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Evaluation of chitosan hydrochloride-alginate as enteric micro-probiotic-carrier with dual protective barriers

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ABSTRACT

In this study, the cells-free and cells-loaded chitosan hydrochloride-alginate (CHC-Alg) microcapsules were firstly fabricated with polyelectrolyte complexes via an orifice-polymerization method. Scanning electron microscope images showed that the CHC-Alg microcapsules had a typical shell-core structure and the model probiotic cells (*Bacillus licheniformis*) were embedded in the core in cells-loaded microcapsules. The microcapsules prepared had good thermal stability and moisture property (3.89%). Cells survival and release studies showed that the number of probiotic cells released from the cells-loaded microcapsules (approx. 6.36 log CFU ml^{-1}) was 6.19 log CFU ml^{-1} when they were performed in the simulated gastric fluid (SGF, pH 2.0) for 1 h and subsequently in the simulated intestinal fluid (SIF, 0.3%) for 4 h. The CHC-Alg microcapsules with favorable swelling performances were helpful to permeate the harsh acid to protect the cells in the SGF (pH 2.0). The CHC-Alg microcapsules effectively protected the model probiotic cells, which was attributed to the "dual protective barriers" of the shell-core structure, that is, the primary barrier of the Alg hydrogel layer formed with a compact polymer matrix and the secondary barrier of the PEC film formed on the surface. The microcapsules prepared could be used as an enteric micro-probiotic-carrier for designing potential probiotic delivery systems.

1. Introduction

Probiotics, primitively defined as "growth-promoting factors produced by live microorganisms" [1], have been widely utilized in food and medicine industry during the past several decades [2]. As is known, probiotics can be used to improve host's intestinal microbial balance when administered in adequate amounts (FAO/WHO, 2002). Diseases, such as bacterial infection or viral diarrhoea, constipation and inflammatory bowel disease caused by gastrointestinal microbial ecosystem associated disorders, could be prevented or treated with proper complex-probiotic-preparations [3–5]. How to develop a probiotic delivery system? Evidently, an effective vehicle is desirable when designing the targeted probiotic preparation for better human health service.

Recently, scientists are committed to categorize the applied probiotics to speed up the research. Three categories were proposed, namely "category 1" products (e.g. yogurts) for lactose-intolerant individuals, "category 2" products for reducing adverse effects of antibiotics or restoring gastrointestinal microbial balance, and "category 3" products referring in particular to recombinant strains [6]. *Bacillus licheniformis* belonging to the "category 2" is a probiotic suitable for clinical treatment of diarrhoea, such as the Zhengchangsheng® capsules [7]. Normally, probiotics like *Lactobacillus plantarum*, *B. licheniformis* are vulnerable to the gastric juice, especially to the strong gastric acid [8]. Thus, vehicles with favorable performances such as harsh acid resistance and colon specificity are vital to develop novel probiotic products, and polyelectrolyte complexes (PECs) may be the good biomaterials to be chosen for the microcarriers preparation.

Great attentions have been paid to the microcarriers that fabricated with the PECs in recent years [9,10]. PEC could be formed with two or more oppositely charged polyelectrolytes. For instance, the PECs formed with chitosan or its derivatives and alginate (Alg) have been verified to be good candidates for drug or probiotic microcarriers [11–13]. These PECs usually have favorable biological properties, such as excellent biodegradability and good biocompatibility, and thus can be used as challenging probiotic delivery vehicles. Rodklongtan et al. [14] evaluated the Alg-chitosan microcapsules as intestinal probiotic delivery carriers. It was found that the microcapsules with semi-interpenetrating polymer networks could effectively protect the *Lactobacillus reuteri* KUB-AC5

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cells against strong acids during both *in vitro* and *in vivo* studies, which means the microcapsules could potentially be used to design intestine-targeted probiotic delivery systems. De Prisco et al.[15] fabricated *Lactobacillus reuteri* DSM 17938 loaded chitosan-Alg microcapsules via vibrating technology. It was confirmed that the chitosan-Alg matrix could improve the tolerance of *Lactobacillus reuteri* DSM 17938 towards certain stress environments, such as gastrointestinal stress and osmotic stress conditions. Similar results have also been reported by Song et al. [16].

Polysaccharides, especially natural macromolecules with polycationic or polyanionic properties, have been widely explored as functional polyelectrolytes materials for preparation of PECs [17,18]. Among them, chitosan and Alg have become the most popular biomaterials in recent years. Chitosan possesses favorable properties, such as non-toxicity, biocompatibility, biodegradability, mucoadhesivity [19] and antimicrobial activity [20]. Besides, as a water soluble derivative of chitosan, chitosan hydrochloride (CHC) combines the intrinsic properties with chitosan and has excellent water solubility under neutral pH conditions [21]. Hereby no organic solvent is needed during the preparation process, which makes the delivery system much simpler [21]. More importantly, CHC can form PECs with some polyanionic electrolytes, such as, sodium cellulose sulfate (NaCS) [22,23] and sodium caseinate [24], which could possibly be used as microcarriers for drugs or probiotics. As a popular polyanionic electrolyte, Alg may form PEC with CHC based on the similar principle. As is known, Alg is the most commonly used gel material in the industry, prospective to be applied in food and medical field [25,26]. Nevertheless, the poor mechanical strength of Alg microgels to gastric or intestinal peristalsis is one of the usual challenges in applications.

