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ORIGINAL PAPER

To prepared fluorescent chitosan capsules by in situ polyelectrolyte coacervation on poly(methacrylic acid)-doped porous calcium carbonate microparticles

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Abstract To prepare hollow microcapsules composed of native chitosan (CS), a templating method is developed using poly(methacrylic acid) (PMAA)-doped porous calcium carbonate ($CaCO_3$) microparticles as sacrificial templates. At first, CS was adsorbed onto PMAA-doped porous $CaCO_3$ microparticles, and then the adsorbed CS was covalently cross-linked with each other by using glutaraldehyde. After the dissolution of the templates, the resultant CS capsules ranged from 2 to 5 μm in diameter. Nitrogen adsorption–desorption analysis are applied to characterize the porous $CaCO_3$ templates, the BET surface area and total pore volume are 160 and 0.50 cm^3/g. The structure and morphology of the CS capsules are characterized by FESEM and TEM. Confocal laser scanning microscopy images reveal that the capsules have been labeled with green FITC. The gradual capsule invagination in response to bulk osmotic pressure created by CS solutions has also been discussed.

Keywords Calcium carbonate · Capsules · Chitosan · Template

Introduction

Hollow spheres and capsules are of great interest due to their potential applications and fundamental importance. Recently hollow spheres and capsules have been introduced as a novel type of nanoengineered multifunctional material [1, 2]. Permeability as a function of the wall materials of the microcapsules has received considerable attention due to an increased interest in biotechnology, medicine, catalysis, environment, food, etc., especially in the field of drug delivery [3–5].

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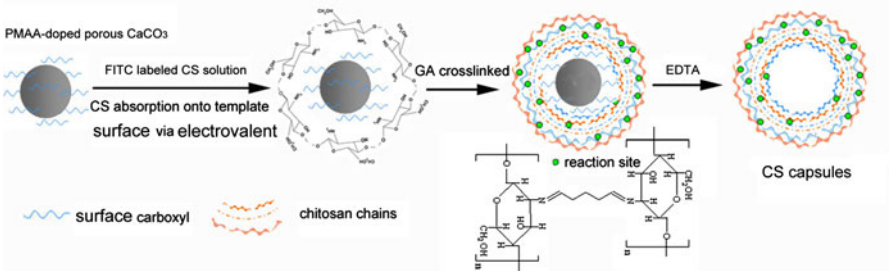
Today, microcapsule systems have the highest potential in the pharmaceutical industry since many different requirements have to be fulfilled to deliver a drug at the right moment, in the right place, and at an adequate concentration. Capsules have been widely used in fields of pharmaceutics, cosmetic, food, textile, adhesive, agricultural industry, the hollow spheres of artificial cells, and protection of proteins, enzymes, DNA, and catalysis [6, 7]. All these based on their isolating property, large inner volume, and tunable permeability [8].

Hollow spheres and capsules can be prepared by a variety of physical and chemical techniques, such as self-assembly, template, phase separation, and emulsion polymerization [9–12]. Of these methods, in the late of 1990s, a new class of multilayer microcapsules was developed a simple and elegant and yet extremely powerful approach to the formation of controlled architecture multilayer polymer films based upon the layer-by-layer (LbL) assembly of oppositely charged polyelectrolytes on a charged surface, irrespective of the shape, and size of the substrate [13]. The LbL method is effective in building up polyelectrolyte multilayers onto solid substrates with controlled thickness and composition [14]. In the past decade, LbL method has been intensively studied, and various polymer capsules with versatile functions have been obtained with different polymers [15]. Stimuli-responsive [16, 17], and degradable [18], are examples of such functional capsules. However, a conventional LbL method requires at least two components to stabilize the assemblies via intermolecular interactions. These multi-component capsules may be inadequate for certain applications requiring the properties of each individual component rather than its complex.

Several modified LbL methods have been developed to construct single-component polyelectrolyte capsules. CS, linear copolymer consisting of β-1,4-linked 2-amino-2-deoxy-D-glucopyranose and 2-acetamido-2-deoxy-D-glucopyranose units, a weak cationic polysaccharide produced by the deacetylation of the natural polymer chitin, has many useful biological properties, such as biocompatibility, biodegradability, and bioactivity [19, 20]. To these ends, microparticles of CS in hundreds of micrometers have been prepared in different ways, including coacervation-precipitation, spray-drying, emulsion cross-linking, emulsion droplet coalescence, reverse micellization, ionic gelation, and sieving method [21]. Complexation between CS and oppositely charged polysaccharides in solution is another way to synthesize CS microparticles; however, this process often leads to the formation of fibers [22].

