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An improved pH-responsive carrier based on EDTA-Ca-alginate for oral delivery of *Lactobacillus rhamnosus* ATCC 53103

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ABSTRACT

A pH-responsive carrier based on an ethylenediaminetetraacetic-calcium-alginate (EDTA-Ca-Alg) system was developed by controlling the release of Ca^{2+}. The system remained in the solution state at neutral pH since EDTA completely chelated the Ca^{2+}. In contrast, a hydrogel immediately formed when the pH was below 4.0, which triggered the in situ release of Ca^{2+} from the EDTA-Ca compound and led to alginate-Ca binding. Taking advantage of the pH sensitivity, we prepared hydrogel microspheres with uniform size to entrap *Lactobacillus rhamnosus* ATCC 53103 through emulsification. In an acidic environment, the hydrogel structure remained compact with negligible pores to protect *L. rhamnosus* ATCC 53103. However, in a neutral intestinal environment, the hydrogel structure gradually disassembled because of the Ca^{2+} release from the hydrogel, which caused cell release. Therefore, a pH-responsive carrier was developed for the protection and the controlled release of cells in gastrointestinal tract, thus providing potential for oral delivery of probiotics.

1. Introduction

Probiotics are defined by the World Health Organization as "live microorganisms which when administered in adequate amounts confer health benefits to the host" (FAO/WHO, 2002). Probiotic bacteria are believed to provide the host with positive health benefits by inhibiting pathogen growth, maintaining health-promoting gut microflora, and stimulating the host's immune response (Figueroa-Gonzalez, Quijano, Ramirez, & Cruz-Guerrero, 2011). With the growing attention to health, studies involving oral administration of probiotic bacteria as nutraceuticals have rapidly increased in recent years (Cook, Tzortzis, Charalampopoulos, & Khutoryanskiy, 2012; Mirzaei, Pourjafar, & Homayouni, 2012). However, the considerable loss of probiotic viability during the critical stages of processing and oral administration is inevitable, especially during gastrointestinal transit, which caused the insufficient survival probiotics upon arrival in the intestinal target (Charteris, Kelly, Morelli, & Collins, 1998). Hence, the retention of a sufficient amount of viable probiotics after administration is still a challenge. Recently, various approaches have been proposed to improve the viability of cells. Microencapsulation, one of feasible methods for cell immobilization owing to the mild preparation process and favorable biocompatibility, is an effective method of protecting probiotic cells from the stresses during processing and gastrointestinal transit (Anal & Singh, 2007; Dong et al., 2013; Yeung, Ucok, Tiani, McClements, & Sela, 2016).

The most common encapsulation agent is alginate (Atia et al., 2016), a linear polysaccharide consisting of 1→4 linked β-(D)-glucuronic (G) and α-(L)-mannuronic (M) acids derived from brown algae or bacterial sources (Augst, Kong, & Mooney, 2006). It is a generally regarded as safe (GRAS) material certified by the Food and Drug Administration (FDA) (George & Abraham, 2006). Alginate gels upon contact with divalent metals (e.g. calcium), forming an "egg box" structure with four G residues (Cook, Tzortzis, Charalampopoulos, & Khutoryanskiy, 2000). For instance, Chandramouli, Kailasapathy, Peiris, and Jones (2004) employed alginate with different concentrations and capsule sizes to embed probiotics, and the result showed the viability of encapsulated bacteria increased with an increase in alginate capsule size and gel concentration. However, owing to the limited stabil-

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ity of alginate hydrogel, many approaches have been employed to improve these oral delivery systems over the years. Cook, Tzortzis, Charalampopoulos, and Khutoryanskiy (2011) utilized chitosan as a coating by forming polyelectrolyte complex (PEC) membranes to increase hydrogel stability. However, the release rate decreased undesirably due to the protective effect of the chitosan coating. Thus, some studies have focused on pH-responsive hydrogels, taking advantage of their different behaviors in gastrointestinal environments. Mei et al. (2014) developed a pH-responsive carrier based on Ca-alginate/protamine microcapsules. Polycationic protamine not only enhances the stability of alginate gels but also exhibits pH-responsive characteristic to guarantee the survival of probiotics in the stomach and their release in the small intestine. However, the introduction of protamine results in both increased cost and a more complicated process; these factors limit industrial production. Moreover, many studies have reported that probiotic microcapsules, which have uneven and oversized diameters, may have poor sensory properties; thus, it is still difficult to use these microcapsules in oral formulations or as additives for food applications (De Prisco & Mauriello, 2016).

In the present study, we successfully fabricated a pH-responsive hydrogel based on an ethylenediaminetetraacetic-calcium-alginate (EDTA-Ca-Alg) system. Due to the reversibility of the EDTA-Ca complexation reaction (Utech et al., 2015), the release of Ca^{2+} could be controlled by pH. This property was used to prepare pH-responsive alginate-Ca hydrogel microspheres to encapsulate *L. rhamnosus* ATCC 53103 by using the emulsification/internal gelation technique. The microspheres with uniform size became denser and more stable in acidic environments and were damaged in neutral pH; these microspheres could protect the cells from the acidic environment of the stomach and subsequently facilitate gradual cell release in the intestinal sections. This controlled-release device with pH-sensitivity can not only eliminate the conflict between stability and controlled release but also provide a new way to produce oral probiotic formulations.

