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Synthesis, characterisation and preliminary investigation of the haemocompatibility of polyethyleneimine-grafted carboxymethyl chitosan for gene delivery

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ABSTRACT

The development of safe and efficient gene carriers is the key to the clinical success of gene therapy. In the present study, carboxymethyl chitosan (CMCS) was prepared by chitosan (CS) alkalisation and carboxymethylation reactions. Then polyethyleneimine (PEI) was grafted to the backbone of CMCS by an amidation reaction. The CMCS–PEI copolymer showed strong complexation capability with DNA to form nanoparticles, and achieved lower cytotoxicity and higher transfection efficiency compared with PEI (25 kDa) towards 293T and 3T3 cells. Moreover, the haemocompatibility of the CMCS–PEI copolymer was investigated through the aggregation, morphology and lysis of human red blood cells (RBCs), along with the impact on the clotting function with activated partial thromboplastin time (APTT), prothrombin time (PT) and thromboelastographic (TEG) assays. The results demonstrated that the CMCS–PEI copolymer with a concentration lower than 0.05 mg/mL had little impact on the aggregation, morphology or lysis of RBCs, or on blood coagulation. Therefore, the copolymer may be a strong alternative candidate as an effective and safe non-viral vector.

1. Introduction

Gene therapy is a promising approach for the treatment of a wide range of diseases because it results in the production of bioactive agents or the cessation of abnormal functions in the cell [1]. Generally, negatively-charged DNA molecules, which exist in a loose form with a relatively large volume, are prone to bind to positively-charged groups, and are mutually exclusive with the negative charges on the surface of the cell membrane. Consequently, they resist circulation in the body. Additionally, blood and cells abound with hydrolases, especially DNA hydrolases that can destroy naked DNA [2]. Gene carriers are needed to effectively deliver genes, and the key to the clinical success of gene therapy lies in the development of safe and efficient gene carriers.

Non-viral gene vectors, especially cationic polymers, are of great interest because of their numerous advantages over viral vectors. These advantages include ease of production, low immune response, low cost, high flexibility for modification and the ability to transfer large DNA molecules [3,4]. Among the cationic polymers, polyethyleneimine (PEI) is one of the most effective non-viral gene carriers because of its high charge density and enhanced "proton sponge effect" in the endolysosome [5]. However, its poor biocompatibility and severe toxicity limit its clinical applications [6]. To develop PEI derivatives as gene carriers with low toxicity, researchers have focused on biocompatible and natural polymers, including polysaccharides such as chitosan (CS) [7], cyclodextrin [8] and dextran [9] or degradable synthetic polymers such as poly(ester amine) [10].

Chitosan is a natural cationic polymer with low cytotoxicity, and good biodegradability and biocompatibility [11]. These features make it perfect as a non-viral gene delivery vector. However, the solubility of chitosan in the physiological conditions of pH 7.4 is poor, and its transfection efficiency is not satisfactory [12]. To overcome these problems, researchers have developed chitosan derivatives such as pegylated chitosan [13], quaternised chitosan [14] and arginine-grafted chitosan [15].

For gene therapy purposes, the biopolymers often need to enter the human body by different routes [16]; the biopolymers or their degradation products then eventually gain entry into the blood circulation. Blood tissue is responsible for the transport of oxygen, nutrients and wastes, coagulation for haemostasis and immunoprotection. Once the biopolymers enter the blood circulation, they instantly become

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associated with numerous blood cells and abundant plasma proteins, which may alter cell membrane structures and protein conformations, perturb blood functions and eventually affect the whole organism [17]. Additionally, the aggregation caused by the interaction between the blood components and the gene carrier will lead to their quick clearance by the endothelial system. The interactions with blood can affect the *in vivo* pharmacokinetic behaviour of the polymer and its ability to leave the blood compartment and enter other tissues [18–20]. Therefore, the blood compatibility of the gene carrier is vitally important.

In this work, in order to obtain a gene carrier with high transfection efficiency and low toxicity, PEI–grafted CMCS (CMCS–PEI) was synthesised and characterised. Since cationic PEI can protect DNA from nuclease degradation and facilitate endosomal escape of polymer/DNA complexes [21–23], thus enhance the transfection efficiency of chitosan-based vectors. The *in vitro* toxicity and transfection efficiency of the copolymer were evaluated on 293T and 3T3 cells. To understand the *in vivo* safety and efficacy of the CMCS–PEI copolymer, the haemocompatibility of the copolymer was first investigated through the aggregation, morphology and lysis of human red blood cells (RBCs), along with the impact on the clotting function with activated partial thromboplastin time (APTT), prothrombin time (PT) and thromboelastographic (TEG) assays.

2. Materials and methods

2.1. Materials

Chitosan (CS, medium molecular weight (Mw), deacetylation degree 75–85%), branched polyethyleneimine (PEI, Mw = 1.8 kDa and 25 kDa), sperm DNA and ethidium bromide (EB, 95%) were purchased from Sigma–Aldrich (Shanghai, China). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were purchased from Aladdin Reagents (Shanghai, China). The Cell Counting Kit 8 (CCK–8) was purchased from Beyotime Biotechnology (Shanghai, China). Chloroacetic acid was supplied by Xiya Chemical (Shandong, China). The encoding green fluorescent protein pEGFP–N1 (GFP) was purchased from Clontech (Mountain View, CA, USA). Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin were obtained from Life Technologies (Shanghai, China). Foetal bovine serum (FBS) was obtained from Sijiqing Biologic Engineering Materials (Hangzhou, China). Blood from healthy consenting volunteers was collected in sodium citrate tubes with a blood: anticoagulant ratio of 9:1. Reagents for the APTT and PT conventional coagulation assays were provided by the First Affiliated Hospital of Jinan University. All other reagents used were of analytical grade.

