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Methylated N-(4-N,N-dimethylaminobenzyl) chitosan coated liposomes for oral protein drug delivery

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ABSTRACT

In the present study, methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan ($TM_{56}Bz_{42}CS$) was synthesised and investigated for oral protein drug delivery by combining it with liposomes entrapped with fluorescein isothiocyanate-conjugated bovine serum albumin (FITC-BSA), a model protein. Liposomes (LPs) composed of 10:2 molar ratios of egg yolk phosphatidylcholine (EPC) and sodium oleate (NaO) were prepared by a thin film hydration method and coated with $TM_{56}Bz_{42}CS$. BSA-loaded, $TM_{56}Bz_{42}CS$-coated liposomes ($TM_{56}Bz_{42}CS$-coated FITC-BSA-LP) were evaluated for their protein transport efficiencies and cytotoxicities in Caco-2 cells. Moreover, the *in vitro* stabilities of the $TM_{56}Bz_{42}CS$-coated LP-BSA were determined by examining the degradation of the protein in simulated intestinal fluid containing 1% w/v pancreatin porcine pancreas. The mean particle size and zeta-potential of the $TM_{56}Bz_{42}CS$-coated LP-BSA were 128 ± 15 nm and 5.38 ± 1.66 mV, respectively. Additionally, the initial FITC-BSA to lipid ratio (2.5% w/w) showed the highest entrapment efficiency percentage (50.13%) and FITC-BSA content (8.08 mg/g of lipid) overall. The results of the FITC-BSA transport showed that the $TM_{56}Bz_{42}CS$-coated FITC-BSA-LP enhanced protein permeability across the Caco-2 cell monolayers with low cytotoxicity. In addition, these liposomes protected against protein degradation in pancreatin. Our studies demonstrated that $TM_{56}Bz_{42}CS$-coated liposomes have the potential to be used in oral protein drug delivery methods.

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1. Introduction

Currently, therapeutic peptides and proteins continue to grow in popularity due to their potential uses in drug therapy. They are used to relieve patient suffering for many conditions, including various cancers (monoclonal antibodies and interferons), heart attack, stroke, cystic fibrosis, Gaucher's disease (enzymes and blood factors), diabetes (insulin), anaemia (erythropoietin), and haemophilia (blood clotting factors). Unfortunately, most of these therapeutic peptides and proteins can only be administered by injection due to their instabilities in the gastrointestinal (GI) tract and poor absorption. Fortunately, various approaches, including absorption enhancers (Radwant and Aboul-Enein, 2002), enzyme inhibitors (Morishita et al., 1992), and chemical modifications (Xia et al., 2000), have been studied to overcome these problems.

In addition to such strategies, liposomes (LPs) have been also thoroughly studied because liposomes have been shown to improve the enteral absorption of peptide and protein drugs (Kissel et al., 2001). Unfortunately, most liposome formulations cannot be used for oral delivery because liposomes dissolve in intestinal detergents such as bile salts, and they are readily degraded by intestinal phospholipases. Furthermore, these disruptions to the liposomal membranes in the GI tract lead to the exposure of the encapsulated material and the subsequent loss of their protective function. To minimise these disruptive influences, accordingly, polyelectrolyte coatings obtained by alternating the deposition of polyanions and polycations emerged as a novel way to functionalise surfaces. This method has been quickly applied to the drug delivery field by combining chitosan (CS), a cationic polysaccharide consisting of *N*-acetyl glucosamine (GlcNAc) and glucosamine (GlcN), with liposomes. Takeuchi et al. showed that the CS-coated liposomes could be formed via ionic interactions between positively charged CSs and negatively charged diacetyl phosphates on the surfaces of the liposomes (Takeuchi et al., 1996). Additionally, CS was used as a stabilising constituent in liposomes (Henriksen et al., 1994).

While CS has many unique activity properties, several studies have also highlighted the potential use of CS through its biocompatibility, biodegradability and mucoadhesivity (Borchard et al., 1996).

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The mucoadhesive property of CS-coated liposomes has been demonstrated to help delay the intestinal transit time to increase the absorption of insulin (Aspden et al., 1997). Moreover, CS has been shown to induce the redistribution of cytoskeletal F-actin and tight junction protein ZO-1 via interactions between its positive charges and the mucosal negative charges; these interactions result in the increased paracellular permeabilities of the hydrophilic macromolecules (Schipper et al, 1997). Unfortunately, CS is a weak base, and in neutral and basic environments, the CS molecule loses its charge and precipitates from solution. Therefore, under these conditions, CS is ineffective as an absorption enhancer, limiting its use in the more basic environments of the intestine and colon.

Recently, our research groups have successfully synthesised a modified CS, methylated *N*-(4-*N,N*-dimethylaminobenzyl) chitosan (TM-Bz-CS). This modified CS is water soluble at physiological pH and has shown *in vitro* absorption enhancing properties (Kowapradit et al., 2008). Based on these findings it is suggested that this modified CS may be favoured for use as a carrier for oral protein drugs. In a previous study, TM-Bz-CS showed an optimal degree of quaternisation (DQ) and extent of substitution (ES) when substituted as follows: $TM_{56}Bz_{42}CS$. Therefore, in this study, the same modified CS-($TM_{56}Bz_{42}CS$)-coated liposomes have been evaluated for their *in vitro* absorption enhancing properties on Caco-2 cell monolayers by using fluorescein isothiocyanate-conjugated bovine serum albumin (FITC-BSA) as the model protein. The stability of BSA while entrapped in the $TM_{56}Bz_{42}CS$-coated liposomes and exposed to conditions that simulate the gastrointestinal (GI) tract was also evaluated to determine the protection properties of $TM_{56}Bz_{42}CS$.

