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Novel Intestinal-Targeted Ca-Alginate-Based Carrier for pH-Responsive Protection and Release of Lactic Acid Bacteria

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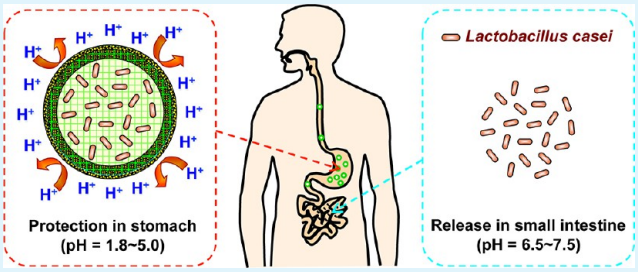
ABSTRACT: A novel intestinal-targeted carrier for pH-responsive protection of lactic acid bacteria in stomach and rapid release of lactic acid bacteria in small intestine is successfully developed. The proposed carrier is composed of a Ca-alginate/protamine (CAP) composite shell and a *Lactobacillus-casei*-encapsulated Ca-alginate (CA) core. The carriers are prepared simply by a coextrusion minifluidic and subsequent adsorption method. The CAP composite shell offers not only improved protection for *Lactobacillus casei* to guarantee the endurance and survival in the stomach but also satisfactory intestinal-targeted characteristics to guarantee the rapid release of *Lactobacillus casei* in the small intestine. In the stomach, where there is an acidic environment, the diffusion channels delineated by the CA networks in the CAP composite shell of the carriers are choked with protamine molecules; as a result, it is hard for the gastric acid to diffuse across the CAP composite shell and thus the encapsulated *Lactobacillus casei* inside carriers can be efficiently protected. However, when they come to the small intestine, where there is a neutral environment, the carriers dissolve rapidly because of the cooperation between protamine and trypsin; consequently, the encapsulated *Lactobacillus casei* can be quickly released. The proposed CAP composite carrier provides a novel mode for developing efficient protection systems, responsive controlled-release systems, and intestinal-targeted drug delivery systems.



KEYWORDS: *pH-responsive carriers, intestinal-targeted probiotics carriers, controlled release, lactic acid bacteria, Ca-alginate (CA), protamine*

INTRODUCTION

Probiotic lactic acid bacteria, as living microorganism supplements, are favored by consumers because of many health benefits on the host. When adequate viable lactic acid bacteria are ingested by the human body, they can play many important functions, such as improving intestinal flora, strengthening immunity, inhibiting tumor growth, promoting nutrient absorption, and so on.[1−5] In daily life, a wide variety of lactic acid bacteria strains are available to consumers in both traditional fermented foods and in supplement forms. After oral ingestion, lactic acid bacteria pass through the mouth, esophagus, and stomach, and finally reach the small intestine and start to exert the functional effect within the body. To fully play the beneficial functions, sufficient viable lactic acid bacteria are required to ensure that they can reach the small intestine. However, lactic acid bacteria are extremely sensitive to low pH gastric acid. If they come into direct contact with the gastric acid in stomach, a large amount of lactic acid bacteria might die before they reach the small intestine.[4,6−10] Therefore, protection of lactic acid bacteria in acidic environments with appropriate methods is of great importance.

Up to now, the main way to protect lactic acid bacteria has been encapsulation.[11−14] Among numerous materials for the encapsulation of lactic acid bacteria, biocompatible alginate is widely used. Many researchers report that encapsulation of lactic acid bacteria in alginate gel can help lactic acid bacteria to resist the gastric acid and guarantee adequate living bacteria to reach the small intestine.[15−21] For example, Lee et al.[21] employ alginate with different concentrations to embed probiotics, and the results show that the death rate of living bacteria in simulated gastric acid decreases and the protection effect improves with the increase of alginate concentration. However, the application of alginate is limited due to its unstable property. So, researchers have made a lot of improvements to improve the stability of alginate. Gombotz et al.[22] report that coating polycationic compound, e.g., chitosan, on the surface of alginate gel can raise the stability of physical and chemical property. Koo et al.[23] also report that the survival of Lactobacillus entrapped in alginate and chitosan can be

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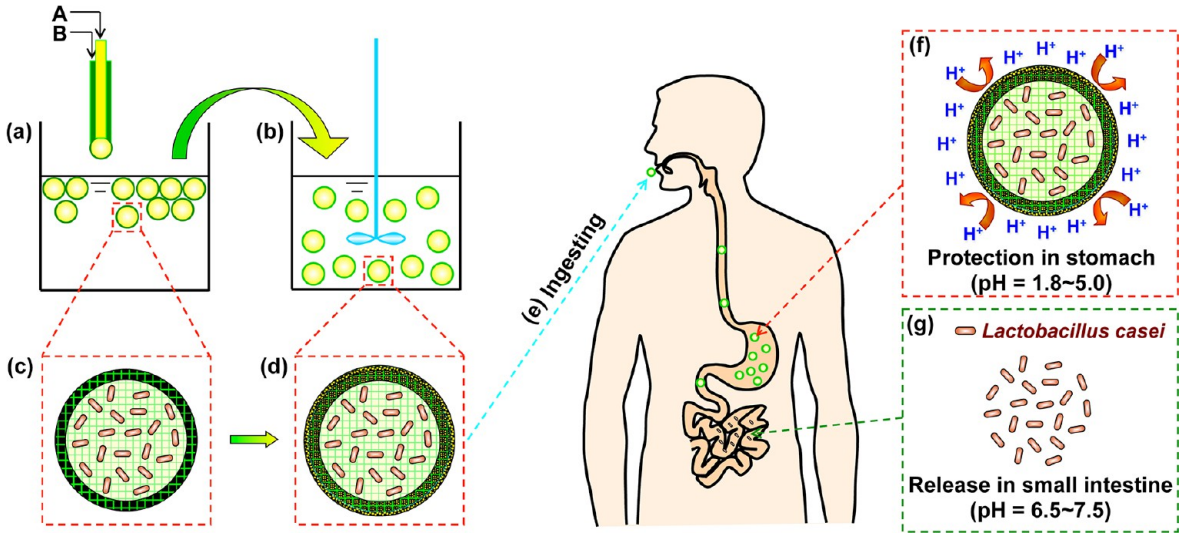

