Carbohydrate Polymers 157 (2017) 311–320

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Chitosan-based DNA delivery vector targeted to gonadotropin-releasing hormone (GnRH) receptor

ARTICLE INFO

Keywords:
Gonadotropin releasing hormone (GnRH)
Chitosan
Gene delivery

ABSTRACT

The main purpose of this study was to investigate the application of modified chitosan as a potential vector for gene delivery to gonadotropin-releasing hormone receptor (GnRHR)-expressing cells. Such design of gene carrier could be useful in particular for gene therapy for cancers related to the reproductive system, gene disorders of sexual development, and contraception and fertility control. In this study, a decapeptide GnRH was successfully conjugated to chitosan (CS) as confirmed by proton nuclear magnetic resonance spectroscopy (^{1}H NMR) and Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR). The synthesized GnRH-conjugated chitosan (GnRH-CS) was able to condense DNA to form positively charged nanoparticles and specifically deliver plasmid DNA to targeted cells in both two-dimensional (2D) and three-dimensional (3D) cell cultures systems. Importantly, GnRH-CS exhibited higher transfection activity compared to unmodified CS. In conclusion, GnRH-conjugated chitosan can be a promising carrier for targeted DNA delivery to GnRHR-expressing cells.

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1. Introduction

The direct use of nucleic acid as a therapeutic agent to treat gene-associated diseases (so-called gene therapy) by transferring exogenous nucleic acids into the appropriate cells has attracted great interest over the past few decades (Badiga et al., 2011). In order to make great progress in gene therapy, it is of paramount importance to develop effective carriers capable of delivering a gene of interest to target cells to achieve sufficient and sustained transgene expression. Viral vectors have received considerable attention and become powerful tools of gene transfer. Unfortunately, the clinical application of viral vectors is limited because of unfavorable immunological features and mutagenic integration for some viruses. These concerns have driven extensive attention to the development of non-viral vectors (Morille, Passirani, Vonarbourg, Clavreul, & Benoit, 2008).

Chitosan (CS) is a linear polysaccharide derived from the deacetylation of chitin found in the exoskeletons of crustaceans. Among non-viral vectors, chitosan has been exploited as gene-delivery vectors due to its excellent biocompatibility and biodegradability (Saranya, Moorthi, Saravanan, Devi, & Selvamurugan, 2011). However, the effectiveness of this system is hindered by its low transfection efficacy and low cell specificity (Prabaharan & Mano, 2005). To overcome this limitation, ligands with high affinity and specificity, such as transferrin-, folate-, mannose-, and galactose-conjugated chitosan, have been integrated into gene delivery vector for receptor-mediated endocytosis (Jiang et al., 2007).

Gonadotropin-releasing hormone (GnRH) is a 10 amino acid peptide produced and secreted by the hypothalamus. GnRH binds to its receptor (GnRHR) on gonadotrope cells in the anterior pituitary, stimulating the biosynthesis and release of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), affecting sex development and reproductive functions (Millar, 2005). In addition to gonadotrope cells, GnRHR was found to be expressed in normal reproductive tissues (e.g. breast, endometrium, ovary, and prostate) (Cheung & Wong, 2008). Interestingly, GnRHR are overexpressed in cancer tissues, either related (i.e. prostate, breast, endometrial, and ovarian cancers) or unrelated (i.e. melanoma, glioblastoma, lung, and pancreatic cancers) to the reproductive system. Therefore, it is possible that targeted gene delivery to GnRHR-expressing cells could be a valuable tool for 1) the treatment of certain gene disorders affecting sexual development and reproductive function (Ono & Harley, 2013), 2) gene therapy of cancer associated with reproductive system (i.e. prostate, breast, endometrial, and ovarian cancers) (Limonta et al., 2012), and 3) contraception and fertility control (Dissen, Lomniczi, Boudreau, Chen, Davidson, & Ojeda, 2012).

