Carbohydrate Polymers 91 (2013) 508–517

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

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

Effects of molecular weight and pyridinium moiety on water-soluble chitosan derivatives for mediated gene delivery

Warayuth Sajomsang[a,*], Pattarapond Gonil[a], Uracha Rungsardthong Ruktanonchai[a], Maleenart Petchsangsai[a], Praneet Opanasopit[b], Satit Puttipipatkhachorn[c]

[a] National Nanotechnology Center, National Science and Technology Development Agency, Pathumthani, Thailand
[b] Pharmaceutical Development of Green Innovations Group (PDGIG), Faculty of Pharmacy, Silpakorn University, Nakhon Pathom, Thailand
[c] Department of Manufacturing Pharmacy, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand

ARTICLE INFO

Article history:
Received 14 May 2012
Received in revised form 16 August 2012
Accepted 16 August 2012
Available online 24 August 2012

Keywords:
Chitosan
Quaternized chitosan
Methylated *N*-(3-pyridylmethyl) chitosan chloride
Molecular weight
Pyridinium/trimethyl ammonium ratio
Gene delivery

ABSTRACT

The aim of this study is to investigate the effects of molecular weight, the pyridinium/trimethyl ammonium (Py/Tr) ratio, the nitrogen atoms (N) in the methylated *N*-(3-pyridylmethyl) chitosan chloride (M3-PyMeChC)/the phosphorus atoms (P) in DNA (N/P) ratio, and the physicochemical properties of nanopolyplexes on transfection efficiency. The water-soluble chitosan derivative, M3-PyMeChC, was used as a non-viral vector to deliver pEGFP-C2 into human hepatoma (Huh7) cell lines. The results revealed that higher molecular weight M3-PyMeChC was able to form complexes completely with DNA at lower N/P ratios than that with lower molecular weights, which led to higher transfection efficiency. Moreover, the M3-PyMeChC with higher Py/Tr ratios showed superior transfection efficiency at lower Py/Tr ratios at all N/P ratios studied. The highest transfection efficiency for the nanopolyplexes occurred for a molecular weight of 82 kDa at a N/P ratio of 5. The results indicated that the hydrophobic effect of pyridinium moiety could enhance gene transfection efficiency, which can be attributed to the dissociation of DNA from nanopolyplexes. High Py/Tr ratios in nanopolyplexes tended to decrease cytotoxicity due to delocalization of positive charge into a pyridine ring while high N/P ratios and molecular weight increased cytotoxicity. Our results showed that the vector was able to spread the positive charge by delocalizing it into a heterocyclic ring, suggesting to a promising approach to mediate higher levels of gene transfection.

1. Introduction

Gene therapy is the insertion of genes into a specific cell and tissue to treat or prevent disease. It may be used to replace a faulty gene or to introduce a new gene whose function is to cure a disease. An ideal gene delivery method needs to meet 3 major criteria: (1) it should protect the transgene against degradation by nucleases in intercellular matrices, (2) it should bring the transgene across the plasma membrane and into the nucleus of target cells, and (3) it should have no detrimental effects (Gao, Kim, & Liu, 2007). There are two approaches for gene delivery: viral and non-viral. Viral delivery is a conventional approach because viruses have evolved to infect cells with high efficacy. However, clinical trials have underscored the safety risks of viral gene delivery due to the possibility of causing cancer and death (Green, Langer, & Anderson, 2008). For this reasons, much attention has been focused on the non-viral approach due to its potentials to overcome many inherent challenges of viral vectors. Numerous non-viral gene vectors which have several advantages over their viral counterparts, including ease of production, low immune response, the ability to transfer large DNA molecules and potential cell targeting properties, have been developed for gene delivery (Anderson, 1992; Li & Huang, 2000). However, a disadvantage of non-viral gene vectors is their low transfection efficiencies compared to viral vectors. In order to improve the transfection efficiency, numerous cationic polymers such as polyethyleneimine (PEI), poly(L-lysines) (PLL), poly(2-(dimethylamino)ethylmethacrylate) (PDMAEMA), and chitosan (Ch) have been studied for *in vitro* as well as *in vivo* applications (Kim et al., 2007). Despite PEI having excellent transfection efficiency, it is not an ideal carrier because it is not biodegradable and it can cause considerable cytotoxicity via necrosis and apoptosis (Boussif et al., 1995). Chitosan is one of the candidates for use as a non-viral vector due to its non-toxic, biocompatible, and biodegradable properties in the human body. Moreover, it has been proposed as a safer alternative to

