In situ fabrication of radiopaque microcapsules for oral delivery and real-time gastrointestinal tracking of *Bifidobacterium*

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**Introduction:** Although oral administration of *Bifidobacterium* is a promising approach for diseases, lack of resistance to harsh conditions and real-time tracking in gastrointestinal system in vivo are still major challenges in basic research and clinical applications.

**Materials and methods:** In this study, we fabricated a chitosan-coated alginate microcapsule loaded with in situ synthesized barium sulfate (CA/BaSO4 microcapsule) for oral *Bifidobacterium* delivery and real-time X-ray computed tomography (CT) imaging. CA/BaSO4 microcapsules containing the *Bifidobacterium* were prepared in situ by one-step electrostatic spraying method, and then coated with chitosan.

**Results:** The results indicated that CA/BaSO4 microcapsules with an average diameter of approximately 200 μm possessed favorable mechanical stability and X-ray attenuation capacity. Encapsulation of *Bifidobacteria* in the CA/BaSO4 microcapsules exhibited superior resistance to cryopreservation and gastric acid environment in vitro. After oral administration in mice, these CA/BaSO4 microcapsules could be real-time visualized by CT imaging and readily reached the intestine to release *Bifidobacteria*.

**Conclusion:** The radiopaque CA/BaSO4 microcapsules provide a novel platform for efficient protection, non-invasive real-time monitoring and intestinal-targeted *Bifidobacterium* delivery.

**Keywords:** alginate, microcapsule, CT imaging, intestinal-targeted, *Bifidobacterium*

**Introduction:**

*Bifidobacterium* are defined as a group of living microorganism supplements that confer health benefits on the host when administered in adequate amounts.1 When body lacks a certain amount of *Bifidobacterium*, it will result in a series of diseases, such as promote harmful bacteria reproduction,2 change intestinal flora caused diarrhea,3 increase tumor growth,4 reduce body immunity and so on.5 *Lactobacillus* case often used as effectors of host functions could play an important role in maintaining human health by controlling other intestinal microorganisms capable of producing harmful effects. *Lactobacillus* case played an important role in prevention of enteric infections, a low dose of which was enough for protection against intestinal infections by secreting IgA into the intestinal lumen, thus providing adequate defenses for mucosal surface. Studies have suggested that secretary immune system of gut might be stimulated most effectively by microbes, which could actively colonize the mucosal surface of the intestinal tract and release adequate protective antigens.6 Thus, delivery of *Bifidobacterium* to influence and modulate microbiome compositions can potentially impact the treatment of human diseases.7 When oral *Bifidobacterium* pass through mouth, pharynx, esophagus, stomach and finally reach intestine, they start to exert the...
functional exogenous *Bifidobacterium* are extremely sensitive to low pH gastric acid. A large amount of *Bifidobacterium* might have died before they reached the intestine if they came into direct contact with gastric acid in stomach. Thus, how to protect *Bifidobacterium* in acidic environments is still a significant challenge.

Up to now, encapsulation is the main way to protect *Bifidobacterium*. Alginate-based microcapsules (AMs) are widely used for encapsulation of *Bifidobacterium*. Many researchers report that encapsulation of *Bifidobacterium* in AMs can help *Bifidobacterium* to resist gastric acid and guarantee adequate living *Bifidobacterium* to reach intestine. However, the application of AMs are limited due to their unstable property. So, researchers have explored a number of methods to improve the stability of AMs. Chitosan as a mucoadhesive polymer is a biocompatible and biodegradable cationic polysaccharide, which is synthesized by partial deacetylation of chitin. The role of primary amino groups, electrostatic attraction, hydrogen bonding and hydrophobic have an effect on aggregation of mucin in the presence of chitosan. Reducing the number of amino groups through their half acetylation not only results in expansion of chitosan’s pH solubility window up to pH 7.4 but also reduces its capacity to aggregate mucin. Gombotz and Wee reported that coating polycationic compound chitosan on the surface of AMs enhanced the stability of physical and chemical property. Malmo et al. also reported that the survival of *Bifidobacterium* entrapped in AMs could be increased through chitosan coating. However, such method results in another problem; although the protection effect in the stomach and intestinal-targeted *Bifidobacterium* delivery is improved to a certain extent, encapsulation of *Bifidobacterium* in AMs coated with chitosan cannot be monitored in the whole digestive system.