Figure 1. Schematic illustration of the preparation process and the design concept of the proposed intestinal-targeted CAP carrier for pH-responsive protection and release of lactic acid bacteria. (a, c) CA beads prepared by a coextrusion method. "A" is Na-alginate solution containing *Lactobacillus casei*, and "B" is pure Na-alginate solution. (b, d) CAP beads prepared by adsorption of protamine molecules. (e) Ingesting CAP beads in mouth. (f) CAP beads offer improved protection for *Lactobacillus* in stomach. (g) CAP beads release *Lactobacillus casei* rapidly in the small intestine.

increased. Nevertheless, as far as the previous literatures are concerned, with either pure alginate or composite alginate gel with polycationic compound, the mechanism of the protection of lactic acid bacteria is to postpone the contact time with gastric acid relying on the mass transfer resistance provided by the embedding materials. However, such a protection approach causes another problem, i.e., although the protection effect in the stomach is improved to a certain extent, the release rate in the small intestine is undesirably decreased.[24]

In this study, we report a novel intestinal-targeted Ca-alginate (CA)-based carrier for pH-responsive protection of Lactic acid bacteria in stomach and rapid release of lactic acid bacteria in small intestine. The preparation procedure and the proposed concept of the pH-responsive protection and release of Lactic acid bacteria with the intestinal-targeted CA-based carrier are schematically demonstrated in Figure 1. The proposed pH-responsive intestinal-targeted carrier for lactic acid bacteria is designed with core−shell structure, i.e., inner lactic-acid-bacteria-encapsulated CA gel core and outer Ca-alginate/protamine (CAP) composite shell. The carrier is prepared simply by integrating the coextrusion minifluidic and electrostatic adsorption technologies (Figure 1a−d).[25,26] Protamine, as a polycationic peptide, not only enhances the stability of alginate property, but also exhibits pH-responsive characteristics for the improved protection of lactic acid bacteria in stomach and the rapid release of Lactic acid bacteria in small intestine due to the electrostatic interactions between CA networks and protamine molecules.[26] When the proposed Lactic acid bacteria-encapsulated carriers are ingested by mouth and go through esophagus, they enter into stomach (Figure 1e). In the stomach, the environmental pH is usually extremely low; therefore, the diffusion channels delineated by the electrically neutral CA networks due to the protonation of carboxyl groups in the CAP composite shell of the carriers are "choked" with protamine molecules because of the electrostatic repulsion between the positively charged protamine molecules, so that the diffusion channels across the carrier shell are in the "closed" state.[26] As a result, it is hard for the low pH gastric acid in stomach to diffuse into the carrier, and thus the Lactic acid bacteria in the carrier are efficiently protected (Figure 1f). Later, when the lactic-acid-bacteria-encapsulated carriers come into small intestine where the ambient pH value is neutral, the carriers dissolve rapidly under the cooperation between protamine and trypsin; consequently, the encapsulated lactic acid bacteria are quickly released in the small intestine (Figure 1g). Thus, the pH-responsive characteristics provided by the proposed CA-based carrier ensure that the encapsulated lactic acid bacteria can be not only protected in stomach efficiently but also delivered in the small intestine rapidly.

EXPERIMENTAL SECTION

Materials. Protamine sulfate from salmon (P4380) was purchased from Sigma. Apoptosis assays kit C1056 was obtained from Biyuntian Biotechnological Institute. Vitamin B_{12} (VB_{12}) was purchased from Shanghai Yuanju Bio S&T Co.. All other chemicals, including sodium alginate (Na-alginate), calcium nitrate, pepsin, trypsin, sodium citrate, sodium chloride, hydrochloric acid (HCl), sodium hydroxide (NaOH), were of analytical grade. Sterile water and sterile operation were used throughout the experiments.

Microorganism and Solution. *Lactobacillus casei* CICC 23185 from National Center for Preservation of China was used throughout the experiments. MRS medium was prepared by dissolving Tween 80 (1 g), casein peptone (10 g), beer extract (10 g), yeast extract (5 g), glucose (5 g), sodium acetate (5 g), ammonium citrate (2 g), KH_2PO_4 (2 g), $MgSO_4{\cdot}7H_2O$ (0.2 g), $MnSO_4{\cdot}H_2O$ (0.05 g) into water, adjusting pH to 6.8, and metering volume to 1000 mL. Normal saline was 0.9% w/v NaCl solution. Simulated gastric acid (pH 1.2) was prepared by diluting 234 mL of HCl to 1000 mL, taking out 16.4 mL of HCl solution into 800 mL of water, adding 3 g of pepsin, and metering volume to 1000 mL. Simulated gastric acid (pH 2.5) was prepared by adjusting the pH of the prior solution to 2.5 with 0.1 mol/L NaOH solution. Simulated intestinal fluid was prepared by dissolving 6.8 g of KH_2PO_4 into 500 mL of sterile water and adjusting pH to 7.0 with 0.1 mol/L NaOH solution, dissolving 10 g of trypsin in a small amount of water, and then mixing the two solutions and metering the volume to 1000 mL.

Preparation of Intestinal-Targeted CA-Based Carriers for pH-Responsive Protection of Lactic Acid Bacteria. *Lactobacillus casei* CICC 23185 was selected as a model lactic acid bacteria to be encapsulated in the pH-responsive intestinal-targeted carriers. Preparation of the *Lactobacillus-casei*-encapsulated carriers includes

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two steps (Figure 1). In the first step, the *Lactobacillus-casei*-encapsulated CA beads were continuously prepared by a coextrusion minifluidic approach with a simple capillary apparatus (Figure 1a).[25−27] There are two main reasons to employ the coextrusion minifluidic apparatus. The first is to guarantee the monodispersity of CA beads. The second is to ensure that no *Lactobacillus casei* is distributed in the shells of Lactobacillus-casei-encapsulated CAP beads in order to avoid bacteria contacting with gastric acid and reduce the death rate of the bacteria. Briefly, *Lactobacillus casei* CICC 23185 were activated twice in MRS solid medium and cultured in MRS liquid medium at 37 °C for 2 days. After centrifugation, 1×10^7 CFU/mL *Lactobacillus casei* solution was mixed with 1% w/v or 2% w/v Na-alginate solution, respectively, which was used the inner fluid. The outer fluid was a 2% w/v Na-alginate solution. The outer and inner fluids were pumped into the outer cylindrical tube and inner square tube at flow rate of 10 and 40 mL/h, respectively. At the nozzle end of the capillary apparatus, water-in-water core−shell droplets were formed at room temperature. Subsequently, the droplets were dripped into a $Ca(NO_3)_2$ solution dropwise, and the gelated *Lactobacillus-casei*-encapsulated CA beads were fabricated.

