Carbohydrate Polymers 130 (2015) 213–221

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Microwave-assisted facile synthesis of a new tri-block chitosan conjugate with improved mucoadhesion

Ravindra V. Badhe[a,b], Rabindra K. Nanda[b], Dharmesh R. Chejara[a], Yahya E. Choonara[a], Pradeep Kumar[a], Lisa C. du Toit[a], Viness Pillay[a,*]

[a] Wits Advanced Drug Delivery Platform Research Unit, Department of Pharmacy and Pharmacology, School of Therapeutic Sciences, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, 7 York Road, Parktown 2193, South Africa
[b] Pharmaceutical Chemistry Department, Dr. D. Y. Patil Institute of Pharmaceutical Sciences and Research, Sant Tukaram Nagar, Pimpri, Pune, Maharashtra 411018, India

ARTICLE INFO

Article history:
Received 4 February 2015
Received in revised form 10 May 2015
Accepted 12 May 2015
Available online 20 May 2015

Keywords:
Microwave
Chitosan
PEGylation
Thiolation
Mucoadhesion
In silico modeling

ABSTRACT

A new chitosan-based tri-block conjugate, *O*-PEG-chitosan-*N*-cysteine was synthesized using microwave irradiation. For synthesis of this derivative, chitosan was modified to a PEG-chitosan conjugate followed by PEG-chitosan-cysteine using 6-*O* PEGylation and 2-*N*-thiolation, respectively. The synthesized derivative was characterized using various analytical techniques such as FT-IR and ^{1}H NMR spectroscopy. The conjugate was also analyzed for its biochemical, biodegradation and mucoadhesive properties. The modified chitosan conjugate exhibited improved mucoadhesion behavior (14.0 h) with greater biodegradation compared to the parent polymer (6.3 h). The in silico modeling corroborated with the in vitro study demonstrating a stable complex between mucin and *O*-PEG-chitosan-*N*-cysteine conjugate ($\Delta E = -60.100$ kcal/mol) compared to mucin and chitosan conjugate. The synthesis proposed herein, involves the use of microwave irradiation which causes a substantial reduction in the reaction time (approximately 2.30 h) compared to conventional method (35 h).

1. Introduction

Chitosan is the second most abundant natural polymer comprising of β-(1–4)-2-amino-2-deoxy-D-glucopyranose units produced by the deacetylation process of chitin from crab shell or shrimps. It has been widely used in various fields because of its membrane permeability and biocompatibility characteristics (Aspden, Mason, & Jones, 1997; Hirano, Seino, Akiyama, & Nonaka, 1989; Hirano, Seino, Akiyama, & Nonaka, 1990; Knapczyk et al., 1989; Lehr, Bouwstra, Schacht, & Junginger, 1992; Takeuchi, Yamamoto, Niwa, Hino, & Kawashima, 1996). From a biopharmaceutical point of view, chitosan has absorption enhancing properties across the intestinal epithelium due to its mucoadhesive behavior. Researchers have shown increasing interest in the use of chitosan and modified chitosan derivatives for the biotechnology, pharmaceutical, textile, food, cosmetics, and agricultural industries for a wide variety of applications due to its non-toxicity, biodegradability and biocompatibility (Li, Dunn, Grandmasion, & Goosen, 1992; Ravi-Kumar, 2000).

Current research is focused on the use of chitosan as a delivery vehicle for drugs, genes, peptides, vaccines and scaffolds for targeted delivery as well as tissue engineering applications (Bernkop-Schnürch, Scholler, & Biebel, 2000; Illum, Jabbal-Gill, Hinchcliffe, Fisher, & Davis, 2001; Liu & Yao, 2002; Sundararajan & Howard, 1999). A number of chitosan derivatives have been prepared and reported thus far using modification/substitution in the primary amine (2-*N*) and 6-*O*-hydroxyl positions of the polymer (Bernkop-Schnürch, Hornof, & Zoidl, 2003; Inmaculada, Ruth, & Angeles, 2010; Ravi-Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004; Sugimoto, Morimoto, Sashiwa, Saimoto, & Shigemasa, 1998). It is well-known that polymers containing thiol groups possess excellent mucoadhesive properties compared with the other polymers (Bernkop-Schnürch, Schwarz, & Steininger, 1999). The interaction via disulfide bond formation between cysteine-rich subdomains of mucus glycoproteins and thiol groups of the thiolated polymers make these polymers most favorable for enhanced mucoadhesive behavior (Snyder, Reddy, Cennerazzo, & Field, 1983). Several thiolated polymers such as carboxymethylcellulose and polycarbophil have already been reported with improved