2. Materials and methods

2.1. Cells and materials

L. rhamnosus ATCC 53103 was obtained from American Type Culture Collection (ATCC 53103). Cells were grown in MRS medium with aeration at 37 °C, 180 rpm for 12 h in shaking incubator. The cell suspensions were subsequently used either as free cells or in microencapsulated form as described below. Sodium alginate (460 kDa molecular weight; 2:1 molar ratio of mannuronic acid to guluronic acid) was obtained from Qingdao Crystal Salt Bioscience and Technology Corporation (Qingdao, China). Both edetate disodium dihydrate and calcium chloride were obtained from Aladdin. All other reagents and solvents were of analytical grade.

2.2. Preparation of pH-responsive alginate hydrogel

First, an EDTA-Ca solution was prepared by mixing 100 mM solutions of disodium-EDTA and calcium chloride (1:1 ratio v/v), and the pH was subsequently adjusted (2.0, 3.0, 4.0, 5.0, 6.0 and 7.0) using sodium hydroxide or hydrochloric acid. The alginate solutions were prepared by dissolving sodium alginate (2 wt%) in deionized water and mixing the solution with 0.1% Dextran blue to form a blue solution. Then, the alginate and EDTA-Ca solution were mixed in a 1:1 ratio (v/v).

2.3. Rheological characterization

The storage moduli (G′) of the hydrogel disks (30 mm in diameter and 2 mm thickness, three samples for each of pH conditions) were measured with a rheometer fitted with parallel dentate anti-skid plates (MCR302, Anton Paar, Austria) by a fixed strain (1.0%) and an angular frequency sweep from 0.01 rad s^{-1} to 100 rad s^{-1}. The samples were loaded on the working stage by a blade and the kinetics of gelation was determined by three repeated measurements of G′ at T = 20 °C at intervals of 2 min. The elastic modulus E of the hydrogel was calculated from the data of the first 1.0% of the strain. The solution viscosity was measured using Brookfield rotational viscometers (DV- II +P, America).

2.4. Preparation of alginate-Ca microspheres entrapping L. rhamnosus ATCC 53103 cells by an emulsification/internal gelation technique

The alginate-Ca microspheres were prepared as described previously with some modifications (Song, Yu, Gao, Liu, & Ma, 2013). 10 mL of an EDTA-Ca-Alg mixture (including the final concentration of 1% w/v alginate and 50 mM EDTA-Ca, pH = 7.2) containing 1×10^{10} CFU/mL *L. rhamnosus* ATCC 53103 was dispersed in 50 mL liquid paraffin containing 0.5% (v/v) Span 85 to form an emulsion by stirring at 200 rpm for 30 min. After emulsification, glacial acetic acid (0.05% v/v) was slowly added to the emulsion and kept for 10 min by stirring at 200 rpm to liberate Ca^{2+} for gelation. The alginate-Ca microspheres were collected by separating funnel and rinsed with distilled water to remove the oil (liquid paraffin) for further experimentation.

2.5. Characterization of size distribution and morphology

The size distribution of microspheres was determined in distilled water by static light scattering using a Mastersizer 2000 (Malvern Instruments, Worcestershire, England), with a size range from 0.2 to 2000 μm.

A confocal laser scanning microscope (CLSM, Leica TCS-SP2, Germany) equipped with a blue laser sources (Ar 488 nm/5 mW) and an inverted microscope (Leica, DMIRE2, Germany), was employed to characterize the morphology of cells in the microspheres, which were stained with the LIVE/DEAD Baclight™ Bacterial Viability Kit (L7012, Invitrogen).

The alginate-Ca hydrogels formed under different pH conditions and alginate-Ca microspheres encapsulating *L. rhamnosus* ATCC 53103 were first fixed in paraformaldehyde (4% w/v) for 4 h at room temperature. Then, the samples were dehydrated with an ethanol concentration gradient (30%, 50%, 70%, 90% and 100%, v/v) for 15 min at each concentration. Finally, the samples were dried overnight in a vacuum. The morphology of the alginate hydrogels and the cells encapsulated in microspheres was observed using a Field Emission Scanning Electron Microscope (FE-SEM; JSM-7800F, JEOL, Japan).

2.6. Characterization of mechanical stability

For mechanical stability measurements, the method described in our previous study was adopted with a minor modification (Chen et al., 2014). 1 mL of microspheres, along with 10 mL hydrochloric acid (pH 2.0–6.0) or PBS buffer (pH 7.0–8.0), was added to triangular flasks with 20 agate balls, and then the flasks were shaken at 120 rpm, 37 °C for 2 h. The microspheres were collected and observed by microscopy, and images were captured using a camera. Image J was employed to analyze the diameters of microspheres, and the swelling rate (S_w) and breakage rate (B_r) were calculated as follows:

$$S_w = \left[\left(\frac{D}{D_0}\right)^3 - 1\right] \times 100\%$$