In the second step, the protamine molecules were adsorbed onto the *Lactobacillus-casei*-encapsulated CA beads to prepare the pH-responsive CAP beads (Figure 1b). At room temperature, a number of *Lactobacillus-casei*-encapsulated CA beads were put into 2.5 mg mL^{-1} protamine solution at pH 5. To achieve satisfactory pH-responsive performance of the beads, the amount of protamine molecules was excessive in the adsorption experiments. The adsorption process lasted 30 min with continuous stirring. The resultant *Lactobacillus-casei*-encapsulated CAP beads were washed with pure water three times and preserved in normal saline at 4 °C.

Morphological Analyses. A digital camera (DMC-LX5GK, Panasonic) was used to observe the morphology of the *Lactobacillus-casei*-encapsulated CA beads and CAP beads in pure water. The size distribution of more than 200 beads in pure water was evaluated by a coefficient of variation (CV), defined by eq 1.

$$CV = 100\% \times \left(\sum_{i=1}^{N} \frac{(D_i - \bar{D})^2}{N - 1} \right)^{1/2} / \bar{D} \tag{1}$$

where D_i is the diameter of the ith bead (m), N is the total number of the beads counted, and $\bar{D}$ is the arithmetic average diameter (m). The diameters of beads in pure water were determined by image analysis software (DP2-BSW, Olympus).

A scanning electron microscope (SEM) (G2 Pro, Phenom) was employed to study the microstructures of the beads in dry state. The beads for SEM observation were first freeze-dried for 48 h, then frozen in liquid nitrogen for 10 min, and fractured mechanically, and finally sputter-coated with gold for 40 s before the observation.

A fluorescence microscope (Carl Zeiss AxioScope Al) was used to study the distribution of the *Lactobacillus casei* in the beads. The beads for fluorescence microscope observation were first washed with pure water for several times and then immersed in apoptosis assays kit C1056 solution at room temperature for at least 24 h. The *Lactobacillus casei* presented blue fluorescence. The excitation wavelength was 346 nm and the emission wavelength was 460 nm.

pH-Responsive Controlled-Release Experiments in Simulated Gastric Acid. Vitamin B_{12} (VB_{12}) was used as a model solute to investigate the diffusional permeability of solutes across the shells of *Lactobacillus-casei-encapsulated* CA beads and the pH-responsive *Lactobacillus-casei-encapsulated* CAP beads at 37 °C. Before the VB_{12} diffusion experiments, a certain number of beads were immersed in a VB_{12} buffer solution at room temperature for at least 72 h. The VB_{12} buffer solution was composed of VB_{12} and pure water. The concentration of VB_{12} was 0.4 mmol L^{-1} and the pH was 5.5. The VB_{12} buffer solution was refreshed periodically to ensure the inner spaces of beads were filled with the buffer solution. At the beginning of the VB_{12} diffusion experiment, the VB_{12} loaded beads (as the donor for diffusion) were immersed into 100 mL of pH 1.2 or pH 2.5 simulated gastric acids (as a receptor for diffusion) at 37 °C. At regular intervals, the concentration of VB_{12} in the surrounding medium was analyzed by using an UV−vis spectrometer (UV-1700, Shimadzu) at a wavelength of 361 nm. Each concentration of VB_{12} at regular intervals was measured at least three times, and the arithmetical mean value was calculated. The diffusional permeability coefficient (P) of VB_{12} across the shells of beads was determined by the concentration increase of VB_{12} in the surrounding medium with time, and calculated with the following equation[27]

$$P = \frac{V_s V_m}{A(V_s + V_m)t} \ln\left(\frac{C_f - C_i}{C_f - C_t} \right) \tag{2}$$

where C_i, C_t, and C_f are the initial, intermediary (at time t) and final concentrations of solute in the surrounding medium respectively (mol L^{-1}), the parameters V_m and V_s are the total volume of beads and the volume of the surrounding medium respectively (L), A is the total surface area of beads (m^2), and t is the time (s).

All the plots of $\ln[(C_f - C_i)/(C_f - C_t)]$ versus t at different pH values were straight lines, and the diffusional permeability coefficient values were proportional to the slopes of the straight lines and could be calculated by eq 3:

$$P = \frac{1}{6} \bar{D} \frac{V_s}{(V_s + V_m)} K \tag{3}$$

where $\bar{D}$ is the average diameter of VB_{12}-loaded beads (m), and K is the slope of the straight line of $\ln[(C_f - C_i)/(C_f - C_t)]$ versus t at each pH value.

Endurance Experiments in Simulated Gastric Acid. To investigate the pH-responsive protection effect of CAP beads, a certain amount of free *Lactobacillus casei*, *Lactobacillus-casei-encapsulated* CA beads and *Lactobacillus-casei-encapsulated* CAP beads were individually immersed into 15 mL of pH 2.5 simulated gastric acid solutions at 37 °C for 2 h with continuous oscillation. At regular intervals, the same amount of free *Lactobacillus casei* solution, *Lactobacillus-casei-encapsulated* CA beads and *Lactobacillus-casei-encapsulated* CAP beads were shifted into 15 mL of 0.06 mol/L sodium citrate solution and the beads were dissolved under continuous oscillation. At last, the viable count of *Lactobacillus casei* was tested by plate count method and the survival was the ratio of viable count and whole count.

