Carbohydrate Polymers 97 (2013) 676–683

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Synthesis and preliminary cellular evaluation of phosphonium chitosan derivatives as novel non-viral vector

Changyun Qian [1], Xiaofen Xu [1], Yunayuan Shen [1], Yanggong Li, Shengrong Guo *

School of Pharmacy, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai 200240, China

ARTICLE INFO

Article history:
Received 26 February 2013
Received in revised form 9 May 2013
Accepted 16 May 2013
Available online 24 May 2013

Keywords:
Chitosan
Phosphonium
Transfection
Gene delivery
Complex

ABSTRACT

In this study, *N*-phosphonium chitosans (NPCSs) with two degrees of substitution were synthesized in a homogeneous system as nonviral gene vectors. Grafted polymer/DNA complexes at various charge ratios were formulated and characterized. Particle sizes of NPCS/DNA complexes were between 110 and 160 nm as determined by dynamic light scattering. Accordingly, scanning electron microscopy photo of NPCS/DNA complexes exhibited a compact morphology. Zeta potentials of these complexes changed as the charge ratio and pH varied. The cytotoxicity assay showed that NPCS polymers were less toxic than branched PEI-25K. Furthermore, gene transfection efficiencies of NPCS/DNA complexes showed that the gene transfection ability of the grafted polymer was much better than chitosan and NPCS with the degree of substitution of 21.5% had comparative gene transfection efficiency to branched PEI-25K. Together, these results suggest that the low toxic NPCS grafted polymers could be used as effective gene delivery vectors.

1. Introduction

Gene therapy is a promising way for curing various genetic diseases (De Luca, Pellegrini, & Mavilio, 2009; Kohn & Candotti, 2009) as well as cancers (Brannon-Peppas & Blanchette, 2012; Gomez-Navarro, Curiel, & Douglas, 1999; Kochanek & Gansbacher, 2010) by delivering genes to specific cells or organs. Successful clinical application of gene therapy requires safe and high efficient gene delivery vectors (Midoux, Pichon, Yaouanc, & Jaffrès, 2009; Sun & Zhang, 2010). Non-viral gene vectors have gained increasing attention as alternatives to viral vectors, since they offer advantages of minimal immune response, stable in storage and ease of large-scale production (Park, Jeong, & Kim, 2006; Srinivas, Samanta, & Chaudhuri, 2009). Among numerous non-viral systems, cationic polymers are widely accepted because of their high ability to form polyelectrolyte complexes with plasmid DNA through charge interaction and self-assembling (Pack, Hoffman, Pun, & Stayton, 2005). To date, cationic gene delivery polymers, including poly(ethylenimine) (PEI) (Zhou et al., 2011), poly(2-dimethylaminoethyl methacrylate) (PDMAEMA) (Mathew, Cao, Collin, Wang, & Pandit, 2012), gelatin (Zorzi, Párraga, Seijo, & Sánchez, 2011) and poly(L-lysine) (PLL) (Zhang, Ma, Su, & Benkirane-Jessel, 2011), have been broadly studied. However, their high cytotoxicity and low biodegradability lead to a risk that these polymers might be accumulated in cells or body, particularly, after repeated administration (Gao, Kim, & Liu, 2007).

Chitosan, a natural linear cationic polysaccharide derived from chitin, is considered as a good candidate for gene delivery vector due to its biocompatibility, biodegradability, low toxicity and high potential ability to complexate DNA (Chopra et al., 2006; Di Martino, Sittinger, & Risbud, 2005). However, in spite of these excellent characteristics, the poor water-solubility and low gene transfection efficiency of chitosan limited its application. It has been suggested that the low transfection efficiency was attributed to the strong interactions between chitosan and DNA (Köping-Höggård et al., 2001; Ross, 2001), resulting in highly stable particles, thereby preventing dissociation within the cell and ultimately precluding translation of the DNA. To improve gene transfection efficiency, a number of chemically modified chitosan derivatives were synthesized in order to obtain desirable physicochemical characteristics (Kievit et al., 2009; Strand et al., 2010; Wang, He, Tang, & Yin, 2011). Although the results showed that the transfection efficiency of the modified chitosan based vectors exhibited superior properties to that of chitosan, further investigations on improved gene transfection efficiency and the structure–behavior relations are still required for the practical application.