In this study, we attempt to fabricate a novel intestinal-targeted and real-time monitoring CA microcapsule for pH-responsive protection of *Bifidobacterium* in stomach and rapid release of *Bifidobacterium* in small intestine. In the formation process of chitosan-coated alginate microcapsules loaded with in situ synthesized barium sulfate (CA/BaSO₄) microcapsules, barium ions not only solidified the alginate droplets, which could be further consolidated by coating with chitosan and removed off via following citric acid liquidation, but also reacted with sulfate ions to synthesize BaSO₄ precipitates in situ acting as radiopaque imaging agent within the resultant microcapsules. After surface coating on the surface of alginate microcapsule, the chitosan improved protection for *Bifidobacterium* to guarantee the survival of *Bifidobacterium* in stomach and readily intestinal mucoadhesive characteristics to guarantee the release of *Bifidobacterium* in the intestine. When the prepared CA/BaSO₄ microcapsules containing *Bifidobacterium* enter the extremely low PH stomach environment, and take advantage of the electrostatic repulsion of the positively charged chitosan, it is hard for the gastric acid in stomach to diffuse across the barium-mediated alginate (BA) microcapsules and then the *Bifidobacterium* in BA microcapsules are efficiently protected. However, the *Bifidobacterium* release quickly from the BA microcapsules to the intestine due to neutral pH environment and mechanical agitation, and this process could be visualized in real time using computed tomography (CT) imaging by radiopaque BaSO₄ nanocluster within microcapsules.

### Materials and methods

#### Materials

Sodium alginate (molecular weight: 216.123, viscosity: 200±20 mpa.s.) was purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Na₂SO₄ (anhydrate, purity ≥99.0%), BaCl₂ (dihydrate, purity ≥99.0%), chitosan (molecular weight: 1,526.454, viscosity: 100–200 mpa.s., deacetylation ≥95%) and liquid paraffin were purchased from Sinopharm Chemical Reagent Co. Ltd. All other chemicals, including 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

*Bifidobacterium* (strain name: TR17, number: CCTCCM 2017265) from National Center for Preservation of China were used throughout the experiments. MRS medium was used to activate *Bifidobacterium*. Simulate gastric acid (pH 2.5) was prepared by adjusting the pH of the prior solution to 2.5 with 0.1 mol/L HCl solution. Simulated intestinal fluid was prepared by dissolving 6.8 g of KH₂PO₄ 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 1,000 mL.

#### Preparation of CA/BaSO₄ microcapsules loading *Bifidobacterium*

CA/BaSO₄ microcapsules loading *Bifidobacterium* were fabricated using three steps as shown in Scheme 1. In the first step, the alginate beads loading *Bifidobacterium* were simultaneously prepared by an electrostatic spraying method.
Briefly, *Bifidobacterium* was mixed with 1% sodium alginate and 0.5% Na$_2$SO$_4$ solution as droplet fluids. Then, the mixed solution was subsequently sprayed into a bath containing BaCl$_2$ (0.6 mol/L) under electrostatic field 20 voltage, and rapidly cross-linked by barium ions to form alginate beads. Meanwhile, BaSO$_4$ nanoclusters were in situ synthesized by the reaction between SO$_4^{2−}$ premixed in the alginate solution and excessive Ba$^{2+}$ in the collection bath. In the second step, chitosan was coated onto the alginate beads by electrostatic attraction. To achieve satisfactory pH-responsive performance and stable core-shell structure, resultant alginate beads loaded with *Bifidobacterium* were immersed in 1% chitosan solution with 1 h of continuous stirring.

In the last step, CA/BaSO$_4$ microcapsules were obtained by immersing alginate beads coated with chitosan into the 1% sodium citrate solution for 5 min with continuous stirring. The prepared CA/BaSO$_4$ microcapsules containing *Bifidobacterium* were washed with pure water three times and preserved in normal saline at 4°C.

To meet the visibility criteria, the minimum content of BaSO$_4$ in microspheres should be approximately 5 wt% for CT and 10 wt% for fluoroscopy. Unlike physically mixing BaSO$_4$ in microcapsules, we introduced NaSO$_4$ in the dispersed phase and assumed that all SO$_4^{2−}$ ions could react with Ba$^{2+}$ ions completely in the collection bath to form BaSO$_4$. Thus, the weight of BaSO$_4$ in the CA/BaSO$_4$ microcapsules can be estimated by the initial NaSO$_4$ concentrations. The range of NaSO$_4$ in the dispersed phase and higher initial NaSO$_4$ concentrations in the dispersed phase yielded higher BaSO$_4$ contents in CA/BaSO$_4$ microcapsules. So, when the concentration of NaSO$_4$ in the dispersed phase was 0, 0.025, 0.05 and 0.1 mol/L, the corresponding content of BaSO$_4$ was estimated to be 0, 0.25 wt%, 0.5 wt% and 1 wt% in the hydrated CA/BaSO$_4$ microcapsules, respectively.

**Morphological analyses**

We used the following three methods to analyze CA/BaSO$_4$ microcapsules.

First, a digital camera was used to observe the morphology of the alginate beads and CA/BaSO$_4$ microcapsules in pure water. The size of beads and microcapsules in pure water were determined by imageJ software.

Second, a fluorescence microscope was used to study the distribution of *Bifidobacterium* in the microcapsules. *Bifidobacterium* was incubated with apoptosis assays kit C1056 solution at room temperature for 30 min before made microcapsules. A confocal laser scanning fluorescence microscope (Zeiss LSM-710) was used to observe and record the fluorescence emission from *Bifidobacterium* inside the microcapsules, which were excited at 346 nm wavelength. *Bifidobacterium* presented blue fluorescence.

