Assembly of multilayer microcapsules on CaCO₃ particles from biocompatible polysaccharides

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Abstract—Multilayer microcapsules were fabricated by layer-by-layer (LbL) assembly of natural polysaccharides onto CaCO₃ particles, following with core removal. The micron-sized CaCO₃ particles were synthesized by reaction between Ca(NO₃)₂ and Na₂CO₃ solutions in the existence of carboxymethyl cellulose (CMC). The incorporated amount of CMC in the CaCO₃ particles was found to be 5.3 wt% by thermogravimetric analysis. Two biocompatible polysaccharides, chitosan and sodium alginate were alternately deposited onto the CaCO₃(CMC) templates to obtain hollow microcapsules. Regular oscillation of surface charge as detected by zeta potential demonstrated that the assembly proceeded surely in a LbL manner. The stability of the microcapsules was effectively improved by cross-linking of chitosan with glutaraldehyde. The chemical reaction was verified by infrared spectroscopy. The microcapsules thus fabricated could be spontaneously filled with positively charged low molecular weight substances such as rhodamine 6G and showed good biocompatibility, as detected by in vitro cell culture.

Key words: Chitosan; sodium alginate; carboxymethyl cellulose; layer-by-layer; microcapsules.

INTRODUCTION

Drug-delivery carriers have attracted a lot of interest during the past decades, since they can deliver low-molecular-weight drugs, as well as large biomacromolecules such as proteins and genes, either in a localized or in a targeted manner [1–3]. To date, many types of carriers, such as hydrogels [4], micelles [5], colloidal particles [6], lipid vesicles [7], polyelectrolyte complexes [8] and polyelectrolyte microcapsules [9], are under investigation, and some of them have been practically used.

Polyelectrolyte multilayer thin films can be constructed onto a variety of substrates by means of sequential self-assembly of positively and negatively charged...
polyelectrolytes, namely the layer-by-layer (LbL) technique, which was initially introduced by Decher and co-workers in 1991 [10]. Later on, the technique was applied onto decomposable colloidal particles by Möhwald and co-workers, followed by core removal to produce hollow microcapsules [11, 12]. The hollow microcapsules have great potential applications as drug-delivery vehicle, biosensors and micro-reactors, since their properties and functionalities can be fine-tuned by varying microcapsule wall thickness, composition and introduction of exterior stimuli [13–17].

Acting as drug-delivery carriers, the hollow capsules have been well studied with respect to controllable loading and release properties. Another important factor is biocompatibility of the capsules, which should be always emphasized for those materials used in vivo, e.g., the drug-delivery carriers. Many efforts have been devoted towards fabricating biocompatible drug carriers using the LbL technique. For example, a direct deposition of biocompatible polysaccharides [18, 19], proteins or lipids [20] onto some drug crystals can conveniently form multilayer thin films on them. Attempts have also been tried to fabricate biopolymer microcapsules by depositing chitosan/chitosan sulfate [21], dextran sulfate/protamine [22], chitosan/alginate [23, 24] and poly(L-lysine)/poly(L-glutamic acid) [25] onto colloids such as melamine formaldehyde (MF), poly(DL-lactic acid) (PLA), mesoporous SiO₂ particles, etc., followed by core removal with suitable pathways.

Although biocompatible building blocks or cores have been independently applied in multilayer capsule fabrication, the capsules composed of natural polymers and templated on biocompatible cores are more promising but have been hardly reported so far. In the present study, natural polysaccharides, chitosan and alginate, are alternately deposited onto CaCO₃ particles to produce hollow capsules with expectable better biocompatibility. The capsules can attract positively charged substances by electrostatic interaction with the pre-encapsulated carboxymethyl cellulose (CMC), an additive used to tune core formation [26].