The main overall aim of this study is to investigate the application of modified chitosan as potential vectors for gene delivery to GnRHR-expressing mammalian cells. Throughout this paper, the abbreviation GnRH-CS will be used to refer to GnRH-conjugated chitosan. In this study, we prepared GnRH-CS. The physiochemical properties of the synthesized GnRH-CS as well as the complex of GnRH-CS and plasmid DNA (pDNA) carrying a gene of interest (GnRH-CS/pDNA complexes) were analyzed, and their cytotoxicity and cell specificity were also characterized. Transfection efficiency was investigated in established mammalian cell line model system expressing GnRHR. We hypothesized that GnRH-CS polymers are capable of directing pDNA into GnRHR-expressing cells via receptor-mediated pathways and exhibit higher transfection activity compared to unmodified CS. A schematic diagram of the

http://dx.doi.org/10.1016/j.carbpol.2016.09.015
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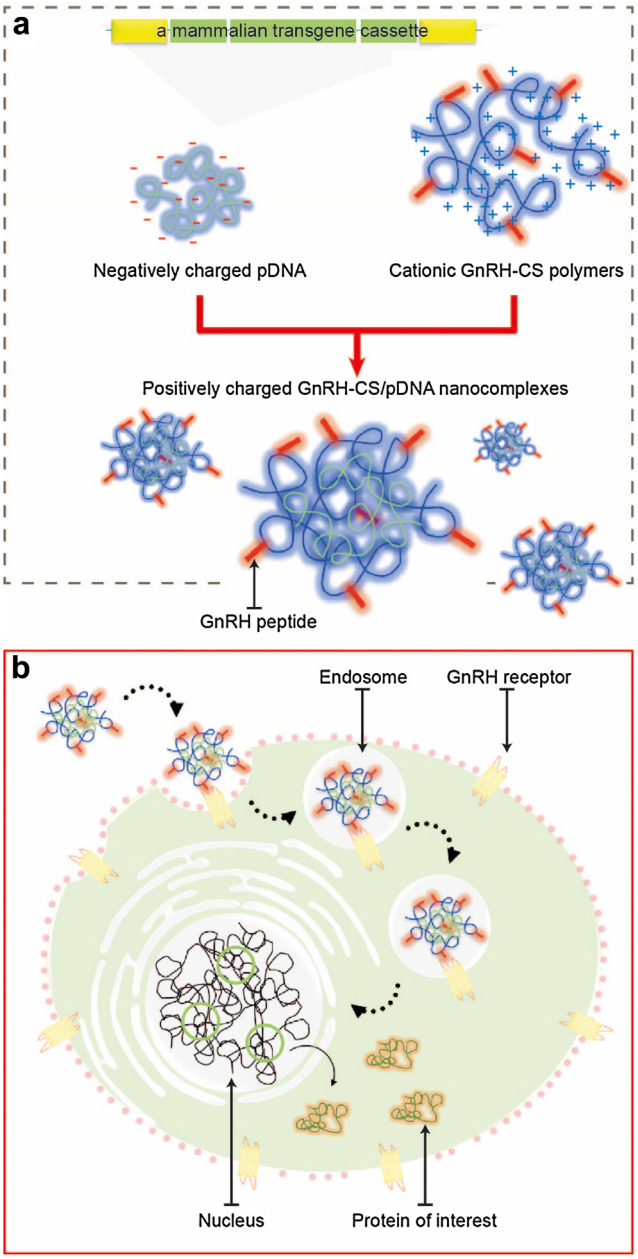

Fig. 1. Schematic illustration showing the formation of GnRH-CS/pDNA complex and its delivery procedure. a) pDNA condensation in the presence of cationic GnRH-CS polymer by electrostatic interactions. b) The proposed mechanism of gene delivery process by GnRH-CS/pDNA complex. GnRH peptides displayed GnRH/pDNA complexes facilitate active targeting of GnRHR-expressing cells via specific interaction between the GnRH motif and the GnRH receptor. Following internalization, the pDNA must be released and transported to the nucleus where gene expression occurs.

GnRH-CS/pDNA complex and its proposed mechanism of trafficking were shown in Fig. 1.