2. Materials and methods

2.1. Materials

The methylated *N*-(4-*N,N*-dimethylaminobenzyl) chitosan ($TM_{56}Bz_{42}CS$) was carried out in accordance with the previously procedure (Kowapradit et al., 2008). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), pancreatin porcine pancreas, sodium oleate (NaO), fluorescein isothiocyanate conjugated bovine serum albumin (FITC-BSA) and BSA (Albumin Fraction V from bovine serum, 69,000 Da) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Egg yolk phosphatidyl choline (EPC) was purchased from Wako Pure Chemical (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA, penicillin–streptomycin antibiotics, fetal bovine serum (FBS), Hanks' balanced salt solutions (HBSS) and phosphate buffer saline (PBS) were purchased from GIBCO-Invitrogen (Grand Island, NY, USA). The Caco-2 cell line was obtained from American Type Culture Collection (Rockville, MD, USA). Transwell (12-well plates) cell culture chambers with a 3.0 μm pore size were purchased from Corning Life Sciences (Massachusetts, USA). All other chemicals were of cell culture and molecular biology quality.

2.2. Preparation of the modified CS-coated liposomes

Negatively charged liposomes (LPs) containing constant amounts of EPC and anionic surfactant (NaO) were formulated using a thin film-hydration method previously described (Opanasopit et al., 2011). Briefly, EPC and NaO (10:2 molar ratio) were separately dissolved in chloroform/methanol (2:1, v/v) and methanol, respectively, and then both materials were deposited in a test tube, where the solvents were evaporated under nitrogen gas flow. The lipid film was placed in a desiccator connected to a vacuum pump for 6 h to remove the remaining organic solvents. The dried lipid film was hydrated with PBS, pH 7.4 with a 0–10% w/w FITC-BSA to lipid ratio. Following hydration, the dispersion was sonicated in a bath sonicator for 10 min and then in a probe sonicator for two cycles of 30 min each. The excess lipid composition and non-entrapment BSA were separated from the vesicles formulation by centrifugation at 15,000 rpm and 4 °C for 15 min. The supernatant was collected for characterisation. BSA was used instead of FITC-BSA in the *in vitro* stability study. For the $TM_{56}Bz_{42}CS$-coated liposomes, a 1:1 (v/v) mixture of liposomes and $TM_{56}Bz_{42}CS$ solution (1.25–20 mM in PBS, pH 7.4) was stirred with a magnetic stirrer at 10 °C for 30 min.

2.3. Characterisation of the $TM_{56}Bz_{42}CS$-coated FITC-BSA-LP

2.3.1. Particle size and zeta potential

The particle size and zeta potential of the BSA solution and liposome formulations were measured at room temperature by a laser diffraction particle size distribution analyser (Partica LA-950, Horiba Scientific, Japan) and a zeta potential analyser (ZetaPlus, Brookhaven Instruments Corporation, NY, USA), respectively. The samples were diluted with distilled water, which was passed through a 0.22-μm membrane filter prior to use. All samples were measured in triplicate.

2.3.2. Entrapment efficiency and FITC-BSA content

Free FITC-BSA was separated from the $TM_{56}Bz_{42}CS$-coated FITC-BSA-LP by using a centrifugal filter unit (Amicon Ultra-4, MWCO 100 kDa). The liposomes were placed in the centrifugal filter, centrifuged at 4000 rpm for 30 min, and washed once with PBS. The concentration of the filtrate, free FITC-BSA, was directly determined by using a fluorescence 96-well plate reader (Universal Microplate Analyzer, Model AOPUS01 and AI53601, Packard BioScience, CT, USA). The excitation and emission wavelengths used were 400 and 535 nm, respectively. The entrapment efficiency percentage (%EE) and content of FITC-BSA in the $TM_{56}Bz_{42}CS$-coated FITC-BSA-LP were calculated using Eqs. (1) and (2), respectively.

$$\%\mathrm{EE} = (C_i - C_f/C_i) \times 100 \quad (1)$$

where C_i is the initial concentration of the FITC-BSA solution added into the $TM_{56}Bz_{42}CS$-coated FITC-BSA-LP and C_f is the concentration of the free FITC-BSA after filtration.

$$\mathrm{FITC-BSA\ content} = \mathrm{Pt(mg)/Mt(g)} \quad (2)$$

where Pt is the total amount of FITC-BSA loaded into liposomes (C_i–C_f) and Mt is the total amount of lipid.

2.4. Cell culture preparation

The Caco-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) at pH 7.4 supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 1% non-essential amino acid solution, and 0.1% penicillin–streptomycin solution and kept in a humidified atmosphere (5% CO_2, 95% air, 37 °C). The cells were grown under standard conditions until 60–70% confluency. Cells from passages 32–40 were used for all of the experiments. The cells were seeded on tissue culture polycarbonate membrane filters (pore size of 3.0 μm) in 12-well Transwell® plates (Costar®, Corning Inc., Corning, NY, USA) at a seeding density of 2×10^4 cells/cm^2. The culture medium was added to both the donor and acceptor compartments, and the medium was changed every second day. The cells were left to differentiate for 15–21 day after seeding. The detected trans-epithelial electrical resistance (TEER) values were more than 600 Ω cm^2 using a Millicell® ERS meter (Millipore, Bedford, MA, USA).

2.4.1. Transport studies of FITC-BSA

The transport of FITC-BSA across the Caco-2 cell monolayer at pH 7.4 was studied. Caco-2 monolayers were grown in Transwell®

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