Chitosan and alginate (Scheme 1) have been widely adopted as carriers to immobilize or encapsulate drugs [27–32], bioactive molecules [33], proteins [34–38] and cells [39–42], for their biocompatible and biodegradable nature. They are

![Scheme 1. Molecular structures of chitosan (a), sodium alginate (b) and carboxymethyl cellulose (c).](image-url)
utilized solely [43, 44] or jointly [45] in the form of colloidal particles or microspheres to transport molecules through mucosa and epithelia because of their high affinity to cell membranes [46]. Chitosan can be regarded as a co-polymer of N-acetylglucosamine and glucosamine, 2-amino-2-deoxy-β-d-glucose [47–49]. Alginate is extracted from seaweed and composed of alternating α-L-guluronic acid and β-D-mannuronic acid residues [27–32, 50]. Carboxymethyl cellulose (CMC) (Scheme 1) is an important cellulose derivative and is widely used in industrial and health care aspects.

MATERIALS AND METHODS

Materials

Chitosan (viscosity 115 cps) was a commercial product of Haidebei Halobios (Ji’nan, China). Sodium alginate (viscosity 250 cps) was obtained from Sigma (St. Louis, MO, USA). Carboxymethyl cellulose, sodium salt (CMC, viscosity 30–80 cps) was purchased from China Curative and Medicine (Shanghai, China). Rhodamine B isothiocyanate labeled chitosan (Rd-chitosan) was synthesized according to the procedure described in the literature [51]. The water used in all experiments was triple distilled. All other chemicals were of analytical grade and used as received.

Synthesis of CaCO₃ microparticles

100 ml 0.025 M Ca(NO₃)₂ solution was mixed with 2 ml 5% CMC, into which 100 ml Na₂CO₃ solution was rapidly poured under ultrasonication. CaCO₃ microparticles were formed immediately and collected through filtration. CMC-incorporating CaCO₃ particles were designated CaCO₃(CMC).

Pure CaCO₃ particles were synthesized similarly without involvement of CMC.

Alternate assembly of chitosan and alginate onto the CaCO₃ particles

Solutions of chitosan (0.5 mg/ml, in 0.5 M NaCl, pH 5.0) and alginate (1 mg/ml, in 0.5 M NaCl, pH 5.0) were obtained by dissolving chitosan and alginate in 0.5 M NaCl solution, respectively. Their pH values were adjusted to 5 with 0.1 M HCl and 0.1 M NaOH. CaCO₃(CMC) particles were washed with 0.2 M NaCl solution (pH 5.0) 3 times before assembly. The multilayers were deposited onto the CaCO₃(CMC) particles by consecutive adsorption of chitosan and alginate using a centrifugation protocol [11]. In a typical fabrication process, the CaCO₃(CMC) particles were incubated in 1 ml chitosan solution for 15 min. The suspension was then centrifuged at 4000 rpm for 5 min and the supernatant was carefully removed with a pipette. Three washes in 0.2 M NaCl solution (pH 5.0) at each interval were conducted before the next alginate adsorption. Following the same adsorption protocol as chitosan, a layer of alginate was assembled. The adsorption
was repeated until 10 layers of polysaccharides were assembled with alginate as the outermost layer.

**Cross-linking of the multilayers by glutaraldehyde and removal of the cores**

After 5 bilayers of chitosan/alginate were assembled, the multilayers on the CaCO₃ particles were treated with 1% glutaraldehyde (GA) solution for 12 h at room temperature to cross-link the chitosan component. The CaCO₃ cores were then removed by incubation in 0.2 M disodium ethylenediaminetetraacetic acid (EDTA) solution 3 times, each for 30 min. Non-cross-linked microcapsules were similarly prepared without GA treatment for comparison.

**Characterization**

**Thermogravimetry.** Thermogravimetric analysis (TGA) was conducted on a Pyris 6 thermogravimetric analyzer (Perkin-Elmer, Norwalk, CT, USA) at a heating rate of 20°C/min from 50 to 900°C. Samples were treated at 100°C prior to TGA analysis.