In this study, quaternary phosphonium chitosan derivatives with two different *DS* were synthesized and characterized using ^{1}H NMR and FT-IR. A series of polymer/DNA complexes with different charge ratios were prepared and their physicochemical properties such as particle sizes, zeta potentials and plasmid DNA-binding abilities were investigated. Morphology of the lyophilized complex was investigated with SEM. And cytotoxicity of polymers was

* Corresponding author. Tel.: +86 21 3420 4793; fax: +86 21 3420 4793.
E-mail address: srguo@sjtu.edu.cn (S. Guo).
[1] Equal contributors.

0144-8617/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2013.05.036

evaluated using MTT assay. A number of variables influencing in vitro gene transfection efficiency such as polymer/DNA charge ratio, degree of substituted phosphonium, pH of culture medium were also investigated.

2. Materials and methods

2.1. Materials

Chitosan with a viscosity-average molecular weight of 150 kDa and 93% degree of deacetylation (DD) was purchased from Zhejiang Yuhuan Ocean Biochemistry Co., Ltd. (5-Carboxypentyl) triphenylphosphonium bromide (CTPB) was purchased from Gracia Chemical Technology Co., Ltd. (Chengdu, China) with a purity of 98%. 1-Ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride (EDC·HCl) and 1-hydroxybenzotrizole (HOBt) were purchased from Shanghai Medpep Co., Ltd. Fetal bovine serum (FBS), penicillin and streptomycin were purchased from Beijing Solarbio Science & Technology Co., Ltd. DMEM was obtained from GIBCO (Grand Island, NY, USA). Other chemicals used were of analytical grade. All chemicals were used without further purification. The pEGFP-N1 plasmid DNAs were a kind gift from Professor Tuo Jin, and then were extracted from *E. coli* and purified with a NucleoBond® Xtra Maxi kit.

2.2. Methods

2.2.1. Synthesis and characterization of N-phosphonium chitosan (NPCS)

NPCS was synthesized according to our previous study (Wang, Xu, Guo, Peng, & Tang, 2011) with a little modification. Briefly, chitosan (0.4 g, 2.44 mmol of glycosyl units) was stirred with two equivalents of HOBt (0.664 g, 4.88 mmol) in 30 mL of H_2O/DMSO (v/v = 2/1) mixture overnight at 15 °C. A certain amount of CTPB in H_2O/DMSO (v/v = 2/1) was added to the chitosan solution followed by the addition of EDC·HCl in DMSO (0.705 g, 4.88 mmol). The reaction was carried out at 15 for 24 h. The mixture was precipitated in diethyl ether/acetone (v/v = 1/2), followed by dialyzing (MWCO = 3500) and lyophilizing to obtain the product NPCS. The degree of substitution (*DS*) of CTPB to monosaccharide residue of chitosan was calculated based on ^{1}H NMR spectra, which was obtained using a MERCURY plus 400 spectrometer. Fourier-transformed infrared (FT-IR) spectra of chitosan and NPCS were recorded on EOUINOX55 spectrophotometer (Bruker, Germany). Samples were prepared as KBr pellet and were scanned against a blank KBr pellet background at wavelength range of 4000–400 cm^{-1} with resolution of 4.0 cm^{-1}.

