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Preparation of fucoidan-shelled and genipin-crosslinked chitosan beads for antibacterial application

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ABSTRACT

In this study, a fucoidan-shelled chitosan bead was developed with the purpose of oral delivery of berberine to inhibit the growth of bacteria. The cross-linking level and swelling property of the beads were affected by the pH value and the composition of the genipin/fucoidan combined gelling agent. The drug release of the berberine-loaded beads was faster in simulated gastric fluid (pH 1.2) than those in simulated intestinal fluid (pH 7.4). Furthermore, a nanoparticles/beads complex system was developed by incorporation of berberine-loaded chitosan/fucoidan nanoparticles in the fucoidan-shelled chitosan beads. The nanoparticles/beads complex served as a drug carrier to delay the berberine release in simulated gastric fluid, with an estimated lag time of 2 h. Our results showed that the berberine-loaded beads and nanoparticles/beads complex could effectively inhibit the growth inhibition of common clinical pathogens, such as *Staphylococcus aureus* and *Escherichia coli*, and have the advantage of continually releasing berberine to inhibit the growth of the bacteria over 24 h.

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1. Introduction

Berberine is an isoquinoline alkaloid present in *Berberis* species that can act as an antimicrobial against a number of pathogenic microorganisms such as bacteria, fungi and viruses. The inhibitory effects against viruses by berberine are associated with inhibition of virus protein trafficking/maturation and interference with the viral replication cycle. Berberine also attenuated inflammatory substances release and viruse protease inhibitor-induced inflammatory response. Especially, berberine can inhibit various bacteria through different biosynthesis pathway and effectively inhibit multidrug efflux pumps against several bacteria (Ball et al., 2006; Stermitz, Lorenz, Tawara, Zenewicz, & Lewis, 2000). Therefore, berberine can overcome multidrug resistant in gram-negative and positive bacteria. Moreover, berberine is able to reduce inflammation and induces apoptosis in a wide variety of human cancer, including breast cancer, leukemia, melanoma, epidermoid carcinoma, hepatoma, pancreatic cancer, oral carcinoma, tongue carcinoma, glioblastoma, prostate carcinoma and gastric carcinoma. However, berberine exhibits poor water solubility and limited absorption in the gastrointestinal tract. Many studies have focused on developing nanofiber, nanoparticle, emulsion, cyclodextrin complex, and microcapsule for oral delivery of berberine to increase effective concentration and absorption in the gastrointestinal tract (Chang et al., 2011; Chou et al., 2013; Huang et al., 2013; Lam et al., 2012).

Chitosan is a cationic polysaccharide with the ability to form polyelectrolyte complexes (PEC) with naturally occurring polyanions such as heparin, hyaluronic acid, and alginate. In addition to their biodegradability, nontoxicity, and biocompatibility, the PECs exhibit other favorable characteristics including pH-sensitivity, receptor affinity, and protein stabilization (Muzzarelli, Greco, Busilachi, Sollazzo, & Gigante, 2012). Chitosan–alginate polyelectrolyte complex was the most commonly used method to encapsulate drugs, proteins, living cells, and probiotics, which have great potential for applications in different areas such as food biotechnology, pharmacy, and medicine (Yang et al., 2013). Since chitosan can be easily dissolved in dilute acid solutions, a major challenge for using the chitosan–alginate PEC beads in the oral delivery of drugs is the poor stability of the drug carriers in the

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gastric acid. Calcium chloride was traditionally used to obtain stable chitosan–alginate PEC beads by forming a cross-linked alginate gel core.

Fucoidan is a polysaccharide mainly composed of fucose residues and sulfate groups. It is extracted from marine brown seaweed which has a backbone composed of sulfated esters of fucose and glucuronic acid. Fucoidan acts as a naturally occurring antioxidant (Hou, Wang, Jin, Zhang, & Zhang, 2012), antitumor agent (Synytsya et al., 2010), and antithrombotic (Zhao et al., 2012). Moreover, fucoidan can regulate the differentiation and mobilization of stem cells (Huang & Liu, 2012), and can enhance the probiotic effects of lactic acid bacteria (LAB) on immune functions (Kawashima, Murakami, Nishimura, Nakano, & Obata, 2012). The negatively charged polyanion can form PECs with the positively charged chitosan to prepare nanoparticles which are very suitable for drug delivery (Huang & Liu, 2012; Huang & Li, 2014; Pinheiro et al., 2015; Wu, Don, Lin, & Mi, 2014; Yu et al., 2013).

However, the chitosan/fucoidan PECs are associated with limitations that restrict its use as a carrier for oral drug delivery. The PECs are instable in low pH due to the protation of carboxylate ions of glucuronic acid residues in fucoidan. Furthermore, unlike alginate, fucoidans cannot react with calcium ion to form stable, cross-linked hydrogels. Up to now, preparation of chitosan/fucoidan PEC beads is still unfeasible. Genipin is a naturally occurring compound in gardenia fruit extract that can form hydrogels, beads, and nanoparticles by cross-linking with chitosan (Mi, Sung, & Shyu, 2002; Mi, Sung, Shyu, Su, & Peng, 2003; Muzzarelli, 2009). Because genipin is about 5000–10,000 times less cytotoxic than glutaraldehyde, cross-linking of chitosan with genipin has potential applications in the preparation of biocompatible materials for biomedical applications (Muzzarelli, 2009).