Dissolution Experiments in Simulated Gastric Acid and Intestine Fluid. To investigate the intestinal-targeted characteristics, we employed pH 2.5 HCl solution and pH 7.0 NaOH solution as the simulated gastric acid and intestine fluid. A certain number of *Lactobacillus-casei-encapsulated* CA beads and *Lactobacillus-casei-encapsulated* CAP beads were individually immersed into 50 mL of pH 2.5 simulated gastric acid solutions at 37 °C for 2 h with continuous oscillation. At regular intervals, the concentration of *Lactobacillus casei* in the surrounding medium was analyzed by UV−vis at a wavelength of 600 nm. Then, the beads were quickly shifted from pH 2.5 simulated gastric acid to pH 7.0 intestine fluid, and the concentration change of *Lactobacillus casei* with time was measured using the above-mentioned method until *Lactobacillus casei* were released completely. The whole process was recorded by a digital camera. The release rate was determined as the percentage of released *Lactobacillus casei* in the surrounding medium within the whole *Lactobacillus casei* in the carriers by measuring the interval concentration of *Lactobacillus casei* in the surrounding medium.

To investigate if the cooperation between trypsin and protamine results in the fast intestinal release, we employed the release experiment using the same buffer without trypsin as a control.

In all experiments, three repeated trials were studied to obtain the mean values and standard deviations.

RESULTS AND DISCUSSION

Morphological Analyses. Figure 2 shows the optical photographs of monodisperse *Lactobacillus-casei-encapsulated* CA beads and *Lactobacillus-casei-encapsulated* CAP beads in pure water. The *Lactobacillus-casei-encapsulated* CA beads are composed of transparent shell and milky core (Figure 2a), in

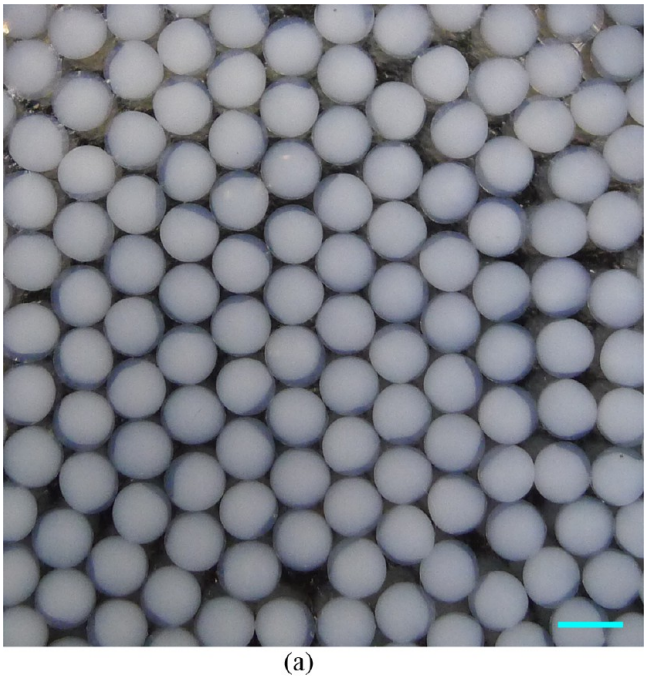

Figure 2. Optical photographs of (a) CA beads and (b) CAP beads in pure water at 25 °C. Scale bars are 4.0 mm.

which the milky core is caused by *Lactobacillus casei*. After the adsorption of protamine molecules onto CA beads, the *Lactobacillus-casei-encapsulated* CAP beads become opaque and slightly bigger than the original *Lactobacillus-casei-encapsulated* CA beads (Figure 2b). The average diameters of *Lactobacillus-casei-encapsulated* CA beads and *Lactobacillus-casei-encapsulated* CAP beads are 3.8 mm and 4.3 mm, respectively, and the coefficient of variation (*CV*, calculated by eq 1) values are 2.7 and 2.6%, respectively, which indicated that the size distributions are very narrow.

Figure 3 shows the SEM images of the cross-sectional views of a typical *Lactobacillus-casei-encapsulated* CAP bead. The cross-section of the *Lactobacillus-casei-encapsulated* CAP bead exhibits irregular loose microstructure due to freeze-drying (Figure 3a). Just as designed, no *Lactobacillus casei* is distributed in the shell of *Lactobacillus-casei-encapsulated* CAP bead in order to avoid bacteria contacting with gastric acid and reduce the death rate (Figure 3b), whereas a large amount of *Lactobacillus casei* are distributed in the CA core of CAP bead, which provides a good biocompatible growing environment for *Lactobacillus casei* (Figure 3c).

Figure 4 shows the fluorescence image of the cross-sectional view of *Lactobacillus-casei-encapsulated* CAP beads. No blue fluorescence exists in the shell and a large amount of blue fluorescence presents in the core of the CAP bead, which again verify the above-mentioned distribution of *Lactobacillus casei* inside the CAP beads.

pH-Responsive Controlled-Release Characteristics in Simulated Gastric Acid. The interactions between alginate and protamine affected by the pH have been discussed in detail in a published paper.[26] When the ambient pH is higher than the critical pH value of CA ($pH_{critical}$, ~4.5), CA networks are negatively charged. The isoelectric point of protamine ($pI_{protamine}$) has been reported to be 10−12. Therefore, when the ambient pH is higher than the $pH_{critical}$ value, the positively charged protamine molecules are adsorbed onto the negatively charged CA networks in the CAP composite shell because of the electrostatic attraction, which results in free spaces delineated by the CA networks in the shell. Thus, the resistance to the solute diffusing across the shells is low, and as a result the diffusional permeability coefficient of the solute across the shells of CAP beads is high. On the contrary, when the ambient pH is lower than the $pH_{critical}$ value, CA networks become electrically neutral while the protamine molecules are still positively charged. In this case, the protamine molecules are desorbed from the CA networks because the electrostatic attraction between CA networks and protamine molecules does not exist anymore. Because of the electrostatic repulsion between the positively charged protamine molecules, the interspaces delineated by the CA networks in the shell are filled with protamine molecules. As a result, the resistance for the solute diffusing across the shell is high, and the diffusional permeability coefficient of the solute across the shell is low.[26]