Third, for scanning electron microscopy (SEM), the CA/BaSO$_4$ microcapsules were sprinkled on the double-sided adhesive and then sprayed with gold powder (100 μ).
Accelerating voltage was 10 kV and the observation time required was as short as possible.

Characterizations of BaSO₄ in microcapsules
An aqueous dispersion of Bifidobacterium-encapsulated CA/BaSO₄ microcapsules was deposited onto clean water and then dried under frozen vacuum drying for 24 h. The crystal structure of BaSO₄ synthesized in situ in BA microcapsules was measured by a powder X-ray diffraction (XRD) instrument with 2θ ranging from 5.0° to 90.0° in a step of 0.02° under Cu Ka radiation.¹³

Endurance experiments in simulated gastric acid
To investigate the protection of microcapsules, same amount of naked Bifidobacterium, alginate bead-loaded Bifidobacterium and CA/BaSO₄ microcapsule-encapsulated Bifidobacterium were individually immersed into 15 mL of pH 2.5 simulated gastric acid solutions at 37°C for 2 h with continuous oscillation at 120 rpm. At regular intervals, the same amount of free Bifidobacterium solution, alginate bead-loaded Bifidobacterium and CA/BaSO₄ microcapsule-encapsulated Bifidobacterium were shifted into 15 mL of 0.06 mol/L sodium citrate solution until the beads and microcapsules were dissolved under vigorous oscillation. Then, these Bifidobacterium were incubated, respectively, on the culture plate for 24 h. At last, the viable count of Bifidobacterium was tested by plate count method and the survival was the ratio of viable count and whole count.²

Dissolution experiments in simulated gastric acid and intestine fluid
To investigate the release characteristics, same amount of Bifidobacterium loaded by alginate beads and Bifidobacterium loaded by CA/BaSO₄ microcapsules were individually immersed into 50 mL of pH 2.5 simulated gastric acid solution at 37°C for 2 h with continuous oscillation at 120 rpm. At regular intervals, the concentration of Bifidobacterium in the surrounding medium was analyzed by UV-vis at a wavelength 600 nm until Bifidobacterium were released completely.²

Animal experiments
All animal experiments in this study were executed according to the protocol approved by the Animal Management Rules of the Ministry of Health of the People’s Republic of China and approved by the Institutional Animal Care and Use Committee of Jiangsu University. Six- to 8-week-old female C57BL/6 mice were purchased from Model Animal Genetics Research Center of Jiangsu University (Zhenjiang, People’s Republic of China). All efforts were made to minimize animals’ suffering and to reduce the number of animals used.

Induction of colitis and treatment
Colitis animal models were induced in C57BL/6 mice with 2.0% dextran sulfate sodium (DSS) (molecular weight 36–50 kDa) dissolved in drinking water (days 1–11). Normal mice were given water. Vehicle control (water), Bifidobacterium (2.4×10⁹ CFU), microcapsules (0.52 g/20 g), sulfasalazine 0.03 g/(kg/d) and CA/BaSO₄ microcapsules containing Bifidobacterium (0.6, 1.2, 2.4×10⁹ CFU) were given orally from day 3 to day 11.³⁴

Clinical scoring and histological analysis
Body weight, stool consistency and the presence of gross blood in feces and at the anus were observed every day. The disease activity index (DAI) was calculated by assigning well-established and validated scores. Briefly, the following parameters were used for calculation: 1) diarrhea (0 points = normal, 2 points = loose stools, 4 points = watery diarrhea); 2) hematochezia (0 points = no bleeding, 2 points = slight bleeding, 4 points = gross bleeding). At day 11 following induction of colitis, animals were sacrificed, the colon was removed and pieces of colonic tissue were used for vivo analysis. For histological analysis, part of the colon was fixed in 4% paraformaldehyde and embedded in paraffin. Sections were stained with H&E and colonic sections was graded as follows: 0) no signs of inflammation; 1) low leukocyte infiltration; 2) moderate leukocyte infiltration; 3) high leukocyte infiltration, moderate fibrosis, high vascular density, thickening of the colon wall, moderate goblet cell loss and focal loss of crypts; and 4) transmural infiltrations, massive loss of goblet cell, extensive fibrosis and diffuse loss of crypts.³⁴–³⁶

In vivo real-time imaging of CA/BaSO₄ microcapsules
The X-ray visibility of resultant CA/BaSO₄ microcapsules was assessed by using a 16-slice spiral CT instrument (SOMATOM
Sensation; Siemens, Berlin, Germany) under a scanning voltage of 80 kV and a current of 120 mA. Mice were kept under standard feeding condition and no feeding was treated for 24 h prior to the following procedure. Briefly, mice were administrated by gavage 0.52 g CA/BaSO₄ microcapsules according to a method described elsewhere. The located efficacy of intestinal of mice and the visibility of CA/BaSO₄ microcapsules were further assessed by CT at designated time intervals.