2.2. Synthesis of the CMCS–PEI copolymer

Carboxymethyl chitosan (CMCS) was prepared by two-step alkalisation and carboxymethylation reactions based on the methods described by Sun et al. [24], but with some improvements. Briefly, chitosan (2.5 g) was suspended in isopropyl alcohol (30 mL) for 1 h; 40% aqueous NaOH solution (10 mL) was then added, and the mixture solution was stirred at 30 °C for 12 h. Then, monochloroacetic acid (12 g) in isopropyl alcohol (20 mL) was added to the alkali-treated chitosan and reacted at 65 °C for 3 h. Again, 40% aqueous NaOH solution (10 mL) was added and stirred for another 3 h. The product was dissolved in water and the remaining precipitate was removed by centrifugation. Excess anhydrous ethanol was added to the solution to precipitate the CMCS, which was redissolved in distilled water and reprecipitated twice more and then dried under vacuum.

The CMCS–PEI copolymer was prepared according to the methods described by Sun et al. [24]. In brief, CMCS (220 mg) was dissolved in 30 mL of distilled water, then branched PEI 1800 (3.0 g) was added. After the solution was stirred at room temperature for 30 min, 10 mL of an aqueous solution containing 192 mg of EDC and 115 mg of NHS were added dropwise to the mixture at pH 5.5. The solution was kept stirring at room temperature for 24 h. Subsequently, the solution was dialysed against deionised water and lyophilised.

2.3. Characterisation of the CMCS–PEI copolymer

Fourier transform infrared (FT-IR) analysis was carried out using the attenuated total reflectance (ATR) technique with a diamond crystal plate (Bruker Optics, Ettlingen, Germany). The spectra were recorded in the absorbance mode from 500 to 4000 cm^{-1}.

The ^1H nuclear magnetic resonance (^1H NMR) spectra were determined on an AV 3000 Supercon spectrometer (Bruker). The chitosan spectrum was measured with CD_3COOD/D_2O as the solvent, and those of CMCS and CMCS–PEI were measured with D_2O as the solvent.

2.4. Preparation of the CMCS–PEI/pDNA complexes

All CMCS–PEI/pDNA nanoparticles were freshly prepared before use. CMCS–PEI was dissolved in distilled water to a concentration of 1 mg/mL. The solution was filtered through 0.45-μm filter paper. CMCS–PEI/pDNA complexes at different weight ratios were formulated by adding different volumes of the CMCS–PEI solution to an equal volume of the pDNA solution, vortexed for 30 s and then incubated for 30 min at room temperature.

2.5. Characterisation of the CMCS–PEI/pDNA complexes

2.5.1. Agarose gel retardation assay

The binding ability of the CMCS–PEI copolymer to pDNA was examined by the gel electrophoresis assay. The complexes containing 1 μg of pDNA at various weight ratios were prepared as described earlier. Then, the complexes were loaded into individual wells of 1% agarose gel in TAE buffer (40 mmol/L Trisacetate, 1 mmol/L EDTA) with ethidium bromide as a DNA visualiser. The gel electrophoresis was carried out at 70 V for 30 min. Visualisation and image capture were accomplished using a UV-transilluminator under a Kodak EDAS 290 digital imaging suite (Kodak, Tokyo, Japan).

2.5.2. Transmission electron microscopy (TEM)

The morphological examination of the complex was performed by high-resolution TEM (JEM–2010HR, JEOL, Tokyo, Japan). CMCS–PEI/pDNA complexes containing 1 μg of pDNA were prepared at a weight ratio of 25, and diluted to 1 mL by distilled water. A drop of the complex solution was deposited on a carbon-coated grid and dried at 37 °C.

2.5.3. Particle sizes and zeta potentials

The particle sizes and zeta potentials of the complexes were determined with a Mastersizer 2000 laser diffractometer (Malvern Instruments, Worcestershire, UK). Prior to the measurements, the CMCS–PEI/pDNA complexes were prepared by adding the appropriate volume of the copolymer solution to 1 μg of pDNA at weight ratios ranging from 1 to 30. The solutions containing the complexes were then diluted to 1 mL by distilled water.

2.6. In vitro cytotoxicity assay

The 3T3 and 293T cell lines were maintained in DMEM medium supplemented with 10% FBS and 1% antibiotics (penicillin–streptomycin, 10,000 U/mL) at 37 °C in a humidified atmosphere containing 5% CO_2.

The cytotoxicity of the CMCS–PEI copolymer was determined on 3T3 and 293T cells by CCK–8 assay. The 3T3 cells were seeded into 96-well culture plates at a density of 1×10^4 cells per well. After 24 h of incubation, the cells were treated with the CMCS–PEI copolymer and PEI, respectively, with final concentrations of 10, 20, 40, 80, 160 and 320 μg/mL for 24 h (100 μL/well). Untreated cells in growth medium