lower molecular weight compounds suffer from lower transfection ability [27,29].

Therefore, it is attractive for the combination of multiple lower molecular weights PEI fragments and biocompatible polymers for gene vectors. Up to now, low molecular weight PEI grafted to dextran, chitosan, polyaspartamine or cyclodextrin as effective gene vector has been reported [30–33]. Our group also developed low molecular weight PEI grafted chitosan with higher transfection efficiency and lower cytotoxicity [34]. However, a clear relationship between structure and transfection efficiency was not elucidated. In this

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study, a series of chitosan based oligoamine polymers was synthesized and the effect of the oligoamine side chains on gene vectors was evaluated systematically.

2. Materials and methods

2.1. Materials

Diethylenetriamine (DETA), triethylenetetramine (TETA), tetraethylenepentamine (TEPA) were obtained from Shanghai Chemical Reagent Co., China. Chitosans (M_w = 5 K, 10 K, 50 K with deacetylation degree 85.5%, 85.3%, 85.3%, respectively) were purchased from Haidebei Marine Bioengineering Co. Ltd., Jinan, China. Linear polyethylenimine (M_n 423) and branched PEI(25 K) were provided by Aldrich-Sigma Chemical Co. Dimethyl sulphoxide (DMSO) obtained from Shanghai Chemical Reagent Co., China was refluxed with anhydrous $MgSO_4$ overnight and distilled before use. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), Dubelcco's phosphate buffered saline (PBS), penicillin-streptomycin and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) trypsin were purchased from Invitrogen Corp. The reporter plasmids, pEGFP-C1 and pGL3-Luc, were supplied by Invitrogen and Promega, respectively. The plasmid DNA was stored at −20 °C until the transfection experiments. All other reagents were of analytical grade and were used as received.

2.2. Synthesis of N-maleated chitosan (NMC)

NMC was synthesized according to our previous study [34]. In brief, 2 g of chitosan with molecular weight of 5 K, 10 K, 50 K respectively, was dissolved in 50 mL of 0.1 M acetic acid, and precipitated with 40 mL of 0.2 M NaOH, then collected by filtration, washed with water to pH 7. The swollen chitosan obtained from above was dispersed in 200 mL DMSO with stirring. DMSO solution containing 3.0 g of maleic anhydride was added into above solution, and the mixture was placed in a 60 °C oil bath for 8 h. The product was precipitated in 500 mL acetone, filtered, washed with acetone and ether three times, and then dried to obtain NMC.

NMC(5 K): ^{1}H NMR (D_2O, ppm) d: δ = 3.33–3.54 (multiplet, D-glucosamine unit, H-3, H-4, H-5, H-6, H-6′); δ = 5.68–5.72 and δ = 6.17–6.20 (–CH = CH–); δ = 1.80 ($COCH_3$). Grafting degree (GD): 65.6%.

NMC(10 K): ^{1}H NMR (D_2O, ppm) d: δ = 3.35–3.58 (multiplet, D-glucosamine unit, H-3, H-4, H-5, H-6, H-6′); δ = 5.72–5.76 and δ = 6.18–6.22 (–CH = CH–); δ = 1.82 ($COCH_3$). GD: 60.4%.

NMC(50 K): ^{1}H NMR (D_2O, ppm) d: δ = 3.35–3.58 (multiplet, D-glucosamine unit, H-3, H-4, H-5, H-6, H-6′); δ = 5.70–5.75 and δ = 6.18–6.22 (–CH = CH–); δ = 1.82 ($COCH_3$). GD: 60.0%.

2.3. Synthesis of NMC with oligoamine side chains

To graft oligoamine onto NMC, 0.2 g NMC was dissolved in 20 mL 0.25% sodium hydroxide solution. The aqueous solution with 3 g of oligoamine was added into the above solution. The mixture was stirred at 60 °C for 24 h. Then hydrochloric acid was added in the mixture with stirring until pH value reached 7.0. The product was dialysized (MWCO: 3.5 K) against distilled water for 3 days and then lyophilized for 3 days. The products are designated as NMC(X)-g-OEI (X refers to the molecular weight of raw chitosan, OEI refers to DETA, TETA, TEPA and PEI(423)).

NMC(5 K)-g-DETA: ^{1}H NMR (D_2O, ppm) d: δ = 3.28–3.60 (multiplet, D-glucosamine unit, H-3, H-4, H-5, H-6, H-6′); δ = 2.53–2.90 (–$NHCH_2CH_2$–); δ = 1.85 ($COCH_3$). GD: 41.3%.

NMC(10 K)-g-DETA: ^{1}H NMR (D_2O, ppm) d: δ = 3.30–3.61 (multiplet, D-glucosamine unit, H-3, H-4, H-5, H-6, H-6′); δ = 2.52–2.91 (–$NHCH_2CH_2$–); δ = 1.86 ($COCH_3$). GD: 40.7%.