In the present study, CHC-Alg microcapsules were firstly prepared via orifice-polymerization method [27], so as to develop probiotic loaded microcarrier with favorable performances, e.g. high probiotic survival and good environmental endurance. *B. licheniformis* was chosen as the model probiotic while calcium chloride as the cross-linking agent. Based on the PEC formed between CHC and Alg, the cells-free and cells-loaded CHC-Alg microcapsules were fabricated, and their morphologies and thermal properties were characterized. The swelling performances and the survival of the microencapsulated cells under conditions of simulated gastrointestinal fluids were evaluated with i*n vitro* experiments.

2. Materials and methods

2.1. Cells and materials

Probiotic cells of *B. licheniformis* IBL32-a were isolated from Zhengchangsheng® capsules *R*, and cultured in Luria-Bertani (LB) medium. Sodium alginate mainly containing β-1,4-D-mannuronic acid and α-1,4-L-guluronic acid was purchased from Sinopharm Chemical Reagent Co., Ltd. (China). The viscosity of a 1%(w/v) Alg solution is 20 mPa s. CHC with 85.6% degree of deacetylation and M_w of 50.0 kDa was supplied by Jinan Haidebei Co., Ltd. (China). The viscosity of a 1%(w/v) CHC solution is 30 mPa s. All other chemicals and reagents were of analytical grade and used without further purification.

2.2. Preparation of cells-free CHC-Alg microcapsules

The cells-free CHC-Alg microcapsules were prepared via orifice-polymerization method [27]. Briefly, Alg (2%, w/v), $CaCl_2$ (6%, w/v) and CHC (0.5%, w/v) solutions were prepared with distilled water, and ultrasonicated for 30 min, respectively. The Alg solution (2 ml) was added dropwise into the $CaCl_2$ solution (20 ml) using a constant flow pump (SPLab02, Baoding Shenchen Precision Pump Co., Ltd. China) (650 μl/min). After reaction for 15 min at room temperature under stirring, the microgels were separated and rinsed with distilled water and then added into CHC solution (20 ml) to react under the same conditions. The cells-free CHC-Alg microcapsules were obtained and freeze-dried (Freeze Drier, FD-1A-50, Beijing Boyikang Laboratory Instruments, Co., Ltd. China) for 12 h.

2.3. Preparation of cells-loaded CHC-Alg microcapsules

The model cells were cultured to late exponential phase with LB medium at 37 ± 0.5 °C, 200 rpm for 4 h, then collected by centrifugation at 4 °C, 6000 rpm for 20 min, and then suspended in sterilized NaCl solution (0.85%, w/v). Alg (2%, w/v), $CaCl_2$ (6%, w/v) and CHC (0.5%, w/v) solutions were also prepared as mentioned above and then filtered through a 0.45 μm membrane filter. The cells suspension and Alg solution were well-mixed under sterile conditions. The cells-loaded CHC-Alg microcapsules were prepared using the procedures as described in section 2.2.

2.4. Morphology of CHC-Alg microcapsules

The morphology of the cells-free and cells-loaded CHC-Alg microcapsules was observed with scanning electron microscope (SEM, S-4800, Hitachi, Japan). The dried microcapsules were sputter-coated with gold before observation. Some samples were cut in half with a blade to observe the inner structures.

2.5. Thermo-gravimetric studies of cells-free CHC-Alg microcapsules

The thermal properties of cells-free CHC-Alg microcapsules were investigated by thermo-gravimetric analysis (TGA, Perkin-Elmer Pyris-1, USA). Samples of cells-free microcapsules (3–13 mg) were heated from 50 to 800 °C at a speed of 10 °C min^{-1} under nitrogen purging. The moisture content (%) was estimated from weight losses over the beginning to 100 °C.

2.6. Differential scanning calorimetry studies of cells-free CHC-Alg microcapsules

Differential scanning calorimetry (DSC, Perkin Elmer Pyris-1, USA) analyses of cells-free CHC-Alg microcapsules were also performed. Samples of cells-free microcapsules (5–7 mg) were sealed in aluminium pans and loaded in sample cells under nitrogen purging, and then scanned from 50 to 300 °C at 10 °C min^{-1}.

2.7. Swelling studies of cells-free CHC-Alg microcapsules

The swelling studies of the cells-free CHC-Alg microcapsules were performed in simulated gastric fluid (SGF, pH 2.0) and simulated intestinal fluid (SIF, 0.3%), respectively. The SGF was prepared with 0.85% (w/v) NaCl solution and regulated to pH 2.0 with HCl while the SIF prepared with 0.3% (w/v) bile salts. The cells-free CHC-Alg microcapsules (10 mg) were incubated in SGF and SIF (20 ml), respectively, and maintained at 37 ± 0.5 °C, 100 rpm up to 8 h. The sample microcapsules were taken out from the fluids at predetermined time intervals and an equal volume of fresh fluid was supplemented into the fluids. The excess water on the surface of the sample microcapsules was absorbed by filter papers, and the sample microcapsules were quickly weighed to a constant weight and then put back into the previous fluids for further studies. The following equation was used to calculate the swelling ratio (SR%):

$$SR\% = \left[(Wwt - W0)/W0\right] \times 100\% \quad (1)$$