Colloids and Surfaces B: Biointerfaces 88 (2011) 722–728

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

Submicroparticles composed of amphiphilic chitosan derivative for oral insulin and curcumin release applications

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ARTICLE INFO

Article history:
Received 14 May 2011
Received in revised form 10 August 2011
Accepted 10 August 2011
Available online 17 August 2011

Keywords:
Amphiphilic chitosan
Mucoadhesion
Tight junction
Hydrophobic drug
Hydrophilic drug

ABSTRACT

Amphiphilic polymers for dual drug delivery have been a focus of research in recent years. We have previously developed and characterized Lauroyl sulphated chitosan (LSCS). Here biological characterizations like mucoadhesion, cytotoxicity, calcium binding, tight junction opening and enzymatic degradation studies were performed to understand its applicability. In vitro drug release properties of both hydrophilic insulin and hydrophobic curcumin were carried out. The biological activity and stability of released insulin were also studied. The stability studies of encapsulated curcumin and uptake studies have also been carried out. LSCS showed strong mucoadhesion and 100% of non-toxicity. LSCS could transiently open tight junctions between Caco-2 cells and thus increase the paracellular permeability. LSCS enhanced calcium binding properties and decreased enzymatic degradation rate retaining insulin activity. LSCS could protect curcumin from photodegradation and could also enter into the cells. From release studies, LSCS was found to be a suitable candidate for both drugs.

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1. Introduction

Biocompatible polymers have attracted more attention in biomedical and biotechnological applications during the past decades. In recent years amphiphilic polymers have been utilized as a potential drug carrier for both hydrophilic and hydrophobic drugs. This type of amphiphilic polymers core shell structure is composed of hydrophobic segment, which acts as an inner core, and a hydrophilic segment which acts as an outer core. Yoshioka et al. have reported the amphiphilic modification of natural polymers [1]. In comparison with common polymers; chitosan exhibited special advantages in drug delivery system [2] because of its favourable characteristics like biocompatibility [3], biodegradability [4], and non-toxicity [5]. CS is a linear copolymer of β-(1,4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose and 2-amino-2-deoxy-β-D-glycopyranose, and is chemically prepared by N-deacetylation of naturally occurring chitin [6].

Among CS derivatives, amphiphilic CS derivatives are type of molecules which have intensively been studied due to their interesting behaviour. Chitosan molecule in its native state has no amphiphilicity. Various amphiphilic chitosan derivatives like carboxymethylhexanoyl chitosan [7], water soluble N-methylated chitosan possessing hydrophobic $–N(CH_3)_2$, $–NH(CH_3)$ and hydrophilic $–N–(CH_3)_3$ groups [8] etc. have been extensively published. Hydrophobic groups of amphiphilic groups ever reported generally contain long alkyl [9,10], long acyl [7], and aryl [11] and that of hydrophilic groups include carboxymethyl [7], sulphate [10], phosphate [12,13], N-trimethyl [14] and polyethyleneglycol [11,15].

To prepare amphiphilic CS derivatives, hydrophobic moieties were introduced into the CS backbone by using different methodologies such as alkylation, acylation and graft polymerisation etc. Long chain acyl derivatives of CS are interesting hydrophobic modification for developing nano/microparticles. N-acylation of CS with various fatty acid chlorides increased the hydrophobic character of the resulting polymers. Fatty acid with 12 carbon atoms had a more pronounced effect on transepithelial transport and this has been previously shown to result in a higher increase in absorption of various hydrophilic drugs in comparison to fatty acids with 8 carbon atoms, 10 carbon atoms and 14 carbon atoms [16,17]. The hydrophobic core provides a loading space for water-insoluble drugs and stabilizes them, whereas the hydrophilic shell protects encapsulated water soluble drugs.

In this study, hydrophilic and hydrophobic drugs were encapsulated onto the amphiphilic CS micro particles and its release properties were compared. Insulin was chosen as a hydrophilic drug and curcumin as a hydrophobic compound. Due to the inconvenience of insulin injections, various approaches have been attempted to formulate insulin for administration by oral routes such as encapsulation of insulin within polymeric particles.

Curcumin has been used in traditional medicine for many centuries in countries such as India and China [18]. In current research,

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doi:10.1016/j.colsurfb.2011.08.007

curcumin has been taken as an imminent herbal drug to instigate multi-targeted therapy, which is needed for treatment of various fatal diseases including cancer [19,20]. It has been reported that free curcumin induces cell cycle arrest and apoptosis in human cancer cell lines derived from a variety of tumours including lung, colorectal, pancreatic and prostate carcinoma, etc. [21–24]. Clinically, it is considered safe while administered at very high doses. Conversely, systemic toxicity at high dose rendered other anticancer drug unsuitable for cancer therapy [25].

Previously, we report the preparation, characterization and blood compatibility studies of LSCS particles [26]. However, the application side being drug delivery has not yet been investigated. Herein, we reported the biological properties of LSCS and the comparative release properties of both the insulin and curcumin drugs.

2. Materials and methods

2.1. Materials

Mucin type III (from porcine stomach), N-benzoyl-L-arginine ethyl ester (BAEE), N-benzoyl-L-arginine p-nitroanilide (BTPNA) and curcumin were obtained from Sigma. Caco-2 cells were purchased from National Center for Cell sciences Pune, India, ELISA kit from Mercodia, Human insulin was purchased from Eli Lilly and Company Pvt. Ltd., India. All other chemicals were of analytical grade and used as received.