Berberine alone isn't potent enough to eradicate bacterial infections and attenuate the inflammations when administered orally because of its poor oral bioavailability (Tan et al., 2013). Generally, high doses of berberine taken is needed in the treatment of impaired intestinal barrier function associated with bacterial infection, bearing the risk of cramping and diarhhea due to taking inappropriately amount of berberine. To overcome the problems encountered during oral delivery of berberine, a fucoidan-shelled chitosan bead was developed in this study to reduce the dissolution rate in the stomach and prolong the intestinal residence time. Chitosan is a mucoadhesive polysaccharide that has a tendency to stick to the intestinal mucosa. The fucose residue in fucoidan has been reported to affect the adhesion of several bacteria to intestinal mucins (Pacheco et al., 2012) as well as inhibit the growth of pathogenic bacteria and their adhesion to murine macrophages (Lutay, Nilsson, Wadstrom, & Ljungh, 2011). Fucose-modified chitosan nanoparticles can target and directly contact with microorganism on the epithelium (Lin et al., 2013). We hypothesize that the fucoidan-shelled chitosan bead developed in the current study for berberine delivery may have the benefit of decreasing the berberine dissolution rate in stomach (pH 1.2) to prolong its retention time in the intestinal tract (pH 7.4) and can deliver high doses of the naturally occurring antibiotic directly to the site of infection for bactericidal activity. Genipin-mediated gelling properties, and pH-dependent stability and swelling of the beads were investigated in this work. We also prepared a nanoparticles/beads complex system by incorporating berberine-loaded chitosan/fucoidan nanoparticles in the fucoidan-shelled chitosan beads. Berberine released from the beads and the nanoparticles/beads complex were examined in simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 7.4). In addition, growth inhibition of *Staphylococcus aureus* and *Escherichia coli* by the berberine-loaded beads and the nanoparticles/beads complex was investigated.

2. Materials and methods

2.1. Materials

Genipin and chitosan (*M.W.* = 300 kDa, 90% deacetylation) was supplied by Challenge Bioproducts Co. (Taichung, Taiwan). Berberine hydrochloride was obtained from Sigma-Aldrich Co. Ltd (USA). Fucoidan (U-fucoidan) from Laminaria japonica was purchased from NOVA Pharma & Liposome Biotech Co., Ltd, Taiwan.

2.2. Characterization of fucoidan

^{1}H and ^{13}C NMR spectra were recorded on BRUKER AVIII-500 MHz FT-NMR in D_2O solutions. Working frequencies were 500.1 MHz for ^{1}H and 125.7 MHz for ^{13}C. 2D 1*H*-13C HSQC were applied for signals assignment. Sulfate content of fucoidan was determined by the turbidimetric method using a barium chloride–gelatin reagent (Dodgson & Price, 1962). Carboxylic acid content was quantitatively analyzed by a titrimetric method. Fucoidan was titrated with sodium hydroxide using phenolphthalein as an indicator (Pegg, Jones, Athauda, Ozer, & Chalker, 2014).

2.3. Preparation of fucoidan-shelled chitosan beads

Chitosan (1.5 g) was dissolved in 100 mL of 1.0% (v/v) acetic acid solution and the pH of the prepared chitosan solution was adjusted to 5.0. The gelling solutions were prepared by mixing and dissolving fucoidan and genipin in deionized water (DI water), and the solution was subsequently sterilized. The chitosan solution was continuously added to the gelling bath using a syringe pump. The gelling bath contained different concentrations of fucoidan (0.2, 0.4, 0.6, and 0.8 wt%) and genipin (0.05, 0.10, 0.15, and 0.25 wt%). The pH values of the gelling solutions were adjusted to 2.0, 4.0, 6.0, and 8.0, respectively. The gel beads were allowed to harden for 12 h and then washed twice with deionized water to remove excess genipin and fucoidan. The cross-linking degrees of the beads were determined by ninhydrin assay according to our previous study (Mi et al., 2003). The sizes of the beads were measured with a Vernier Caliper.

2.4. Fourier transform infrared spectroscopy (FTIR)

To investigate the interactions between chitosan and fucoidan, the gel beads were grounded to a powder and FTIR spectra of the powdered sample were recorded on a Perkin Elmer, RXI FTIR System in the region between 400–4000 cm^{-1}. FTIR analysis was performed by mixing the powdered sample with KBr and then pressing the mixture into a disk for spectral analysis.

2.5. pH-dependent swelling properties

The swelling studies were conducted by weighing the beads in a dry state and subsequently immersing the beads into beakers containing 500 mL of swelling medium (pH 1.2, 6.0, and 7.4, respectively). The samples were kept in an isothermal bath (at 37 °C) with constant shaking at 100 rpm. Dry beads were swollen at pH 1.2, 6.0, and 7.4 for 24 h. The swollen beads were separated from the medium at specific time intervals and then were immediately blotted dry and weighed. The swelling percentage of a test sample (each group was repeated five times) was calculated by the weight change of the beads from the following expression:

$$\text{Swelling} \quad (\%) = \left[\left(\frac{W_{\text{swollen}}}{W_{\text{initial}}}\right) - 1\right] \times 100$$

where W_{swollen} is the weight of the swollen beads and W_{initial} is the initial weight of the dry beads.

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