Carbohydrate Polymers 103 (2014) 566–572

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Synthesis and characterization of PEG-conjugated quaternized chitosan and its application as a gene vector

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ARTICLE INFO

Article history:
Received 21 September 2013
Received in revised form
23 December 2013
Accepted 25 December 2013
Available online 3 January 2014

Keywords:
Chitosan
Quaternization
PEGylation
Nanocomplexes
Gene vector

ABSTRACT

Poly(ethylene glycol)-conjugated *N*-(2-hydroxy) propyl-3-trimethyl ammonium chitosan chloride (PHTAC) derivatives were prepared by incorporating PEG molecules onto quaternized chitosan backbone. The copolymers were characterized by FTIR, ^{1}H NMR and XRD. Agarose gel retardation assay indicated that PHTAC had good plasmid DNA (pDNA) binding capability and the particle sizes of PHTAC/pDNA complexes determined by DLS were about 200 nm. Cytotoxicity assays in HeLa and 293T cells showed that PHTAC had low cytotoxicity. In vitro luciferase assay showed that PHTAC with PEGylation degree of 9% (PHTAC-1) had good transfection efficiency about 5.3-fold higher than quaternized chitosan, which was comparable with PEI (25 kDa). These results suggest that PHTAC-1 is a promising candidate as an efficient nonviral gene vector.

1. Introduction

Gene therapy has developed rapidly in recent years (Green, Langer, & Anderson, 2008; Verma & Somia, 1997). As the prerequisite of successful gene transfer, a suitable gene vector needs to be safe and efficient. Among various vectors, cationic polymers possess capabilities to form compact complexes with DNA and induce efficient cell uptake and endosomal escape, thus have been an intriguing interest for researchers (Guo & Huang, 2012; Zhang, Zhao, Zhao, & Wang, 2010).

Chitosan is a natural polysaccharide possessing biocompatibility, biodegradability and low toxicity, and has been employed in gene delivery (Merkel, Zheng, Debus, & Kissel, 2012; Muzzarelli, 2010a). However, the poor solubility in physiological pH significantly limits its further application as an effective gene carrier. Quaternization, one of the common strategies to improve the performance of chitosan, can improve DNA binding capability of chitosan. In addition, cellular uptake capacity can be facilitated through the electrostatic affinity between quaternized chitosan and cell membranes, which is helpful to better transfection efficiency (Mao et al., 2007; Thanou, Florea, Geldof, Junginger, & Borchard, 2002). Xiao et al. (2012) reported that *N*-(2-hydroxy) propyl-3-trimethyl ammonium chitosan chloride was more efficient in transfection efficiency than chitosan in HeLa cells, ranging from 12.6-fold to 584.2-fold. Faizuloev et al. (2012) discovered that quaternized chitosan with degree of quaternization 90 ± 3% was 4-fold more efficient than PEI (25 kDa) in gene transfection efficiency. Xiao et al. (2013) found that in HepG2 cells galactosylation of quaternized chitosan yielded significant higher transfection efficiency than galactosylated chitosan.

However, higher quaternization degrees tend to have apparent cytotoxicity and excessive DNA condensing capacity, which deter DNA dissociation and limit the gene expression level (Faizuloev et al., 2012; Kean, Roth, & Thanou, 2005; Xiao et al., 2012). Thus further effort is still needed to improve the performance of quaternized chitosan.

PEG has unique physical characteristics and biological properties, which leads it a valid element employing in gene delivery (Pasut & Veronese, 2012). PEG modification can alter the physical properties of polymer/pDNA complexes and reduce the cytotoxicity of polymers. Besides, PEGylation is deemed to enhance colloidal stability and maintain polyplex sizes under different conditions, potentially resulting in increased cell uptake efficiency in vitro (Germershaus, Mao, Sitterberg, Bakowsky, & Kissel, 2008).

Therefore, the current investigation aims at combining the advantages of quaternization and PEGylation of chitosan while minimizing the short-comings. Based on theses purposes, three PHTAC copolymers with different PEGylation degrees were

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http://dx.doi.org/10.1016/j.carbpol.2013.12.072

synthesized. PHTAC/pDNA complexes were prepared and characterized in terms of DNA condensation efficiency, particle size, zeta potential, cell viability and in vitro transfection efficiency.

2. Materials and methods

2.1. Materials

Higher molecular weight chitosan was purchased from Golden shell Biochemical Co. Ltd. (Zhejiang, China). Polyethylenimine (PEI; branched, M_w = 25 kDa), *N*-hydroxysuccinimide (NHS), *N*,*N*′-dicyclohexylcarbodiimide (DCC) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Aldrich. Poly(ethylene glycol) monomethylether (mPEG; M_w = 1.9 kDa) was purchased from Alfa Aesar. Glycidyl trimethylammonium chloride (GTA) was obtained from Dongying Guofeng Fine Chemical Co. Ltd. (Shandong, 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 unless otherwise specified.