NMC(50 K)-g-DETA: ^{1}H NMR (D_2O, ppm) d: δ = 3.32–3.6 (multiplet, D-glucosamine unit, H-3, H-4, H-5, H-6, H-6′); δ = 2.50–2.99 (–$NHCH_2CH_2$–); δ = 1.89 ($COCH_3$). GD: 39.3%.

NMC(5 K)-g-TETA: ^{1}H NMR (D_2O, ppm) d: δ = 3.29–3.60 (multiplet, D-glucosamine unit, H-3, H-4, H-5, H-6, H-6′); δ = 2.51–2.88 (–$NHCH_2CH_2$–); δ = 1.86 ($COCH_3$). GD: 39.7%.

NMC(10 K)-g-TETA: ^{1}H NMR (D_2O, ppm) d: δ = 3.31–3.61 (multiplet, D-glucosamine unit, H-3, H-4, H-5, H-6, H-6′); δ = 2.49–2.90 (–$NHCH_2CH_2$–); δ = 1.88 ($COCH_3$). GD: 38.5%.

NMC(50 K)-g-TETA: ^{1}H NMR (D_2O, ppm) d: δ = 3.30–3.62 (multiplet, D-glucosamine unit, H-3, H-4, H-5, H-6, H-6′); δ = 2.51–2.87 (–$NHCH_2CH_2$–); δ = 1.83 ($COCH_3$). GD: 36.5%.

NMC(5 K)-g-TEPA: ^{1}H NMR (D_2O, ppm) d: δ = 3.35–3.54 (multiplet, D-glucosamine unit, H-3, H-4, H-5, H-6, H-6′); δ = 2.50–2.87 (–$NHCH_2CH_2$–); δ = 1.85 ($COCH_3$). GD: 34.7%.

NMC(10 K)-g-TEPA: ^{1}H NMR (D_2O, ppm) d: δ = 3.40–3.63 (multiplet, D-glucosamine unit, H-3, H-4, H-5, H-6, H-6′); δ = 2.58–2.95 (–$NHCH_2CH_2$–); δ = 1.93 ($COCH_3$). GD: 35.0%.

NMC(50 K)-g-TEPA: ^{1}H NMR (D_2O, ppm) d: δ = 3.32–3.75 (multiplet, D-glucosamine unit, H-3, H-4, H-5, H-6, H-6′); δ = 2.53–2.89 (–$NHCH_2CH_2$–); δ = 1.82 ($COCH_3$). GD: 30.7%.

NMC(5 K)-g-PEI(423): ^{1}H NMR (D_2O, ppm) d: δ = 3.28–3.74 (multiplet, D-glucosamine unit, H-3, H-4, H-5, H-6, H-6′); δ = 2.56–2.85 (–$NHCH_2CH_2$–); δ = 1.79 ($COCH_3$). GD: 21.6%.

NMC(10 K)-g-PEI(423): ^{1}H NMR (D_2O, ppm) d: δ = 3.28–3.50 (multiplet, D-glucosamine unit, H-3, H-4, H-5, H-6, H-6′); δ = 2.57–2.86 (–$NHCH_2CH_2$–); δ = 1.79 ($COCH_3$). GD: 20.6%.

NMC(50 K)-g-PEI(423): ^{1}H NMR (D_2O, ppm) d: δ = 3.29–3.48 (multiplet, D-glucosamine unit, H-3, H-4, H-5, H-6, H-6′); δ = 2.47–2.82 (–$NHCH_2CH_2$–); δ = 1.82 ($COCH_3$). GD: 16.2%.

2.4. Buffer capacity

The buffer capacity of cationic copolymers was determined by acid-base titration assay as described by Benns et al. [35,36]. Briefly, 0.2 mg/mL of each sample solution was prepared in 30 mL 150 mM NaCl solution. The sample solution was firstly titrated by 0.1 M NaOH to a pH of 10, and then 0.1 M HCl solution with particular volume was added to the solution to obtained mixtures with different pH values which were determined using a microprocessor pH meter.

2.5. Preparation of NMC-g-OEI/DNA complexes

NMC-g-OEI was dissolved in NaCl solution (150 mM, which is mimicking physiological saline environment) with a concentration of 2 mg/mL and then filtered using a 0.22 μm filter. A plasmid DNA stock solution (120 ng/μL) was prepared in 40 mM Tris–HCl buffer solution. Nanoparticles were prepared by adding copolymer solution to equal volumes of DNA solution (containing 1 μg DNA) at various weight ratios with gentle vortexing and incubated at 37 °C for 30 min before use.

2.6. Cell viability assay

Evaluation of the cytotoxicity of polymers as well as polymer/DNA complexes was performed by MTT method [37]. Cells were seeded in the 96-well plate at a density of 6000 cells/well in 100 μL DMEM containing 10% FBS. After incubation for 24 h, polymer solutions (2 mg/mL) were added to the culture medium, while polymer/DNA