Herein, we present an alternative method for the preparation of single-component FITC labeled CS capsules by using MAA hybrid porous $CaCO_3$ microparticles as templates. $CaCO_3$ can be dissolved under mild conditions with ethylenediaminetetraacetic acid (EDTA), the negatively charged PMAA corona could induce spontaneous deposition of CS onto the $CaCO_3$ shell. Then, the CS shell was cross-linked with GA. In addition, the porous $CaCO_3$ microparticles have the ability to adsorb diverse macromolecules, such as polyelectrolytes, proteins, and polysaccharides [23–25]. Scheme 1 shows the typical preparation procedure of the cross-linked CS capsules, involving the following steps: (1) CS adsorption onto and penetration into PMAA hybrid porous $CaCO_3$ microparticles (termed as the first coating), (2) covalent cross-linking of adsorbed CS with GA, and (3) the removal of $CaCO_3$ microparticles with EDTA. As an additional advantage, this preparation



Scheme 1 Schematic illustration of the preparation process of the cross-linked CS capsules by using porous $CaCO_3$ microparticles as templates: the CS coating (step 1), cross-linking with GA (step 2), and dissolution of $CaCO_3$ templates with EDTA (step 3)

technique does not require any organic solvent, including in the preparation of templates.

Experimental section

Materials

Methacrylic acid (MAA), potassium persulfate (KPS), glutaraldehyde (GA), sodium carbonate (Na_2CO_3), calcium chloride dihydrate ($CaCl_2{\cdot}2H_2O$), disodium ethylenediaminotetraacetate dehydrate (EDTA), and other reagents were all from Shanghai Chemical Reagents Co., Ltd. China. MAA was distilled under vacuum before use. KPS was re-crystallized before use. Chitosan (CS) was obtained from (San Huan Ocean Biochemical Co. Ltd. China). Its degree of deacetylation and the molecular weight were determined to be 95% and 5.0×10^5, respectively. Fluorescein isothiocyanate (FITC) was from Sigma-Aldrich. All other agents were analytical grade and used as received. All used water was obtained from a Milli-Q® Gradient System from Millipore equipped with a Quantum™ cartridge, have a resistivity of 18.2 MΩ cm. The pH of solutions was adjusted with NaOH if necessary.

Synthesis of PMAA-doped porous $CaCO_3$ microparticles

PMAA-doped $CaCO_3$ microparticles as following described. The PMAA were prepared via traditional emulsifier-free polymerization of MAA. In a typical experiment, MAA (5.0 g) were added to 95 mL of Milli-Q® water in a 250 mL three-neck round-bottom flask equipped with a mechanical stirring, a nitrogen inlet, and a condenser. After the mixture was deoxygenated by bubbling nitrogen gas at room temperature for 1 h, the flask was placed in a 70 °C oil bath, and stirred mechanically at 300 rpm. An aqueous solution of KPS (0.100 g in 5 mL) was subsequently added to the reacting medium, all the reaction under a nitrogen atmosphere. The reaction was continued for 12 h to assure the largest reaction. The above PMAA solution (10 mL) was added dropwise into 10 mL 0.33 M

Na_2CO_3 aqueous solution under vigorous agitation. After an additional 2 h stirring, 10 mL 0.33 M $CaCl_2$ aqueous solution was rapidly poured into the mixed solution under vigorous agitation. The agitation was stopped after 30 s. Thirty minutes later, the precipitated $CaCO_3$ particles were collected and washed twice with water by centrifugation at 500×g for 1 min, further washed and collected by filtration, and then dried under vacuum at room temperature.

Preparation of FITC-labeled CS and labeled cross-linked CS capsules

The synthesis of FITC-labeled CS was based on the reaction between the isothiocyanate group of FITC and the primary amino group of CS [26]. Dehydrated methanol (100 mL), followed by 2.0 mg/mL of FITC in methanol (50 mL), were added into 1% w/v CS hydrochloride in 0.1 M acetic acid solution (100 mL). After 3 h of reaction in the dark at ambient temperature, the FITC-labeled CS was precipitated in 0.2 M NaOH and dialyzed in four Liters of distilled water for 6 days under darkness before freeze drying (Dynavac Engineering, Auckland, New Zealand).