* Corresponding author. Tel.: +27 117172274.
E-mail address: viness.pillay@wits.ac.za (V. Pillay).

http://dx.doi.org/10.1016/j.carbpol.2015.05.027
0144-8617/

mucoadhesion behavior on introducing thiol moieties (Bernkop-Schnürch, 2002).

Chitosan-based conjugates, chitosan–thioglycolic acid have been reported with improved mucoadhesion (Kast & Bernkop-Schnürch, 2001). In addition, PEGylated chitosan derivatives have also been reported with improved water solubility (Casettari et al., 2012; Werle, Takeuchi, & Bernkop-Schnurch, 2009), reduced cytotoxicity and with enhanced permeation of macromolecules (Casettari et al., 2010; Mao et al., 2005). Chitosan has also been reported as pH tolerant and mucoadhesive for drug delivery via attachment of PEG units or thiol containing moieties to the chitosan backbone (Hauptstein, Bonengel, Griessinger, & Bernkop-Schnürch, 2014).

The study therefore focused on synthesizing a 6-*O*-PEGylated-2-*N*-thiolated chitosan conjugate with improved mucoadhesion, enhanced permeation and improved water solubility properties. In the present study a new facile route of synthesizing 6-*O*-PEGylated-2-*N*-thiolated chitosan has been developed using microwave irradiation, which substantially reduced the reaction time. To our knowledge, microwave-assisted synthesis of 6-*O*-PEGylated-2-*N*-thiolated chitosan with enhanced mucoadhesive property has not been reported so far.

2. Materials and methods

2.1. Materials

Chitosan (Mw = 400 kD, degree of deacetylation 93%), poly(ethylene glycol) monomethyl ether (mPEG, Mw = 600 D), methyl iodide, triphenyl phosphate, L-cysteine and phthalic anhydride were purchased from Fluka Chemie (St. Gallen, Switzerland). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) and hen egg lysozyme were purchased from Sigma–Aldrich (Wienerbergstraße, Austria). Anhydrous *N*,*N*-dimethylformamide (DMF), sodium hydroxide (NaOH) and methanol were obtained from Thermo Fisher Scientific (Ottawa, ON, Canada). All the other chemicals used were of analytical grade.

2.2. Methods

2.2.1. Synthesis of *O*-PEGylated chitosan-N-cysteine

2.2.1.1. Preparation of N-*phthaloyl chitosan.* A mixture of chitosan (5.00 g, 31.0 mmol) and phthalic anhydride (PHA) (13.8 g, 93.1 mmol) in dimethylformamide (DMF) (100 ml) was exposed to microwave irradiation for 9 min at level 3 (240 W, 35%) in a microwave synthesizer (Raga Microwave Systems, Pune, India – Model-RG 31L, Mono-mode system, operating frequency 2450 MHz with 1.1 cub. ft. cavity and 140 W to 700 W power levels) at a temperature of 130 °C under a nitrogen atmosphere. Upon completion of the reaction the mixture became clear and viscous and the precipitate was obtained by pouring the solution into ice water followed by filtration (Whatman filter paper 41). The precipitate was then heated with ethanol to remove impurities followed by filtration. Further, the final precipitate (*N*-phthaloyl chitosan) was air dried at room temperature (21 °C) (Nishimura, Kohgo, & Kurita, 1991).