The pH-responsive diffusional permeation characteristics of the solute VB_{12} across the shells of CA beads and CAP beads are systematically investigated in pH 1.2 and pH 2.5 simulated gastric acids at 37 °C. Figure 5 shows the diffusional permeation characteristics of VB_{12} across the shells of CA beads and CAP beads with time in pH 1.2 and pH 2.5 gastric acids. In pH 1.2 gastric acid (Figure 5a), the slopes of the two straight lines of $\ln[(C_f - C_i)/(C_f - C_t)] \approx t$ are very close to each other, which indicate that the diffusional permeation characteristics of VB_{12} across the shells of CA beads and CAP beads are almost the same, whereas in pH 2.5 gastric acid (Figure 5b), the slope of the straight line of CAP beads is much lower than that of CA beads, which indicates that in pH 2.5 gastric acid, the diffusion of VB_{12} molecules across the shell of CAP beads is much more difficult than that across the shell of CA beads. Although it has been reported that alginate gel is also pH sensitive, which shrinks at lower pH and swells at higher pH,[28] the shrinking of CA does not significantly affect the mass-transfer characteristics of VB_{12} molecules across the shell of CA beads. However, the diffusion of VB_{12} molecules across the shell of CAP beads is much more difficult than that across the shell of CA beads. Therefore, it can be concluded that the pH-sensitive swelling/shrinking behavior of alginate nearly does not contribute to the pH-responsive diffusion of solute molecules

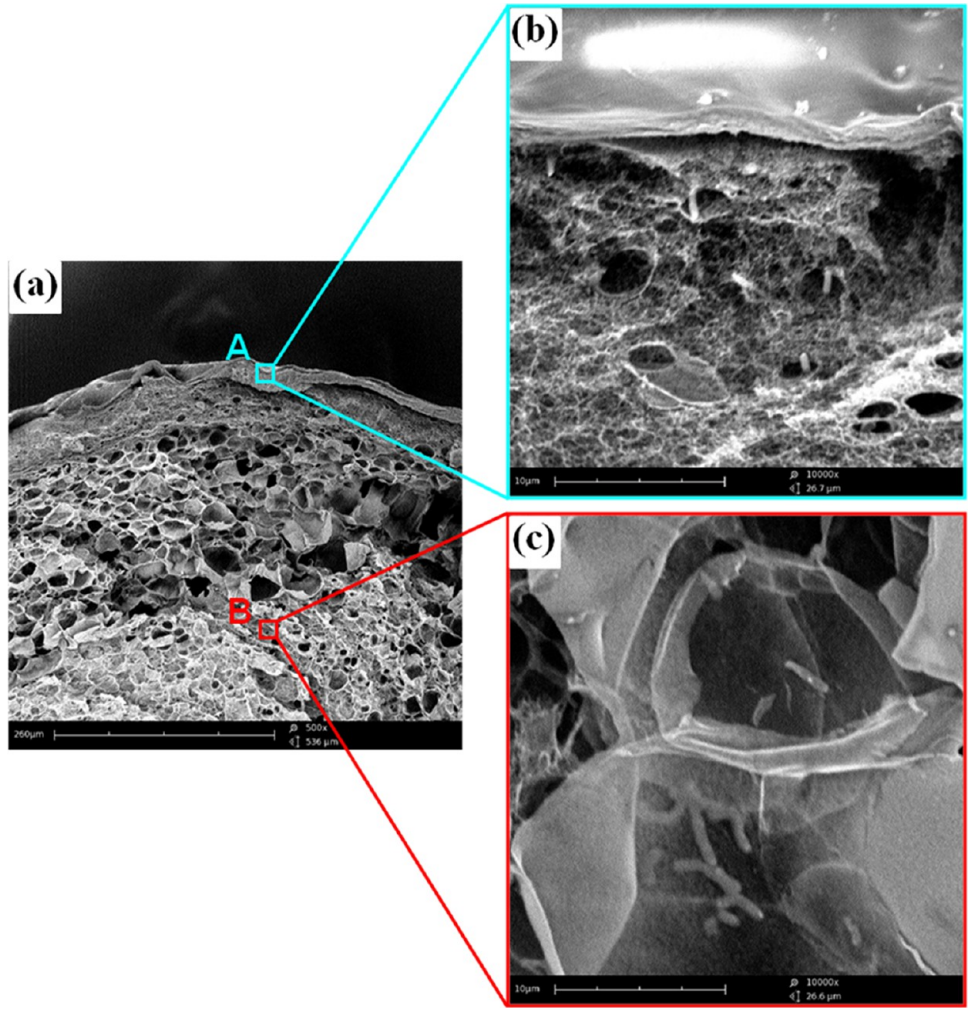

Figure 3. SEM images of the cross-sectional views of a typical CAP bead. (a) Cross-sectional view of a CAP bead. (b) Enlarged view of "site A" ("shell" zone) in a. (c) Enlarged view of "site B" ("core" zone) in a. Scale bar in a is 200 μm, and those in b and c are 10 μm.

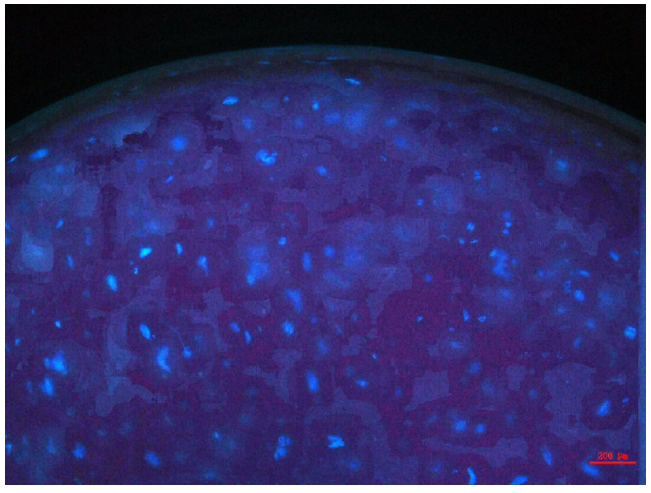

Figure 4. Fluorescence image of cross-sectional view of a typical CAP bead. Scale bar is 200 μm.

across the bead shells, and the shells of CAP beads mainly contribute to the pH-responsive protection for *Lactobacillus casei* surviving in gastric acid.