0.5 g the above-mentioned FITC-labeled CS was dissolved in 25 mL of 2.0 wt% acetate acid under stirring and made into 2.0 wt% CS solution. Then, the PMAA-doped $CaCO_3$ microparticles latexes (5.0 wt%) were dropped into the CS solution with burette under stirring for 1 h. The mixture was subjected to centrifugation for 5 min and re-dispersed in CS solution and centrifuged. This dispersion–centrifugation-collection cycle was repeated four times to assure enough CS were adsorbed on the surface of $CaCO_3$ microparticles.

The resulting CS absorbed on PMAA-doped $CaCO_3$ microparticles was treated with 3.0 mL 2.5% GA for 2 h at 40 °C. After washing with water, the coated particles were incubated in 0.2 M EDTA solution (pH 7.0, adjusted by NaOH) for 30 min under shaking. The resultant capsules were centrifuged at 1,500 g for 5 min with three washings in fresh EDTA solution. Finally, the capsules were washed thrice with water. The products were dialyzed in a filter membrane with a molecular weight cut off of 7,000 Da against a large amount of water for 6 days. The procedure was repeated to guarantee the complete removal of small molecular substances. To obtain CS capsules, products were obtained by freeze-drying.

Characterization

Nitrogen adsorption–desorption measurement was carried out with a BELSORP-mini (BEL Japan, Inc., Osaka, Japan). Before the measurement, the sample was dried for 3 h at 200 °C under a stream of nitrogen. The surface area and the pore-size distribution were calculated by the Brunauer–Emmett–Teller (BET) and the Barrett–Joyner–Halenda (BJH) methods, respectively. Transmission electron microscopy (TEM) measurements were carried out on a Japan Hitachi Model H800 microscopy with an accelerating voltage of 200 kV. Samples were prepared by dropping a suspension onto Formvar-coated copper grids. The morphology observation of the samples was carried out on a field-emission scanning electron microscopy (FESEM, JEOL JSM-6700) at an accelerating voltage of 10 kV. For observation the morphology

of capsules via FESEM, silicon (Si) wafers were cleaned in a piranha solution (70/30 v/v of concentrated H_2SO_4/30% H_2O_2. During the process, should be carefully because piranha solution reacts violently with organic compounds and should not be stored in closed containers, thoroughly rinsed with water, and then blown dry with nitrogen gas. During the FESEM, we used platinum as the sprayed layers. Confocal laser scanning microscopy (CLSM) confocal images were taken with a Bio-Rad Radiance 2100 confocal laser scanning microscope, equipped with a 100× oil immersion objective. FITC was used to stain the microcapsules.

Results and discussion

Characterization of $CaCO_3$ templates microparticles

In this study, porous PMAA-doped $CaCO_3$ microparticles were employed as templates for the preparation of the CS capsules. The porous structure of prepared $CaCO_3$ microparticles, which allows the infiltration of macromolecules into the templates, was confirmed by nitrogen adsorption–desorption measurement. Figure 1 shows the nitrogen adsorption–desorption isotherms of PMAA-doped $CaCO_3$ microparticles. Specific surface area and the average pore diameter were determined from the nitrogen adsorption isotherms by the Brunauer–Emmett–Teller (BET), and Barret–Joyner–Halenda (BJH) methods, respectively [27]. The N_2 adsorption–desorption isotherms exhibit typical type-II hysteresis with a sharp increase in nitrogen uptake at high-relative pressure $P/P_0 \sim 0.9$ and a wide hysteresis loop at $P/P_0 > 0.2$. In addition, the mesopore size distribution curves of is determined from the adsorption branch of the isotherms (seeing the top—left insets in Fig. 1). The mesopore-size distributions exhibit a sharp peak centered around 16 nm, respectively, implying a uniform mesopore size. The BET surface area and total pore volume are 160 and 0.50 cm^3/g.