**Results and discussion**

**Morphological analyses**

CA/BaSO₄ microcapsules have been successfully synthesized in situ using barium-mediated gelation, chitosan coating and sodium liquidation route. At first, we chose electrostatic spraying and metal ion cross-linking to form the BA beads, as shown in Figure 1A. During this process, barium ions served dual-function roles in formation of alginate gelation and BaSO₄ nanoclusters. Then, the polycationic chitosan molecules were coated on the surface of these BA beads via polyanionic alginate molecules due to electrostatic interaction. Finally, alginate gel in the BA beads interiors was liquified by sodium citrate-mediated competitive chelation, but transparent shell was still kept in good condition due to chitosan-alginate complexes’ interaction. Consequently, CA/BaSO₄ microcapsules with hollow structure were obtained by virtue of liquidation of internal alginate gel and surface electrostatic interaction, as shown in Figure 1B and C. It was worth noting that the average diameters of BA beads and CA/BaSO₄ microcapsules were 150 μm and 200 μm, respectively. The as-prepared CA/BaSO₄ microcapsules became slight bigger than the original BA beads, which might be caused by slight swelling in the liquidation process.

To characterize the spatial structure of microcapsules and assess the viability of loaded *Bifidobacterium*, fluorescent staining was carried out, as shown in Figure 1D, in which viable *Bifidobacterium* exhibited blue fluorescence. A large number of bright blue fluorescence was obviously observed in the interior of microcapsule, indicating the presence of lots of *Bifidobacterium* with favorable viability. It was worth noting that almost no blue fluorescence appeared on the edge of the microcapsule. The differential distribution of *Bifidobacterium* in the microcapsule further confirmed well-defined core-shell structure. This closed hollow structure provided a superior environment for probiotic growing against external harsh conditions. SEM was performed to study the morphology of CA/BaSO₄ microcapsules. In Figure 1E, it was revealed that CA/BaSO₄ microcapsules possessed irregular loose spherical shape, which ascribed to the freeze-drying process. In Figure 1F, SEM image showed cross-sectional views of typical *Bifidobacterium*-loaded CA/BaSO₄ microcapsules, demonstrating the existence of huge hollow structure in the microcapsule interior. In addition, chitosan post-coating prevented *Bifidobacterium* distributing on the external surface and leaking from microcapsule. In view of space advantage, this core-shell structure will be conducive to avoid interior *Bifidobacterium* (*Bifidobacterium* marked by red circle) contacting with external harsh surrounding and reduce the death rate, resulting in the formation of a favorable closed environment.

The crystal structure of BaSO₄ nanoclusters synthesized in situ was identified by XRD, as shown in Figure 2A. The main diffraction peaks and relative intensities in the XRD patterns

**16S rRNA gene sequence analysis**

Sequences generated from pyrosequencing barcoded 16S rRNA gene PCR amplicons were quality filtered. The sample of genomic DNA was extracted by using the US MoBio PowerSoil HTP 96 Well Soil DNA Isolation Kit. The purity and concentration of DNA was tested by the Ondrop instrument and agarose gel electrophoresis. A total of 2.5 ng diluted genomic DNA was used as a template. American Promega GoTaQ Hot Start Colorless Master Mix and high fidelity enzyme with specific primers Barcode were employed to ensure the amplification efficiency and accuracy of PCR. Pico Green fluorescence quantitative analysis was performed to detect the concentration of DNA of PCR products. The product was purified by the QIAquick PCR Purification Kit. The amplification of targeting genes was performed by the 16S V4 region primer 515F (5′-GGACTACHVGGGTWTCTAAT-3′), 806R (5′-GGACTACHVGGGTWTCTAAT-3′), which were tested by the fluorescence quantitative and Agilent 2200 TapeStation electrophoretic platform. MiSeq was further tested when the results were satisfied. Sequences were removed if they were shorter than 200 nucleotides and contained primer mismatches, ambiguous bases, uncorrectable barcodes or homopolymer runs in excess of six bases. The remaining sequences were collected and analyzed using the open source software package Quantitative Insights Into Microbial Ecology. 16S rRNA gene sequences were assigned to operational taxonomic units (OTUs) using UCLUST with a threshold of 97% pair-wise identity and then classified taxonomically using the Ribosomal Database Project classifier 2.0.1. 37,38

**Statistical analysis**

All data were presented by mean ± standard deviation. Statistical comparisons were statistically analyzed by one-way ANOVA. The values of *P*<0.05 were considered statistically significant.