Figure 6 shows the effect of the pH value on the diffusional permeability coefficient of CA beads and CAP beads (P, calculated by eqs 2 and 3) in pH 1.2 and pH 2.5 gastric acids. The P values of VB_{12} across shells of CA beads and CAP beads in pH 1.2 are 11.93 and 12.53 μm s^{-1}, respectively. In a pH 1.2 environment, the protamine molecules are degenerated by the strong acid; as a result, the pH-responsive characteristics formed by protamine molecules and CA gels cannot be active any more. So, just like that of CA beads, the shell of CAP beads cannot provide improved protection for *Lactobacillus casei* in pH 1.2 gastric acid either. However, in pH 2.5 gastric acid, the P values of VB_{12} across shells of CA beads and CAP beads are 13.68 and 0.61 μm s^{-1}m respectively. The P value of CA beads is 20 times larger than that of CAP beads. Obviously, the shells of CAP beads provide excellent protection function, and the diffusional mass transfer across shells of CAP beads in pH 2.5 gastric acid is much slower than that of CA beads. When the ambient pH is 2.5, the protamine molecules can maintain their properties and the diffusion channels delineated by the electrically neutral CA networks are "choked" with protamine molecules because of the electrostatic repulsion between the positively charged protamine molecules; therefore, the diffusion channels are in the "closed" state (Figure 1f). Consequently, the P value of VB_{12} across shells of CAP beads is much smaller than that of CA beads. Thus, the strong acid can be efficiently prevented from entering into the CAP beads in pH 2.5 gastric acid environment.

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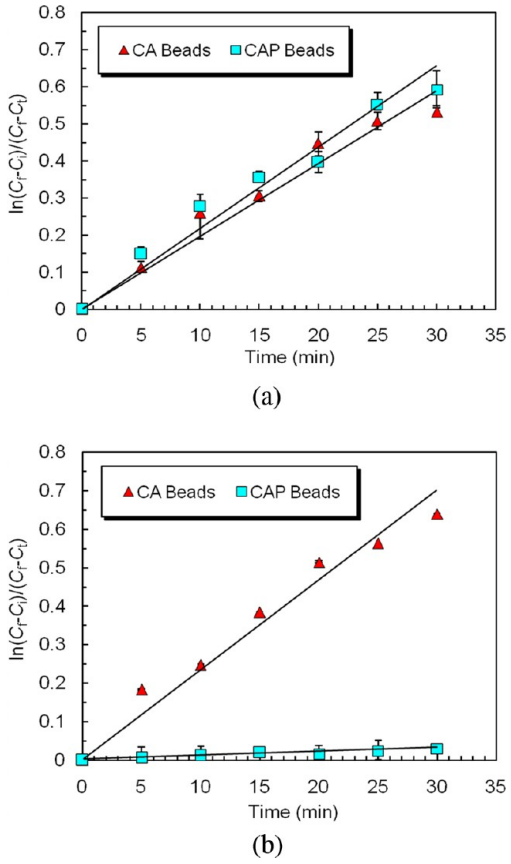

Figure 5. Plots of the diffusional permeation characteristics of VB_{12} across the shells of CA beads and CAP beads with time in (a) pH 1.2 and (b) pH 2.5 simulated gastric acids.

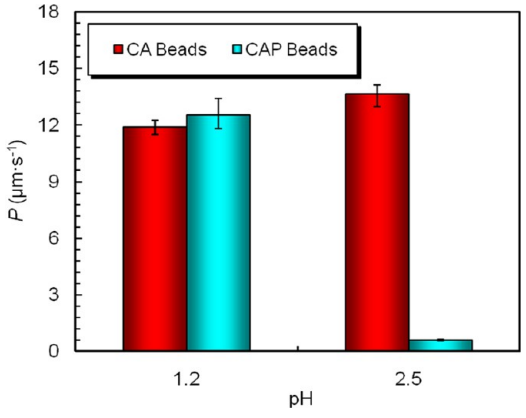

Figure 6. Effect of the pH value on the diffusional permeability coefficient (P) of VB_{12} across the shells of CA beads and CAP beads in simulated gastric acids.

Protection Characteristics in Simulated Gastric Acid. Along with the change of food ingredients, food intake, and human body, the pH value of the gastric acid in the human body is usually about 1.8−5.0.[29] According to the above-mentioned results of VB_{12} diffusional experiments, the pH 2.5 simulated gastric acid is chosen as the model gastric acid to investigate the protection characteristics of *Lactobacillus casei*.

Figure 7 shows the viable count and survival of free *Lactobacillus casei* and *Lactobacillus casei* encapsulated in CA beads after immersed in pH 2.5 gastric acid for 1 and 2 h. For free *Lactobacillus casei*, after being immersed in gastric acid for 1 h, no viable bacteria persist any more. The results indicate that *Lactobacillus casei* is very sensitive to gastric acid. For *Lactobacillus casei* encapsulated in CA beads after immersed in pH 2.5 gastric acid for 1 and 2 h, the viable counts are 1.89×10^6 and 0.078×10^6 CFU/mL, respectively, and the survivals are 18.96 and 0.78%, respectively. Obviously, the CA gels offer certain protection to *Lactobacillus casei* in pH 2.5 gastric acid, but the protection effect is unsatisfactory.