*2.2.1.2. Synthesis of chitosan-*O*-mPEG graft copolymer.* A mixture of the synthesized *N*-phthaloyl chitosan (0.9 g, 3.35 mmol), MPEGI (4.5 g, 6.7 mmol) [prepared using a method described by Liu, Gan, Chen, Liu, & Zhao 2010] and Ag_2O (1.55 g, 6.7 mmol) in 60 ml DMF was exposed to microwave irradiation for 12 min at level 1 (140 W, 20%) in a microwave synthesizer at temperature 60 °C. A mixture of hydrazine monohydrate (40 ml) and water (80 ml) were added to the reaction mixture and was exposed once again to microwave irradiation for 12 min at level 2 (210 W, 30%) at a temperature of 90 °C. To remove excess hydrazine monohydrate, the mixture was evaporated using a rotary evaporator. The solution was then diluted with water and evaporated thrice to completely remove hydrazine monohydrate, and the residue was diluted with water and dialyzed against distilled water for 96 h using Himedia dialysis tubing (MWCO 12 kD, HIMEDIA Lab. Pvt. Ltd., Mumbai, India). The dialyzed solution was concentrated in a vacuum oven until a solid residue was formed (*O*-PEGylated chitosan) (Gorochovceva & Makuska, 2004).

2.2.1.3. Synthesis of the O-*PEGylated chitosan*-N-*cysteine conjugate.* The covalent attachment of cysteine to the *O*-PEGylated chitosan at the 2-*N* position was achieved by the formation of amide bonds between the primary amino group of the *O*-PEGylated chitosan and a carboxylic acid group of the cysteine. *O*-PEGylated chitosan (500 mg) was dissolved in 1% aqueous hydrochloric acid and adjusted to pH 5 with 1 N NaOH. In addition, 2 g of cysteine was dissolved in 50 ml demineralized water in a separate flask and the carboxylic acid moieties of the cysteine were activated for 20 min by the addition of EDAC (50 mM). Furthermore, this solution containing activated cysteine was poured to the *O*-PEGylated chitosan solution and incubated for 2 h under intermittent sonication at room temperature. Thus the total time for synthesis of *O*-PEGylated chitosan-*N*-cysteine conjugate was 2 h 36 min. The resulting *O*-PEGylated chitosan-*N*-cysteine conjugate was isolated in the dark by dialyzing using dialysis tubing (MWCO 12 kD; cellulose membrane; HIMEDIA Lab. Pvt. Ltd., Mumbai, India) for 3 days at 10 °C against 0.05 N HCl, followed by twice against 1% NaCl solution to reduce ionic interactions between the cationic polymer and the anionic cysteine. The sample was then dialyzed exhaustively twice against 0.01 N HCl to adjust at pH 4 followed by drying using lyophilization (Badhe, 2012; Clausen & Bernkop-Schnürch, 2000; Schmitza, Grabovaca, Palmbergera, Hofferb, & Bernkop-Schnürcha, 2008).

2.2.2. Physicochemical property characterization

2.2.2.1. Solubility. The solubility of *O*-PEGylated chitosan-*N*-cysteine graft copolymer was tested in organic solvents like acetone, ethyl acetate, ethanol, chloroform, DMSO, DMF, aliphatic hydrocarbons, carbon tetrachloride and toluene, distilled water as well as in 0.1 M acetate buffer (pH 4.00), 0.1 M phosphate buffer (pH 7.00) and 0.1 M borate buffer (pH 10.00). The samples were immersed in each solvent at a concentration of 5 mg/ml.

2.2.2.2. Quantification of PEG units (PEG%). The content of PEG units in the *O*-PEGylated chitosan-*N*-cysteine graft copolymer was determined as per a method described by Gorochovceva and Makuska (2004). The method provided linear response over a range of 0.05–0.4 mmol of MPEG-600 to construct a calibration curve. The content of PEG units (PEG%) in the copolymer was calculated using Eq. (1).

$$\mathrm{PEG} = \frac{n \times 600}{m} \times 100 \quad (1)$$

where n = concentration of MPEG-600 in the sample solution determined from the calibration curve, mol/l; m = the sample weight.

2.2.2.3. Quantification of thiol groups. The degree of modification, i.e. the quantity of thiol groups immobilized on the *O*-PEGylated chitosan-*N*-cysteine conjugate, was determined as per the method adopted by Clausen and Bernkop-Schnürch (2000), in which the *O*-PEGylated chitosan-*N*-cysteine graft was dissolved and reacted with Ellmans reagent. The developed color was compared with a standard cysteine curve at 450 nm. Cysteine was used as standards to construct the calibration curve for calculation of the quantity of thiol groups immobilized on the grafted polymer.