**In situ fabrication of radiopaque microcapsules**

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**Results and discussion**

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displayed a main broad diffraction peak at 43.02°, which matched well with the reference of orthorhombic BaSO₄ crystal structure. This result implied that BaSO₄ crystallites were formed in situ between sodium alginate and barium chloride during the formation of alginate gelation process. The x-ray attenuation capability of CA/BaSO₄ microcapsules was characterized by CT scanning. As shown in Figure 2B, CT signal intensity gradually increased with an increase of NaSO₄ concentration from 0 mg/mL to 7.5 mg/mL. We found that CA/BaSO₄ microcapsules gave much higher signal intensity and produced similar contrast with iodoxanol solution. The results revealed that 5 mg/mL concentration of NaSO₄ can provide an equivalent contrast ability relative to clinical iodoxanol. The minimum dose in administration is highly favorable as it significantly reduces side effects. In Figure 2C, we can observe lots of barium sulfate nanoclusters of which

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**Figure 1** Morphology characterization of CA/BaSO₄ microcapsules. Optical images of (A) BA beads and (B) CA/BaSO₄ microcapsules in pure water at room temperature. Micrographs of CA/BaSO₄-loading Bifidobacterium under (C) bright field and (D) fluorescence field. Bifidobacterium were stained by Hoechst (D). SEM images of surface (E) and inside (F) CA/BaSO₄ microcapsules. Bifidobacterium are marked by red circle.

**Figure 2** (A) XRD patterns of CA/BaSO₄ microcapsules measured with 2θ ranging from 5° to 90.0° in a step of 0.02° under a Cu Kα radiation. (B) CT images of CA/BaSO₄ microcapsules using various concentrations of NaSO₄ as injection liquid arranged from 0 mg/mL to 7.5 mg/mL (b–e: 0, 2.5, 5, 7.5 mg/mL); phosphate buffered saline was used as the negative control (a) and iodoxanol solution was used as the positive control (f). (C) SEM images of barium sulfate nanoclusters.

**Abbreviations:** XRD, x-ray diffraction; CA/BaSO₄, chitosan-coated alginate microcapsule loaded with in situ synthesized barium sulfate; BA, barium-mediated alginate; SEM, scanning electron microscopy.
the average size was 679.06 nm using statistical analysis in the SEM image by imageJ.

The protective effect of CA/BaSO₄ microcapsules on Bifidobacterium in vitro

The protective effect of CA/BaSO₄ microcapsules on viability of the Bifidobacterium was evaluated under simulated gastric acid conditions. Along with the change of ingredients, food intake and human body, the pH value of the gastric acid in human body is usually about 1.8–5.0.⁴⁴ Therefore, the pH 2.5 simulated gastric acid was chosen as the model gastric acid to investigate the protection effect of CA/BaSO₄ microcapsules on Bifidobacterium. Figure 3 shows the viable count and survival of free Bifidobacterium, Bifidobacterium encapsulated by BA beads and those by CA/BaSO₄ microcapsules after being immersed in pH 2.5 gastric acid for 1 and 2 h. After 1 h of immersion, the viable counts of naked Bifidobacterium and Bifidobacterium encapsulated in BA beads were 26.6×10⁶ and 33.6×10⁶ CFU/mL, respectively, whereas the viable counts of naked Bifidobacterium and Bifidobacterium encapsulated in CA/BaSO₄ microcapsules exhibited higher value of 40.9×10⁶ and 32.6×10⁶ CFU/mL, respectively. Then, the viable count of each group slightly decreased after 2 h, but the tendency did not change. The survival of naked, BA beads and CA/BaSO₄ microcapsule-loaded Bifidobacterium was 36.1%, 45.6% and 55.7%, respectively, after 1 h of immersion. The same tendency appeared after 2 h of immersion, and the survivals were 19.8%, 34.2% and 44.3%, respectively. The results indicated that Bifidobacterium were very sensitive to gastric acid, and BA beads offered certain protective effect. However, compared with BA beads, CA/BaSO₄ microcapsules exhibited the best performance for protecting Bifidobacterium against undesirable gastric acid. For BA beads, the gastric acid can easily diffuse into the core of the beads and kill Bifidobacterium, resulting in low rate of the survival of Bifidobacterium. However, because of the charge repulsion of polycation chitosan located on the surface of microcapsules, it is hard for gastric acid to diffuse into the microcapsules and the survival of Bifidobacterium is much higher.

The release of Bifidobacterium in CA/BaSO₄ microcapsules in simulated intestinal fluid

The release of Bifidobacterium from CA/BaSO₄ microcapsules was investigated by the dissolution of microcapsules under simulated gastric acid and simulate intestine conditions. As depicted in Figure 4, the release rate of Bifidobacterium from BA beads and CA/BaSO₄ microcapsules was measured after immersion in pH 2.5 gastric acid for 2 h and then in intestinal fluid. In pH 2.5 simulated gastric acid, no Bifidobacterium were detected in both bead and microcapsule groups, which demonstrated the favorable stability of beads and microcapsules under acidic environment. However, the release of Bifidobacterium in beads and microcapsules increased following the increase of pH. In pH 6.0 simulated jejunum fluid, only small amount of Bifidobacterium released from BA beads and CA/BaSO₄ microcapsules. When shifted into pH 7.0 simulated ileum fluid, Bifidobacterium were rapidly released from BA beads and CA/BaSO₄ microcapsules. The release rates of Bifidobacterium in BA beads and CA/BaSO₄

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**Figure 3** (A) Viable count and (B) survival of free Bifidobacterium, Bifidobacterium in BA beads and Bifidobacterium in CA/BaSO₄ microcapsules after being immersed in pH 2.5 gastric acid. *P<0.05, **P<0.01.