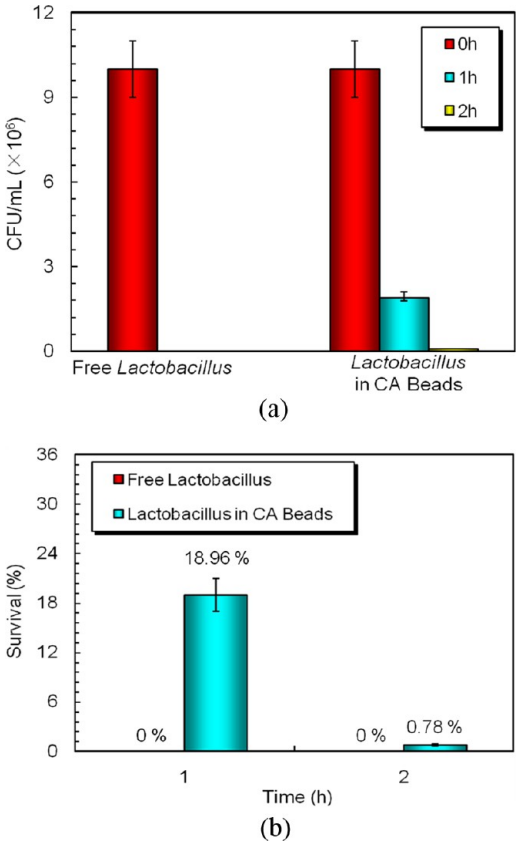

Figure 7. (a) Viable count and (b) survival of free *Lactobacillus* and *Lactobacillus* in CA beads after being immersed in pH 2.5 gastric acid.

Figure 8 shows the viable count and survival of *Lactobacillus casei* encapsulated in CAP beads after immersed in pH 2.5 gastric acid for 1 and 2 h, in which two kinds of beads are prepared with 1% w/v and 2% w/v Na-alginate in the inner fluids, respectively. After immersed in pH 2.5 gastric acid for 1 and 2 h, the viable counts of *Lactobacillus casei* encapsulated in CAP beads with 1% w/v Na-alginate in the inner fluids are 3.86×10^6 and 0.97×10^6 CFU/mL, respectively, and the survivals are 38.63 and 9.63%, respectively, whereas the viable counts with 2% w/v Na-alginate in the inner fluids are 4.66×10^6 and 3.25×10^6 CFU/mL, respectively, and the survivals are 46.57 and 32.41%, respectively. Comparing with that of CA beads, the protection effect of CAP beads increases to a large extent. With increasing the concentration of Na-alginate in the inner fluid, the protection effect of CAP beads also increases to some extent. As mentioned above, when the ambient pH is 2.5, the diffusion channels in the shells of CAP beads are in the "closed" state; as a result, the strong gastric acid is prevented from entering into the CAP beads. Therefore, the shells of CAP beads provide improved protection for the encapsulated *Lactobacillus casei*.

During the adsorbing process, the protamine molecules are not only adsorbed on the surface of CA beads but also inside

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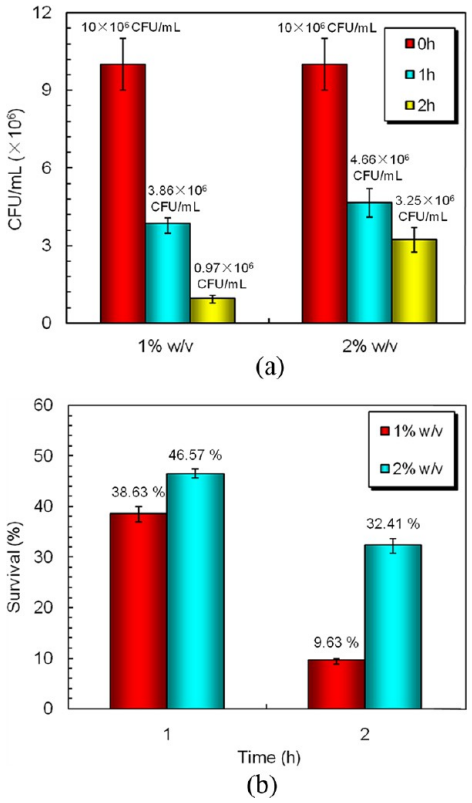

Figure 8. (a) Viable count and (b) survival of *Lactobacillus* in CAP beads prepared with 1% w/v and 2% w/v Na-alginate solutions after immersed in pH 2.5 gastric acid.

the CA networks, and those inside the CA networks mainly contribute to the pH-responsive performance of CAP beads. However, it is hard to accurately distinguish the exact portion that inside the CA networks from the total adsorbed amount of protamine molecules. Therefore, the accurate data on the effect of the amount of protamine molecule per volume of CAP on the pH-responsive performance of the beads are not available yet.

Generally, the pH value of gastric acid in stomach of human body is about 1.8−5.0.[29] In the designed system, when the ambient pH is lower than the $pH_{critical}$ value of CA networks (~4.5), the diffusional permeability coefficient of the solute across the shell of CAP beads is low; as a result, the beads offer the pH-responsive protection to encapsulated *Lactobacillus casei*. On the contrary, when the ambient pH is higher than the $pH_{critical}$ value of CA, the diffusional permeability coefficient of the solute across the shells is high. In this case, although the shell of CAP beads cannot significantly prevent gastric acid from entering the beads, the *Lactobacillus casei* will not be seriously affected by the gastric acid with pH >4.5. Therefore, the CAP beads can offer efficient protection for *Lactobacillus casei* at a pH value ranging from 1.8 to 5.0. However, for the fasted state of the stomach where the pH is as low as 1−1.2, the CAP beads cannot offer improved pH-responsive protection for lactic acid bacteria because of the degradation of protamine as mentioned above.

Intestinal-Targeted Characteristics in Simulated Intestinal Fluid. The intestinal-targeted characteristics of CAP beads are investigated by studying the dissolution of beads in simulated gastric acid and intestine fluid. Figure 9 shows the release rate of *Lactobacillus casei* from CA beads and CAP beads

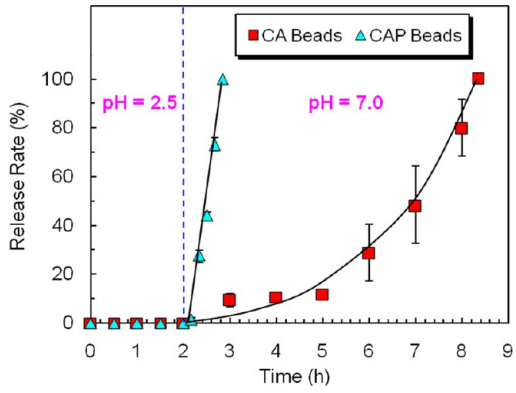

Figure 9. Release rate of *Lactobacillus* from CA beads and CAP beads after being immersed in pH 2.5 gastric acid and pH 7.0 intestinal fluid in turn.