2.2. Methods

We have already reported the procedure for the preparation of LSCS [26]. Molecular weight of the LSCS derivative was determined by using viscometry and it was found to be 288.63 kDa. We have again optimised the particle size of LSCS to 299 ± 41.79 dnm. LSCS nanoparticles were prepared by the ionic gellation method. To the 20 ml of 0.5% LSCS solution in 3% acetic acid solution, added 20 ml of 0.5% tripolyphosphate solution drop wise. Particles were then separated by centrifugation at 14000 rpm for 20 min. The particles were washed with distilled water and dried by lyophilisation.

2.3. Biological characterisations of lauroyl sulphated CS (LSCS)

2.3.1. Mucoadhesion studies

2.3.1.1. Mucin binding study. Mucin binding experiments were performed to assess the amount of porcine mucin adsorbed on the micro particles [27]. CS and LSCS (10 mg) were dispersed in the 1 ml mucin solution (5 mg/ml) in phosphate buffer of pH 7.4, vortexed, and incubated at 37 °C for 2 h. Mucin solution without polymer was used as standard. The mucin-particle dispersions were centrifuged at 4000 rpm for 2 min, and the supernatant was used for the measurement of the free mucin content by Lowry protein assay using Varian 50 conc UV-Vis spectrophotometer (USA) at 750 nm [28]. Percentage of mucin bound on the particles was graphically represented with respect to mucin.

2.3.1.2. Mucoadhesion study with rat intestine. Ex-vivo mucoadhesion studies were performed on freshly excised rat intestinal mucosa according to a method described previously [27]. Excised jejunum portion of the rat intestine was flushed with normal saline to remove luminal contents. Around 10 cm of intestinal tissue was cut open and placed on a polyethylene support with help of cyanoacrylate adhesive. 10 mg of samples (CS and LSCS) were uniformly spread on the mucosal surface and were allowed to interact with mucus gel layer for 5 min. Tissue was then mounted on a platform at an angle of 45° and washed with saline phosphate buffer (pH 7.4) under a constant flow rate (10 ml/min). Particles washed away from the mucosal surface were collected and dry weight was assessed. The weight of the dried particles was compared with the weight of the particles applied to the mucosal surface.

2.3.2. Cytotoxicity

To check the toxicity of the derivative, MTT assay was done on caco-2 cell lines. Cells were seeded in 24 well plates at a density of 5×10^5 cells/well and incubated for 24 h. The medium was replaced with Hanks Balanced Salt Solution (HBSS) and incubated for 2 h. After incubation, HBSS was replaced with HBSS containing CS and LSCS at a concentration of 2 mg/ml/well. The cells were then incubated for 24 h. The medium containing polymers were removed and MTT assay was done [29]. Untreated caco-2 cells, which was used as control served as 100% cell viability. Tests were performed in duplicate for each sample.

2.3.3. Visualization of tight junctions

Caco-2 cells were seeded in 4 well plates at a density of 5×10^5 cells/well and incubated for 24 h. Cells were maintained under normal incubation conditions and used for experiments 6 days post-seeding. Growth medium was replaced with 1 ml HBSS transport medium and cells were equilibrated with HBSS 2 h before the experiment. Medium was then replaced with HBSS containing samples (CS and LSCS) at a concentration of 2 mg/ml and incubated for another 2 h. The particles were removed by washing the cells three times with PBS. The cells were fixed with 300 μl of 4% paraformaldehyde for 20 min at room temperature and thereafter the cells were permeabilized with 0.2% Triton X-100 in blocking solution, made of 1% (w/v) bovine serum albumin (BSA) in PBS, for 20 min. The cells were then washed with PBS three times and blocked with 300 μl of 1% BSA for 30 min. Cells were again washed with PBS and tight junction proteins were stained with 300 μl of rhodamine phalloidin for 20 min. The staining solution was then removed and the cells were washed with PBS. Images were obtained using Carl Zeiss LSM Meta 510 inverted confocal laser scanning microscope (Carl Zeiss, Germany), equipped with He/Ne laser 543. The visualization of rhodamine phalloidin was done using excitation and emission wavelengths of 543 and 605 nm, respectively [30].

2.3.4. Calcium binding studies

Particles (CS and LSCS) were weighed (5 mg) and dispersed in 1 ml of 1 mM $CaCl_2$ solution. $CaCl_2$ solution without polymer was used as standard. The system was incubated for 1 h and was centrifuged at 7000 rpm. The calcium binding efficiency of the particles was evaluated with 200 μl supernatant using calcium assay kit (Enzyme Technologies PVT LTD, Mumbai, India) [31].

2.3.5. Enzymatic degradation of LSCS particles

2.3.5.1. Trypsin inhibition study. Trypsin inhibition study was performed with BAEE substrate. CS and LSCS particles were dispersed in phosphate buffer with pH 7.6 to get a concentration of 0.1% w/v and 1 ml of this dispersion was used for the assay. Mixture of BAEE in phosphate buffer (pH 7.6), polymer dispersion and 30 U of trypsin solution (in 10 mM HCl) was incubated at 37 °C for 30 min and the enzymatic action was stopped by the addition of 1% trichloroacetic acid solution. Supernatant was analysed by measuring the absorbance for residual trypsin activity at 253 nm using a UV/visible spectrometer (Varian Cary 50 Conc). Control used was without polymer presence and percentage of inhibition was calculated relative to control trypsin.

2.3.5.2. Chymotrypsin inhibition study. The chymotrypsin inhibition assay was performed using the chromogenic substrate BTPNA. Polymer dispersion of CS, LSCS were prepared in Tris–HCl buffer of pH 7.8 to get final concentration of 0.5%, w/v solutions. Mixture of BTPNA, polymer dispersion and 40U of chymotrypsin solution

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