**Abbreviations:** BA, barium-mediated alginate; CA/BaSO₄, chitosan-coated alginate microcapsule loaded with in situ synthesized barium sulfate.

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International Journal of Nanomedicine 2018:13

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In situ fabrication of radiopaque microcapsules
microcapsules were 70% and 90%, respectively, before entering into simulated cecum fluid. When immersed into simulated cecum fluid, Bifidobacterium in CA/BaSO₄ microcapsules were released completely, but only 90% of Bifidobacterium in BA beads were released. After BA beads were immersed in simulated small intestine fluid for 3 h, Bifidobacterium released complete from BA beads. It was worth noting that the release rate in microcapsules was much higher than that in beads; it took only about 8 h for CA/BaSO₄ microcapsules to release all of the encapsulated Bifidobacterium, but about 12 h for the BA beads. The results demonstrated that CA/BaSO₄ microcapsules exhibited much more satisfactory intestinal-targeted delivery characteristics than BA beads.

**CA/BaSO₄ microcapsule-mediated Bifidobacterium delivery for DSS-induced colitis in vivo**

To validate the advantage of CA/BaSO₄ microcapsules in oral administration, DSS-induced mice colitis model was chosen as the animal model. DSS-induced colitis showed significant appearance of diarrhea/loose feces and visible fecal blood, resulting in significant DAI elevation. When the animal model was established, these mice were administrated 2.4×10⁹ CFU naked Bifidobacterium, various amounts of Bifidobacterium encapsulated by CA/BaSO₄ microcapsules of 0.6×10⁹, 1.2×10⁹, 2.4×10⁹ CFU and sulfasalazine (clinical drug as positive control). Compared with DSS treated group, the body weight could be dramatically improved after treating with 2.4×10⁹ CFU Bifidobacterium, microcapsules containing 0.6×10⁹, 1.2×10⁹ and 2.4×10⁹ CFU Bifidobacterium, as shown in Figure 5A. DSS-colitis mice showed much higher DAI scores compared with the other five groups. The DAI data further verifying CA/BaSO₄ microcapsules containing distinctive amounts of Bifidobacterium exhibited similar therapeutic effect with sulfasalazine administration, as shown in Figure 5B. After 11 days of treatment, all DSS-colitis mice showed a significant reduction in their colon length compared with the control groups (7.6±0.4 cm). A total of 0.6×10⁹ Bifidobacterium encapsulated by CA/BaSO₄ microcapsules group had the shortest length (5.1±0.3 cm), followed by 1.2×10⁹ Bifidobacterium encapsulated by CA/BaSO₄ microcapsules (5.8±0.2 cm), 2.4×10⁹ Bifidobacterium encapsulated by CA/BaSO₄ microcapsules (6.5±0.3 cm), free 2.4×10⁹ Bifidobacterium (5.5±0.2 cm) or sulfasalazine (6.4±0.4 cm) group, as shown in Figure 5C. Interestingly, the colon length was gradually restored with the increasing amount of Bifidobacterium when treatment with CA/BaSO₄ microcapsules encapsulated 2.4×10⁹ CFU Bifidobacterium the colon length was recovered to 85.5% compared to the normal group. Compared with CA/BaSO₄ microcapsules encapsulated with 2.4×10⁹ CFU Bifidobacterium, only 72.4% of the colon length was recovered after treating with free 2.4×10⁹ CFU Bifidobacterium, as shown in Figure 5D. These results further verified that gastric acid could influence the activity of Bifidobacterium. To some extent, CA/BaSO₄ microcapsules can protect Bifidobacterium in gastric acid.

In order to further verify the therapeutic outcome, histological evaluation was carried out to observe the change of anatomical structure of colon wall, as shown in the Figure 6. The normal and CA/BaSO₄ microcapsules group mice had no marked microscopic lesions on histological evaluation of tissues. Compared with normal tissue, DSS treatment induced distortion of crypts, loss of goblet cells (see regions marked by yellow arrows), infiltration of mononuclear cells, submucosal lymphoid hyperplasia, extension around intestinal glands (see regions marked by blue circle) and severe mucosal damage in the colon specimens of colitis (see regions marked by green arrows). However, these pathological changes were gradually alleviated after CA/BaSO₄ microcapsules were mediated by Bifidobacterium treatment in a dose-dependent manner. Decreased inflammatory signs were also observed in colonic tissues harvested from DSS-treated mice with Bifidobacterium. Interestingly, with the increasing concentration of Bifidobacterium, the therapeutic effect was improved. The protective effects against colitis were almost the same between mice treated with microcapsules containing 2.4×10⁹ CFU Bifidobacterium and those treated with sulfasalazine. It has suggested that the secretory immune system of the
In situ fabrication of radiopaque microcapsules