The morphologies of $CaCO_3$ templates are investigated by FESEM. Figure 2 shows the morphology of uncoated $CaCO_3$ templates and CS absorbed onto the surface of $CaCO_3$ microparticles ($CaCO_3$@CS). The surface morphology of the

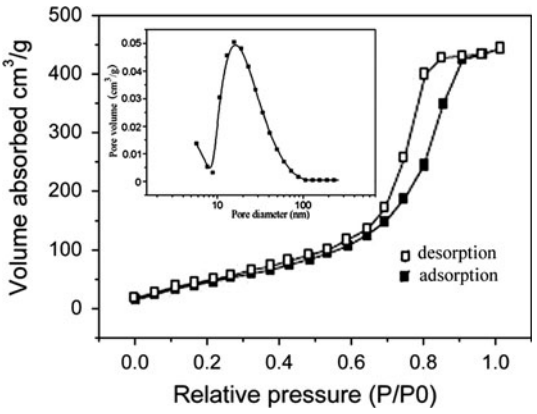


Fig. 1 BET measurement of as-synthesized PMAA-doped $CaCO_3$ microparticles. N_2 adsorption–desorption isotherms; and *insets* pore-size distribution curve

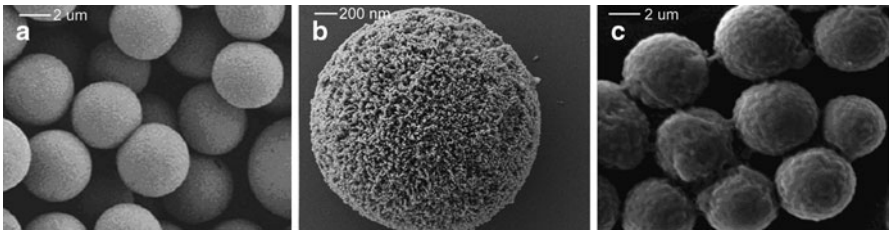


Fig. 2 FESEM images of **a** PMAA-doped $CaCO_3$ microparticles templates, **b** a magnified picture of template, **c** CS absorbed onto the surface of $CaCO_3$ microparticles

uncoated $CaCO_3$ microparticles was shown in Fig. 2a. Figure 2b shows us a magnified picture of $CaCO_3$ template microparticles which has porous structure. A rough and granular porous surface was observed, this morphology was appreciably changed after the coating of CS chain (Fig. 2c). This result is reasonable considering that CS has been aggraded onto $CaCO_3$ surface via electrostatic interaction between carboxyl of PMAA and amino of CS. We selected GA as the cross-linking agent to covalently link CS strands, thus, GA would react with the amino groups of CS and leads to the cross-linking of CS molecules. The diameter of $CaCO_3$ templates and $CaCO_3$@CS hybrid microparticles estimated from FESEM images via software Image J was 4.0 ± 0.5 μm (mean value $\pm$ SD, $n = 100$ particles).

Characterization FITC-labeled cross-linked CS capsules

Since the CS has been labeled with FITC, the $CaCO_3$@CS hybrid microparticles, and CS capsules could be observed under CLSM. Figure 3a displays the confocal images of $CaCO_3$@CS hybrid microparticles. Figure 3b shows that the CLSM images of CS capsules fabricated via sacrificial PMAA-doped $CaCO_3$ templates, which can give us evidence to illustrate that CS polysaccharide has been labeled with green FITC. Figure 3c shows the typical TEM graphs of the CS capsules which are made from the PMAA-doped $CaCO_3$ as the sacrificial templates. Obviously, all the cross-linked CS capsules exhibit the hollow inner structure. The size of the capsules lies in the range from 2 to 3.5 μm. The average particle diameters of the capsules are about (3 μm). From the data, we can see that the average diameters of the capsules are smaller than the data gotten from confocal images. This can be explained that the CS capsules will shrink in some degree during the gradual drying process in air and under the effect of electron beam of electron microscope [22]. The shell thickness of capsules is about $\sim$30 nm. Figure 2d shows FESEM images of CS capsules. From the images, we can see the capsules have been collapsed. Some microspheres keep the spherical shape perfectly. Figure 3e (inset in Fig. 3d) shows the morphology $CaCO_3$@CS hybrid microparticles when the templates can't be removed entirely.