2.2. Depolymerization of chitosan

Chitosan was depolymerized according to the literature (Mao et al., 2004; Muzzarelli, 2009, 2010b). The degree of deacetylation was measured by potentiometric titration as 85% and the molecular weight was determined by the intrinsic viscosity method using 2% HAc/0.2 M NaAc as solvent system. Data were obtained at 25 ± 0.05 °C in triplicate. The viscosity–average molecular weight (M_w) of chitosan was calculated as 120 kDa using Mark–Houwink equation:

$$[\eta] = KM_w{}^{\alpha}$$

where $[\eta]$ is the intrinsic viscosity, K and α are constants for given solute–solvent system and temperature, which are determined as 1.38×10^{-5} and 0.85 respectively by the literature (Kasaai, Arul, & Charlet, 2000).

2.3. Synthesis of N-(2-hydroxy) propyl-3-trimethyl ammonium chitosan chloride (HTAC)

HTAC was prepared by a modified method of Lim and Hudson (2004). 1 g of chitosan was dispersed in 20 mL of deionized water. After stirring at 85 °C for 30 min, 1.8 mL of GTA solution (1 g/mL) was added dropwise into the mixture. The reaction mixture was further stirred for 10 h at the same temperature. After using acetone to precipitate the product, the light yellow product was collected and washed by acetone several times. Then the collected product was dissolved in distilled water and dialyzed (MWCO = 3500) against distilled water for 5 days and finally lyophilized.

2.4. Synthesis of N-hydroxysuccinimide terminated poly(ethylene glycol) (NHS-mPEG)

NHS-mPEG was prepared according to the literature (Mao et al., 2005). Briefly, 2.5 g (1.3 mmol) of pre-dried mPEG was dissolved in 20 mL of anhydrous toluene, and 0.64 g of maleic anhydride (6.5 mmol) was added under argon protection. The mixture was stirred for 48 h at 70 °C under argon atmosphere. Then toluene and excess maleic anhydride were removed by distillation and sublimation under vacuum. After that, the above intermediate (0.54 mmol) and NHS (2.7 mmol) were dissolved together in 20 mL of anhydrous dichloromethane. Then the flask was cooled by ice-water bath and DCC (0.54 mmol) was added under protection of argon. After sealing the flask, the reaction mixture was stirred for 1 h in ice-water bath, and further 24 h at room temperature. The 1,3-dicyclohexylurea precipitation was eliminated by filtration. The filtrate was then added to 50 mL of diethyl ether and cooled for 2 h at 4 °C. The precipitated product was purified by redissolving in dichloromethane and reprecipitating with diethyl ether for three times. Finally, the product obtained by vacuum drying was stored under argon protection at room temperature.

Table 1
Properties of PHTAC copolymers.

Samples	NHS-mPEG/HTAC (w/w)	DQ[a] (%)	DP[b] (%)
PHTAC-1	2.5:1	41	9
PHTAC-2	5.0:1	41	13
PHTAC-3	7.5:1	41	27

[a] Number of quaternary ammonium groups per glucosamine unit, determined by ^{1}H NMR data.
[b] Number of PEG chains per glucosamine unit, determined by ^{1}H NMR data.

2.5. Synthesis of poly(ethylene glycol)-conjugated HTAC (PHTAC)

HTAC was dissolved in deionized water (10 mg/mL) and NHS-mPEG was dissolved in anhydrous DMSO (50 mg/mL). Then appropriate volumes of the above two solutions were mixed and stirred for 24 h at room temperature. As shown in Table 1, the weight ratios of HTAC to NHS-mPEG were varied to obtain three samples with different degrees of PEGylation (DP). After stirring for 24 h, the solution was dialyzed (MWCO = 14,000) against distilled water for 5 days and lyophilized.

2.6. Characterization of polymers

Fourier transform infrared (FTIR) spectra were carried out with 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 determined on a Bruker AV-400 NMR spectrometer at 400 MHz using D_2O or $CDCl_3$ as solvents.

X-ray diffraction spectra of the samples in the powder form 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.

2.7. Plasmid DNA preparation

The reporter plasmid encoding luciferase (pGL3-Control, Promega) was propagated in DH5-α *Escherichia coli* 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) and stored at −20 °C.

2.8. Preparation of PHTAC/DNA complexes

PHTAC samples were dissolved in PBS buffer (pH 7.4) with a concentration of 1 mg/mL and then the solutions were filtered using a 0.22 μm filter. The pDNA stock solutions (0.1 mg/mL) were prepared in Tris–HCl buffer. 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.