Figure 5 CA/BaSO₄ microcapsule-mediated Bifidobacterium delivery for DSS-induced colitis treatment in mice. (A) Loss of basal body weight under different treatments during the disease process. a, normal; b, DSS; c, CA/BaSO₄ microcapsule-encapsulated 0.6×10⁹ CFU Bifidobacterium; d, CA/BaSO₄ microcapsule-encapsulated 1.2×10⁹ CFU Bifidobacterium; e, CA/BaSO₄ microcapsule-encapsulated 2.4×10⁹ CFU Bifidobacterium; f, free 2.4×10⁹ CFU Bifidobacterium; g, sulfasalazine; h, free CA/BaSO₄ microcapsules. (B) DAI under different treatments. (C) The macroscopic appearances and length of colons (D) from each group of mice under different treatments (n=6 per group). Data are presented as mean ± standard error of the mean. *P<0.05, **P<0.01 vs DSS-treated alone group at the same day.

Abbreviations: CA/BaSO₄, chitosan-coated alginate microcapsule loaded with in situ synthesized barium sulfate; DSS, dextran sulfate sodium; DAI, disease activity index.

Figure 6 Pathological analysis of colon tissues after distinct treatments using H&E staining assay, magnification =100x. (A) Normal control, (B) CA/BaSO₄ microcapsule treatment, (C) DSS induction, (D) sulfasalazine treatment after DSS induction, (E) 0.6×10⁹ Bifidobacterium-loading CA/BaSO₄ microcapsule treatment after DSS induction, (F) 1.2×10⁹ Bifidobacterium by microcapsule treatment after DSS induction, (G) 2.4×10⁹ Bifidobacterium by microcapsule treatment after DSS induction. (H) 2.4×10⁹ Bifidobacterium treatment after DSS induction. The blue circles denote infiltration of mononuclear, submucosal lymphoid hyperplasia, and extension around intestinal glands; yellow arrows denote the distortion of crypts and loss of goblet cells; the green arrows denote the severe mucosal damage in the colon.

Abbreviations: CA/BaSO₄, chitosan-coated alginate microcapsule loaded with in situ synthesized barium sulfate; DSS, dextran sulfate sodium.
The gut might be stimulated most effectively by *Bifidobacterium*, which could actively colonize the mucosal surface of the intestinal tract and release adequate protective antigens.\(^{34-36}\) In an inflammatory response, the accumulation of cells within the tissues consisted mainly of neutrophils and monocytes. T-lymphocyte suppressors, which were also involved, played a critical role in the infectious process. It has also been shown that the induction of inflammation and the development of clinical impairment could be prevented by immunosuppression resulting from the appearance of T cells, predominantly of the CD8\(^+\) T-lymphocyte suppressor subset.\(^6\)

**Real-time tracking of CA/BaSO\(_4\) microcapsules using CT imaging in vivo**

Capability of CA/BaSO\(_4\) microcapsules in vivo was also evaluated after oral administration by CT imaging. In Figure 7, 3D renderings of CT images of the GI tract showed the real-time translocation of CA/BaSO\(_4\) microcapsules through the GI tract in which CT scanned images were obtained at designated time intervals. The main organs of the upper digestive system including stomach, duodenum and a few loops of the small intestine started appearing bright at 2 min after incorporation of oral CA/BaSO\(_4\) microcapsules, and the CT value of the stomach increased remarkably (denoted as white regions marked by red arrow). After 30 min of administration, the small intestinal was lighted up due to the presence of much more CA/BaSO\(_4\) microcapsules (denoted as white regions marked by yellow arrow). After 1 h and 2 h of administration, the CT signal of small intestine showed an obvious increase, revealing the excellent imaging of the CA/BaSO\(_4\) microcapsules. More importantly, arrangement and sequence of small intestinal loops could be described clearly. After 3 h of administration, vast majority of CA/BaSO\(_4\) microcapsules was significantly emptied from the small intestine, and the large intestine began to be filled with CA/BaSO\(_4\) microcapsules (see white regions marked by yellow and blue). CA/BaSO\(_4\) microcapsules remained visible after 24 h, which indicated that CA/BaSO\(_4\) microcapsules were located in the sigmoid colon and rectus, and almost all CA/BaSO\(_4\) microcapsules were excreted from body after 2 days, ensuring the neglectable side effect. This observation further demonstrates that the good visibility of CA/BaSO\(_4\) microcapsules under x-ray is beneficial not only for observing the *Bifidobacterium* migration in the digestive tract but also for evaluating the engraftment of *Bifidobacterium* in the intestine.\(^{40-42}\)

**Fecal microbiota composition analysis**

In order to systematically assess the performance of CA/BaSO\(_4\) microcapsules in delivering *Bifidobacterium* for oral administration, fecal microbiota compositions were analyzed using pyrosequencing analysis. As shown in Figure 8,

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**Figure 7** CT scan images of mice at designated time intervals after oral CA/BaSO\(_4\) microcapsules. Red, yellow and blue arrows, respectively, denote stomach, small intestine and large intestine.