2.2.2. Preparation of polymer/DNA complexes

Chitosan/DNA, NPCS/DNA complexes were prepared at various charge ratios (N/P), which were expressed as the molar ratio of the amine groups in chitosan or its derivatives to the phosphate groups in DNA molecules. First, DNA solution of 20 μg/mL was prepared in deionized water. NPCS was dissolved in 15 mM sodium acetate buffer (pH 5.5). Chitosan was first dissolved in 0.5% acetic acid, and then adjusted to pH 5.5 with NaOH. Equal volumes of DNA and polymer solutions were mixed with the final N/P from 1/1 to 16/1. The mixtures were vortexed for 30 s then incubated at 37 °C for 60 min prior to further analyses.

2.2.3. Complex particle size and zeta potential measurements

The hydrodynamic diameters of complexes were measured by dynamic laser scattering (DLS), using a Malvern Zetasizer Nano-S with scattering angle of 173° at 25 °C. The zeta potential of complexes were measured by Particle Size Analyzer (90 Plus Model, Brookhaven Instruments) at 25 °C. All the analyses were run in triplicate.

2.2.4. Morphology study

The polymer/DNA complexes were prepared according to the conditions described above. 100 μL of complex suspension was deposited onto a silicon slide. The lyophilized samples were coated with gold for 1 min before observing the morphology of complexes with a scanning electron microscope (SEM, JEOL, Tokyo, Japan).

2.2.5. Gel electrophoresis assay

20 μL polymer/DNA complexes were prepared as described above before the adding of loading buffer. Then, the samples were applied to a 1% agarose gel in TAE buffer (40 mM Tris/HCl, 1 mM EDTA, pH 7.4) containing 0.6 μg/mL of ethidium bromide at 90 V for 25 min. DNA retardation was observed by irradiation with UV light (Tanon 2500, Tanon Science and Technology Co., Ltd., Shanghai, China).

2.2.6. MTT assay for polymer cytotoxicity

HEK 293 and HeLa cells were seeded in the 96-well plate at a density of 8000 cell/well and grown in a humidified atmosphere of 5% CO_2 at 37 °C for 24 h. The growth medium was replaced with 200 L complete DMEM culture medium that contained desired amount of the test polymers. Incubations were made for 4 h before removal of media containing polymers and then MTT solution (0.5 mg/mL final concentration) in serum-free DMEM medium was added to each well and incubated for 4 h under normal growing conditions. Afterwards, the medium was removed and 200 L DMSO was added. The plate was mildly shaken for 10 min to ensure the complete dissolution of formazan. The absorbance was measured at 570 nm using an ELISA plate reader (Varioskan Flash). The relative cell viability was calculated as: cell viability (%) = $(OD_{sample}/OD_{control}) \times 100$, where untreated cells were considered as control. Each value was averaged from 4 independent experiments.

2.2.7. In vitro gene transfection

HEK293 and HeLa cells were seeded in 24-well plates at a density of 5×10^4 cells/well in 1 mL of complete medium and incubated for 24 h at 37 °C in 5% CO_2. When the cells were grown to the 70–80% confluence, the culture medium was replaced with 1 mL medium containing 200 μL of complexes (different N/P ratios of polymer/DNA complexes). EGFP-N1 was used as the reporter plasmid to assay the transfection efficiency. After incubation for 4 h, the medium was replaced with fresh complete medium and the cells were incubated for another 48–72 h (post-transfection time). The cells were then analyzed for green fluorescence protein expression with a fluorescence microscope (Ti-U, Nikon, Japan). Finally, cells were collected and resuspended in PBS (pH 7.4). The EGFP expression levels were quantified by using the flow cytometer (FACSCalibur, BD, USA) in the fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm and analyzed with CellQuest software. A total of 10,000 events were collected for each sample.

3. Results and discussion

3.1. Synthesis and characterization of NPCS

Chitosan is an important biodegradable material for a variety of biological applications, but the poor water-solubility and low gene transfection efficiency of chitosan limited its application. To improve the transfection efficiency of chitosan, we established a procedure to synthesize phosphonium grafted chitosan polymers under homogeneous condition. By varying the feed ratio of CTPB