The capsule invagination was realized here by incubating the capsules in a series of CS solutions of different concentrations. When the osmotic pressure in the bulk solution is larger than in the interior, the solvent is squeezed out if the elastic restoring

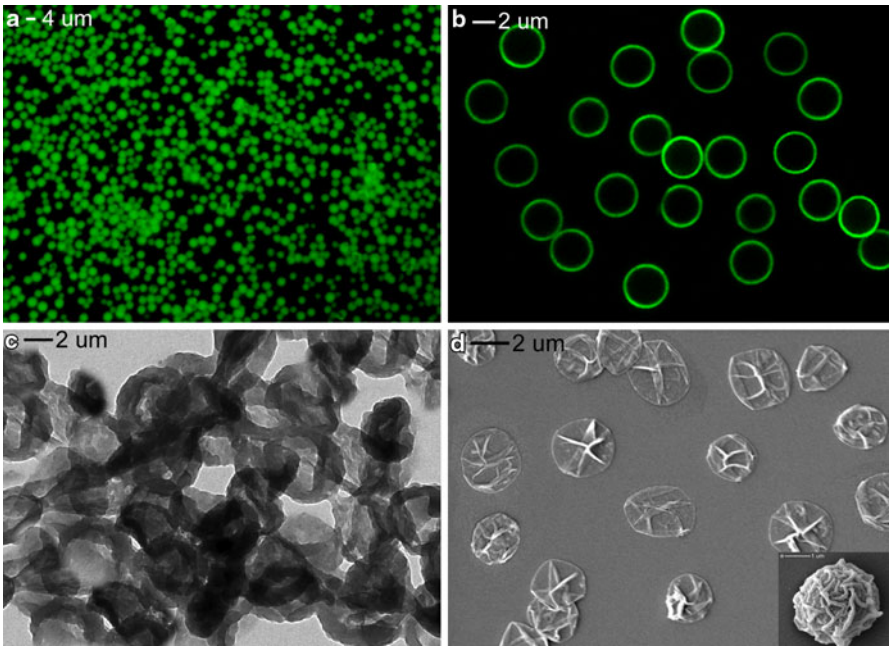


Fig. 3 **a** Confocal images of $CaCO_3$@CS the cross-linked CS absorbed onto templates via GA (the core have not removed). **b** Confocal images of cross-linked CS capsules labeled with FITC; **c** TEM and **d** FESEM images of the cross-linked CS capsules. **e** FESEM of images of $CaCO_3$@CS when the templates have not been removed entirely

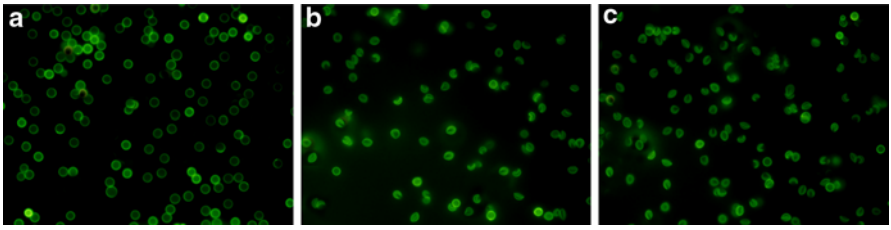


Fig. 4 CLSM images showing the gradual capsule invagination in response to bulk osmotic pressure created by CS solutions, whose concentration is **a** 0%, **b** 0.2%, and **c** 0.75% (wt). *Scale bar* 4 μm

force can not compensate the arising hydrostatic pressure difference. Figure 4 shows that the capsules underwent a shape transition from spherical Fig. 4a to a cup shape at a certain CS concentration Fig. 4b. At still higher CS concentrations, the capsules shrunk further and lost most of their internal volume Fig. 4c.

Conclusion

In this study, we described a method for the preparation of cross-linked FITC-labeled CS capsules by using PMAA-doped porous $CaCO_3$ microparticles as

sacrificial templates. By using this method, fluorescent single-component CS capsules were obtained by adsorption of FITC labeled and by the subsequent cross-linking with GA, and the removal template with EDTA. Results from CLSM, FESEM, and TEM revealed the formation of capsules. CLSM images showing the gradual capsule invagination in response to bulk osmotic pressure created by different concentration CS solutions. This templating method would be applicable to a wide range of other polyelectrolytes by reacting with a suitable cross-linking agent. We expect that this method will be useful to prepare single-component polymer capsules. Toward potential applications in biology and medicine, encapsulating drugs and chemicals in the capsules will be explored in the future.

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