**Abbreviations:** CT, computed tomography; CA/BaSO\(_4\), chitosan-coated alginate microcapsule loaded with in situ synthesized barium sulfate.
we found a high microbial diversity in the mice feces and detected 48 species, but the diversity of mice intestinal microbiota with different treating patterns was different. Using parametric test for comparisons, we could find differences in richness and biodiversity among normal group, DSS group, sulfasalazine group, and microcapsules containing 0.6×10^9, 1.2×10^9, and 2.4×10^9 CFU Bifidobacterium groups at the OTUs cutoff of 0.03. All of rarefaction curves tended to approach the saturation plateau. Samples from different groups were plotted in the upper part. This rarefaction curve indicated a large variation in the total number of OTUs in different samples, but the sequence coverage was still sufficient to capture the diversity of the bacterial communities. The same tendency was found in species accumulation curves and Shannon-Wiener curves; it tended to approach the saturation plateau, which meant that the database of 16S rRNA gene sequences was very abundant to reflect the vast major of microbial information. The relative distributions of OTUs in the fecal from each group as a family and genus taxonomical level were explored, as shown in Figure 9. The results show that fecal microbial composition of all mice mostly comprised Bacteroidetes and Firmicutes. Other less abundant phyla were Proteobacteria, Actinobacteria and Tenericutes. Control mice had greater abundances of Bacteroidetes and lower abundances of Firmicutes than DSS-induced mice, which reflected the dominance of OTUs as genus Allobaculum. After being administrated with CA/BaSO_4 microcapsules with various Bifidobacterium, the mice have greater abundances of Bacteroidetes and lower abundances of Firmicutes compared to DSS group mice. With increasing Bifidobacterium, the level of Bacteroidetes was elevated and Firmicutes were declined in all mice, indicating gradual recovering to normal level. We performed a nearest shrunken centered classification analysis to investigate which OTUs accounted for differences in composition of the gut microbiota. This analysis assesses how well a mouse microbiota is assigned to its treatment group based on its composition.
Unclassified *Rikenellaceae* was the most abundant in DSS group, which was 2-fold more than normal group. With increasing *Bifidobacterium*, the level of unclassified *Rikenellaceae* genus was decreased and *Allobaculum* genus was increased in all mice. Interestingly, at the genus level, *Allobaculum* was the most abundant division in eight groups. *Parabacteroides, Rikenella, Pseudomonas,* unclassified *Rikenellaceae, Turicibacter* and *Intestimonas* were detected in the group treated by CA/BaSO₄ microcapsules with 2.4x10⁸ *Bifidobacterium* was nearly in the same place compared with the normal group. *Bifidobacterium* has been widely used and may prevent pathogens from proliferating in the intestinal tract and in the culture environment.

### Conclusion

In this study, we have successfully developed a chitosan-coated alginate microcapsule in situ loading the barium sulfate nanoclusters for oral *Bifidobacterium* delivery and real-time x-ray CT imaging. This synthesized CA/BaSO₄ microcapsule exhibited uniform size distribution and huge hollow core-shell structure, which were beneficial for probiotic loading. During the preparation process, barium ions played dual roles in formation of alginate gelation and barium sulfate nanoclusters. After citric acid-mediated liquidation, the stable transparent shell was formed by electrostatic interaction between chitosan and alginate. The in vitro experiments demonstrated that CA/BaSO₄ microcapsules possessed strong capability to protect the *Bifidobacterium* from simulated gastric acid attacking and keep them in a good condition under this low pH environment. This protection may be ascribed to the electrostatic repulsion among the positively charged chitosan molecules, which prevented the gastric acid diffusing across the composite shell. However, the CA/BaSO₄ microcapsule could rapidly release *Bifidobacterium* in neutral intestinal fluid. In addition, the in situ synthesized BaSO₄ nanoclusters endowed the microcapsule with ultrahigh efficiency in absorbing x-ray. The in vivo experiment further confirmed that the radiopaque CA/BaSO₄ microcapsule not only provided a novel platform for efficient protection but also afforded satisfactory intestinal-targeted and non-invasive real-time monitoring character to guarantee the rapid release of *Bifidobacterium* in the intestine. Finally, using high-throughput sequencing technologies analysis, *Bifidobacterium* as a group of living microorganism supplements not only altered intestine microbiota composition in bacterial family and genus levels but also alleviated the DSS-induced colitis in mice.

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### Disclosure

The authors report no conflicts of interest in this work.

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Fang et al

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