after immersed in pH 2.5 gastric acid for 2 h and then pH 7.0 intestinal fluid. In pH 2.5 simulated gastric acid, no *Lactobacillus casei* are released. However, the lack of release does not mean that the *Lactobacillus casei* in CA beads and CAP beads are protected. Although no bacteria are released during exposure to gastric conditions, the gastric acid can diffuse into the shell of the beads and kill some *Lactobacillus casei*. Especially, for CA beads, the gastric acid can easily diffuse into the beads and the rate of survival of the bacteria is very low (Figure 7). However, because of the pH-responsive protection characteristic of CAP beads, it is hard for the gastric acid to diffuse into the beads and the rate of survival of the bacteria is much higher (Figure 8). In pH 7.0 simulated intestinal fluid, it takes about 380 min for the CA beads to release all of the encapsulated *Lactobacillus casei*; however, it takes only about 50 min for the CAP beads to release all of the encapsulated *Lactobacillus casei*. The results demonstrate that the CAP beads exhibit much more satisfactory intestinal-targeted delivery characteristics than the CA beads.

The photos in Figure 10 exhibit the dissolution processes of CA beads and CAP beads in pH 7.0 intestine fluids. The CA bead gradually swells first and then disintegrates at last at about 380 min, while the CAP bead dissolves gradually from the beginning, and the whole dissolution process takes about only 50 min, which is much shorter than that of the CA bead. In the release experiments using the same buffers without trypsin, the results show that the CAP beads cannot completely dissolve for more than 1 day. Therefore, it can be concluded that the fast intestinal release is the result of the cooperation between trypsin and protamine. It has been reported that the process of trypsin degrading substrates need to utilize Ca^{2+}.[30,31] So, when the CA beads enter the small intestine, the disintegration phenomenon of CA gels will occur just because the Ca^{2+} are used by trypsin. For the CAP beads, the protamine molecules adsorbed on the CA gels offer more substrates to trypsin and the degradation process of trypsin needs more Ca^{2+} than that in the case of CA beads. Consequently, the CAP beads dissolve much more quickly than the CA beads, and thus *Lactobacillus casei* are released in small intestine much more quickly.

CONCLUSIONS

A novel intestinal-targeted CA-based carrier for pH-responsive protection of lactic acid bacteria in stomach and rapid release of Lactic acid bacteria in small intestine has been successfully developed. The proposed carrier is composed of inner *Lactobacillus-casei*-encapsulated CA core and outer CAP

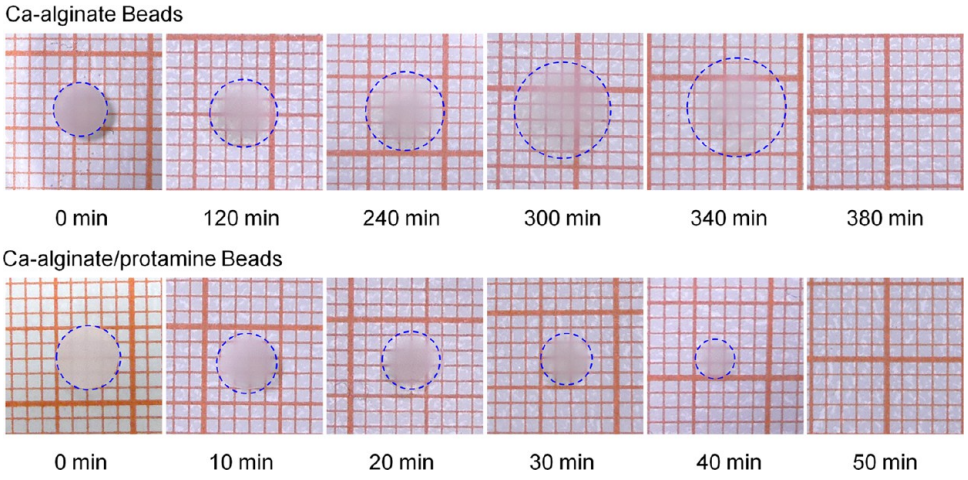

Figure 10. Photographs of dissolution processes of CA beads and CAP beads after being immersed in pH 7.0 intestinal fluids.

composite shell. The CAP composite shell of the carrier offers not only improved protection for *Lactobacillus casei* to guarantee the endurance and survival of *Lactobacillus casei* in the stomach but also satisfactory intestinal-targeted characteristics to guarantee the rapid release of *Lactobacillus casei* in the small intestine. When the proposed *Lactobacillus-casei*-encapsulated carriers enter the stomach with extremely low pH, the diffusion channels delineated by the electrically neutral CA networks in the CAP composite shells are "choked" with protamine molecules because of the electrostatic repulsion between the positively charged protamine molecules; as a result, it is hard for the gastric acid in stomach to diffuse across the CAP composite shells and then the *Lactobacillus casei* encapsulated in the carriers are efficiently protected. When the *Lactobacillus-casei*-encapsulated CAP carriers come into the small intestine with neutral pH, the carriers dissolve rapidly due to the cooperation between protamine and trypsin. Consequently, *Lactobacillus casei* encapsulated inside the CAP carriers are quickly released in the small intestine. After immersed in pH 2.5 simulated gastric acid for 2 h, the survival of *Lactobacillus casei* encapsulated in the CAP beads prepared with 2% w/v Na-alginate is as high as 46.57%; while, that in ordinary CA beads is as low as 0.78%, which is about 60 times lower than the former. The whole release process of *Lactobacillus casei* encapsulated in the prepared CAP beads in pH 7.0 simulated intestinal fluid takes only 50 min; however, it takes about 380 min for the ordinary CA beads, which is 7.6 times longer than the former. The results show that the proposed CAP beads show significant pH-responsive protection and intestinal-targeted characteristics for *Lactobacillus casei*. The intestinal-targeted CAP carrier developed in this study provides a potential and novel mode for developing smart protection systems, responsive controlled-release systems, and intestinal-targeted drug delivery systems.

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Notes

The authors declare no competing financial interest.

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