* Corresponding author at: National Nanotechnology Center, Nanodelivery System Laboratory, National Science and Technology Development Agency, Thailand Science Park, Pathumthani 12120, Thailand. Tel.: +66 2 564 7100; fax: +66 2 564 6981.
E-mail address: warayuth@nanotec.or.th (W. Sajomsang).

0144-8617/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2012.08.053

other non-viral carrier such as cationic lipids and other cationic polymers (Jayakumar et al., 2010; Weecharangsan, Opanasopit, Ngawhirunpat, Rojanarata, & Apirakaramwong, 2006). However, the applications of chitosan are still limited due to its insolubility in water. In addition, the low specificity and transfection efficiency of chitosan has to be overcome before it can be used in clinical trials (Kim et al., 2007).

Since pyridinium amphiphiles have been shown to be non-toxic for *in vitro* gene delivery (Ilies et al., 2004; Pijper et al., 2003; Van Der Woude et al., 1997), the need to improve the water solubility and gene transfection efficiency of chitosan has led to the development of the pyridinium chitosan derivatives (Opanasopit et al., 2008; Sajomsang, Ruktanonchai, Gonil, Mayen, & Opanasopit, 2009). In this study, methylated *N*-(3-pyridylmethyl) chitosan chloride (M3-PyMeChC) was used as a model gene vector and the effects of molecular weight ranging from 82 to 18 kDa, pyridinium/trimethyl ammonium (Py/Tr) ratios of 16 ± 2 and 0.8 ± 2, and their physicochemical properties on gene transfection efficiency were investigated.

2. Materials and methods

2.1. Materials

The chitosan (Ch), which was purchased from Seafresh Chitosan Lab (Bangkok, Thailand), had a molecular weight of 276 and 16 kDa and degree of deacetylation (DDA) of $94 \pm 2\%$. Sodium cyanoborohydride and polyethylenimine (PEI), with a molecular weight of 25 kDa, were purchased from Aldrich (Milwaukee, USA). Iodomethane, 3-pyridinecarboxaldehyde, sodium iodide, and 1-methyl-2-pyrrolidone (NMP) were sourced from Fluka (Deisenhofen, Germany). Sodium nitrite and hydrochloric acid were purchased from Carlo Erba Reagent (Italy). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma-Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), Trypsin–EDTA, penicillin–streptomycin antibiotics, and fetal bovine serum (FBS) were obtained from GIBCO-Invitrogen (Grand Island, NY, USA). The human hepatocellular carcinoma (Huh7) cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). All other chemicals were of cell culture and molecular biology grade.

2.2. Synthesis of 3-PyMeCh

N-(3-pyridylmethyl) chitosan (3-PyMeCh) with different molecular weights and degrees of N-substitution (DS) were prepared by reductive amination according to the previously reported procedure (Sajomsang, Rungsardthong Ruktanonchai, Gonil, & Warin, 2010). Briefly, chitosan was dissolved in 1% (v/v) of acetic acid, and then ethanol was added. After that, 3-pyridinecarboxaldehyde at different mole ratios was added and stirred at room temperature for 24 h. The pH of the solution was adjusted to 5 with 15% (w/v) of NaOH. Subsequently, $NaCNBH_3$ was added and stirred at room temperature for 24 h, followed by pH adjustment to 7 with 15% (w/v) of NaOH. The reaction mixture was then dialyzed in deionized water (DI) for 3 days in order to remove any impurity and freeze-dried to produce powdered 3-PyMeCh. The DS was determined by ^{1}H NMR spectroscopy.