**Microelectrophoresis.** The electrophoretic mobility was measured using a Zetasizer (Mastersizer 2000, UK). The mobility $u$ was converted into a $\zeta$-potential using the Smoluchowski equation ($\zeta = u\eta/\varepsilon$, where $\eta$ and $\varepsilon$ are viscosity and permeability of the solution, respectively). All measurements were performed in 0.2 M NaCl solution with a pH value of 5.0. Each datum was averaged from 3 measurements.

**Confocal laser scanning microscopy (CLSM).** CLSM images were obtained using a Bio-Rad Radiance 2100 confocal laser scanning microscope, equipped with a 100× oil immersion objective with a numerical aperture of 1.4. For visualization of the capsules under fluorescence mode, Rd-chitosan was employed as a building block of the 9th layer. Unlabeled capsules were used for loading experiment. To observe the spontaneous loading, a drop of capsule suspension was put on a thin glass slide, mixed with a tiny amount of rhodamine 6G (Rd6G) and observed immediately.

**Fourier transformed infrared spectroscopy (FT-IR).** FT-IR spectra were acquired using a Vector 22 FT-IR spectrophotometer (Bruker Optics, Switzerland). Dried microcapsules were milled with KBr powder and then pressed into pellets.

**Scanning electron microscopy (SEM).** CaCO₃(CMC) microparticles were adhered onto a conductive adhesive tape and air dried for SEM (SIRION-100, FEI) characterization. CaCO₃(CMC) microparticles were milled between glass slides for cross-section observation. Energy dispersive analysis of X-ray (EDAX) was acquired by attachment of GENENIS4000 assembled in the SEM apparatus.
Transmission electron microscopy (TEM). The capsule structure was observed by transmission electron microscopy (TEM; Jeol JEM-200CX, Japan). Copper grids sputtered with carbon films were used to support the microcapsules. 10 µl of the microcapsule suspension was put on the copper grids and air-dried before measurement.

Scanning force microscopy (SFM). A drop of the capsule suspension was put onto newly cleaved mica and dried in air, and was observed using a Seiko Instruments scanning force microscope (SFM, SPI3800N Probe Station and SPA400 SPM Unit) in a dynamic force mode under ambient condition. Silicon tips with a resonance frequency $f_0$ of 150 kHz and a spring constant of 16 N/m were utilized. The scanning frequency was 1 Hz.

Cytotoxicity evaluation. Cross-linked (chitosan/alginate)$_5$ microcapsules, either treated by lysine overnight to eliminate aldehyde groups or not, were sterilized in 75% ethanol and washed in PBS 6 times before cell culture. The concentration of the microcapsules was adjusted to $(2.5 \pm 0.2) \times 10^7$/ml in PBS. Human dermal fibroblasts were isolated from foreskins and were routinely cultured [52]. The fibroblasts were incubated in a culture medium consisting of 10% (v/v) fetal calf serum (FCS, Sijiqing Biotech., China) and 90% (v/v) DMEM (Gibco-BRL), supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin in humidified air containing 5% CO$_2$ at 37°C. 200 µl human dermal fibroblast suspension was seeded in a well of a 96-well polystyrene plate, with a final cell number of $7.5 \times 10^4$ per well. After 24 h, 20 µl microcapsule solution was added to each well seeded with the fibroblasts. The final capsule concentration was high enough for close contact between the cells and the capsules. The culture medium was changed every 3 days. The cell proliferation was measured using the methylthiazoletetrazolium (MTT) method [53]. The absorbance was recorded at a wavelength of 570 nm by a microplate reader (Bio-Rad model 550). Three parallel experiments were conducted and data were expressed as mean ± standard deviation.