2. Materials and method

Gonadotropin-releasing hormone (GnRH) was purchased from Sigma. Chitosan (CS) was purchased from OilZac Technologies Co., Ltd. The degree of deacetylation (DDA = 98%) was determined by ^{1}H NMR method (Lavertu et al., 2003). The number-averaged molecular weight (M_n), weight average molecular weight (M_w), and polydispersity index (PDI; M_w/M_n) of CS were determined to be 5483 g/mol, 11158 g/mol and 2.03, respectively using gel permeation chromatography (GPC). Succinic anhydride, acetic acid, 1-Hydroxybenzotriazole hydrate (HOBt), N-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), and *N*-Hydroxysuccinimide (NHS). All chemicals and reagents were used without further purification. Dialysis tubing (MWCO = 3500 Da) was purchased from Cellu Sep T1 (Membrane Filtration Products, Inc.). Syringe fillers were purchased from Sartorius Stedim Biotech. MilliQ Plus (18.2 MΩ, Millipore) purified water was used to make all aqueous solutions. The Dulbecco's modified eagle medium (DMEM), antibiotics, and fetal bovine serum (FBS) were purchased from Gibco. MGC Mouse GnRH receptor cDNA (Clone ID: 30249439) was purchased from Dharmacon. LIVE/DEAD® Viability/Cytotoxicity kit and Lipofectamine™ 3000 were obtained from Invitrogen. CellTiter-Glo® Luminescent Cell Viability assay system and Steady-Glo® Luciferase assay system were provided by Promega.

2.1. Polymer characterization

All samples were characterized by using ^{1}H nuclear magnetic resonance spectroscopy (^{1}H NMR) and Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR). The ^{1}H NMR spectra were measured on a Bruker AVANCE 500 MHz spectrometer (Bruker, Switzerland), using D_2O/CD_3COOD as solvent at a 10 mg/ml concentration of polymers. The GnRH-CS with different the degrees of amidation (DADs) value, GnRH-CS1 and GnRH-CS2, for DAD 0.014 and 0.018, respectively were used in this study. All measurements were performed at 300 K, using the pulse accumulation of 64 scans and LB parameter of 0.30 Hz. The degree of N-succinylation (DNS) value of NSCS was calculated using the following equation 1, while the degree of amidation (DAD) value of GnRH-CS was calculated using the following equation 2. All DNS and DAD were determined using ^{1}H nuclear magnetic resonance spectroscopy (^{1}H NMR). ATR-FTIR spectra were collected on a Nicolet 6700 spectrometer (Thermo Company, USA) using a single-bounce ATR-FTIR Smart Orbit accessory with a diamond internal reflection element (IRE), at ambient temperature (25 °C).

2.2. Synthesis of N-succinyl chitosan

N-Succinyl chitosan (NSCS) was synthesized by *N*-succinylation CS with succinic anhydride. Briefly, 1.00 g of CS (6.17×10^{-3} mol) was dissolved in 50 mL of 1% (v/v) aqueous acetic acid, and then 0.03 g of succinic anhydride (0.05 meq/GlcN) was added. The reaction mixture was stirred at room temperature for 24 h and dialyzed against deionized (DI) water for three days to remove free succinic anhydride. The NSCS was then obtained by lyophilization. The degree of N-substitution (DS) of NSCS units in the polymers was determined using ^{1}H-NMR spectroscopy.

2.3. Synthesis of NSCS conjugated with GnRH

The conjugation of N-succinyl chitosan (NSCS) with gonadotropin hormone (GnRH) was carried out by using EDC/NHS as a coupling agent in water as shown in Fig. 2. Briefly, 0.30 g of NSCS (1.79×10^{-3} mol) was suspended in 30 mL of DI water that containing 0.24 g of 1-hydroxybenzotriazole hydrate (HOBt) (1.0 meq/GlcN). The reaction mixture was stirred at room temperature for 24 h, and the clear solution was obtained. Afterwards, 0.34 g of EDC (1.0 meq/GlcN) and 0.21 g of NHS (1.0 meq/GlcN) were added and stirred at room temperature for 1 h. Then, 1.50 mg (0.72×10^{-3} meq/GlcN) or 3.50 mg (1.65×10^{-3} meq/GlcN) of gonadotropin hormone (G) was added into the solution. The reaction mixture was stirred at room temperature for 24 h. The clear solution was dialyzed with DI water for 3 days to remove impurity. The N-succinyl CS conjugated with gonadotropin hor-

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