2.3. Methylation of 3-PyMeCh

A mixture of 3-PyMeCh and NMP was stirred at room temperature for 12 h. Then 15% (w/v) of sodium hydroxide and sodium iodide were added and stirred at 60 °C for 15 min. Subsequently, iodomethane was added in three portions at 3 h intervals and stirred at 60 °C for 24 h. The reaction mixture was precipitated in acetone. The precipitate was then dissolved in 15% (w/v) of NaCl in order to replace the iodide ions with chloride ions. After that the suspension was dialyzed with DI water for 3 days to remove inorganic materials and then freeze-dried to obtain a yellow cotton-like 3-PyMeChC power. The chemical structures of M3-PyMeChC with different pyridinium/trimethyl ammonium (Py/Tr) ratios are shown in Fig. 1.

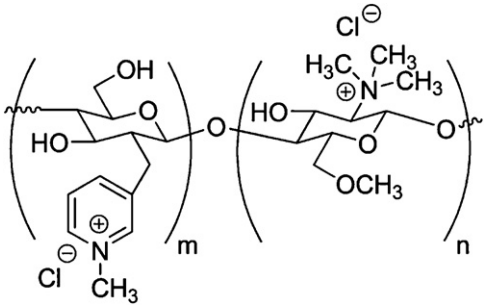

Fig. 1. Chemical structure of M3-PyMeChC with Py/Tr = 16 ± 2 when $m \gg n$ and Py/Tr = 0.8 ± 2 when $m < n$.

2.4. Analytical methods

2.4.1. ATR-FTIR and ^{1}H NMR spectroscopy

All attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectra were collected with a Nicolet 6700 spectrometer (Thermo Company, USA) using the single-bounce ATR-FTIR spectroscopy (Smart Orbit accessory) with a diamond internal reflection element (IRE) at the ambient temperature (25 °C). These spectra were collected using rapid-scan software in an OMNIC 7.0 utilizing 32 scans and a resolution of 4 cm^{-1}. The ^{1}H NMR spectra were measured on a Bruker AVANCE 500 MHz spectrometer (Bruker, Switzerland). All measurements were performed at 300 K, using pulse accumulation of 64 scans and a LB parameter of 0.30 Hz. 1% (v/v) D_2O/CF_3COOD and D_2O were utilized as solvents for 5–10 mg chitosan and its methylated derivatives, respectively.

2.4.2. Determination of molecular weight

The weight average molecular weight (M_w), number average molecular weight (M_n), and M_w/M_n of chitosan and its methylated derivatives were determined using the gel permeation chromatography (GPC). The equipment consisted of a Waters 600E Series generic pump, an injector, ultrahydrogel linear columns (M_w resolving range 1–20,000 kDa), a guard column, standard pollulans (M_w of 5.9–788 kDa), and a refractive index detector (RI). All samples were dissolved in acetate buffer with a pH of 4 and then filtered through VertiPure 0.45 μm nylon syringes filters (Vertical Chromatography Co., Ltd., Thailand). The mobile phases, 0.5 M AcOH and 0.5 M AcONa (acetate buffer pH 4), were used at a flow rate of 0.6 mL/min at 30 °C. Then an injection volume of 20 μL was used.

2.4.3. Determination of size and zeta potential

The *Z*-average hydrodynamic diameter, polydispersity index (PDI) and surface charge of the nanopolyplexes were determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK). The mixtures (1 mL) containing 1 μg of pEGFP-C2 were prepared at N/P ratios ranging from an amount that exhibited incompletely binding to an excess of M3-PyMeChC. The refractive index of chitosan and the viscosity of DI water for were used in the calculations. All samples were measured at 25 °C in triplicate.