RESULTS AND DISCUSSION

CaCO$_3$ microparticles were chosen as the templates for capsule fabrication because of ease of removal and non-toxicity [54, 55]. The non-toxicity of template is greatly appreciated for the microcapsules used in biological field. Acting as assembly templates, the CaCO$_3$ particles should be controlled with respect to their shape and morphology [56]. Various additives, including low-molecular-weight compounds [57–59], synthetic polymers [60–63] and biomacromolecules [64–66] have been used to control the crystallization of the CaCO$_3$ particles. More information on crystallization and morphological control over the CaCO$_3$ particles by additives can be found in some reviews [67, 68]. Here, CMC was added into
the reaction system to control the shape and morphology of the resultant CaCO$_3$ particles. CaCO$_3$ microparticles with spherical shape and narrow size distribution (2–4 µm) were obtained (Fig. 1a). The magnified image (Fig. 1a, inset) shows that

Figure 1. (a) SEM images of CaCO$_3$(CMC) microparticles. Inset: higher magnification image. (b) Cross-section image of a particle.
the CaCO₃ particle surface is composed of many tiny particles. The cross-section image (Fig. 1b) reveals a porous inner structure and a denser skirt, indicating that the big particle is formed by aggregation of those nanoparticles under the mediation of CMC [69]. Here, CMC complexes with Ca²⁺ and, thus, can control the growth dynamics of CaCO₃ particles. Moreover, it can also act as a glue to bind the nanoparticles together, forming spherical microparticles ultimately.

Energy dispersive analysis of X-ray (EDAX) measures a higher C content (20.1 wt%) on cross-sectional surfaces of the CaCO₃(CMC) particles than that (18.1 wt%) of the pure CaCO₃ particles, demonstrating qualitatively the incorporation of the CMC. Quantification of the CMC was performed by thermogravimetry analysis (Fig. 2). Compared to a very minimal weight loss (<1.5%) of the pure CaCO₃ particles in the temperature range 50–520°C, a considerable large amount (11.2%) of mass was lost for the CaCO₃(CMC) particles. The weight loss of 5.9% in the temperature range 50–240°C should represent the evaporation of water, whereas the weight loss of 5.3% in the temperature range 240–520°C corresponds to decomposition of the organic polymer, CMC. The sharpest decreases in weight loss were extrapolated to be 680 and 689°C for pure CaCO₃ and CaCO₃(CMC) particles, respectively, indicating that the decomposition temperature of CaCO₃ is hardly influenced by incorporation of the CMC [70, 71].

To ensure successful deposition of the polysaccharides onto the CaCO₃ templates and to avoid agglomeration of the particles, the self-assembly conditions were...
optimized by tuning the pH values of the chitosan and the alginate solutions. An optimal pH 5.0 was selected because both polymers are highly charged at this condition [72–74]. Because of the existence of CMC, the $\zeta$-potential of CaCO$_3$(CMC) at pH 5.0 was $-38.5 \pm 1.9$ mV (Fig. 3). This is different from the pure CaCO$_3$ particles, since they are positively charged at this pH value ($pK_a = 8.5$ [75]). After deposition of a layer of chitosan, the $\zeta$-potential still exhibited a negative value, $-7.7 \pm 3.5$ mV, which might be caused by the incomplete coverage of the particles by chitosan molecules or the interpenetration of the pre-captured CMC molecules. Regular charge reversal was observed since the second circle of deposition, suggesting stepwise adsorption of the chitosan/alginate multilayers. After cross-linking, the $\zeta$-potential of the microparticles ($-8.8 \pm 2.3$ mV) was almost unchanged, demonstrating that the outmost layer should be still abundant with alginate.

After removal of the CaCO$_3$ cores hollow capsules were obtained, as evidenced by confocal laser scanning microscopy (CLSM) (Fig. 4). No microscopically detectable breakages could be found on the walls of both the GA cross-linked (Fig. 4b) and the non-cross-linked control (Fig. 4a) capsules. The slight larger size of the capsules than their templates should be the result of the capsule swelling during the core removal procedure, an effect of transient higher osmotic pressure within the capsule interiors [76]. After dried in air, the microcapsules were further
Figure 4. CLSM images of (chitosan/alginate)$_4$/Rd-chitosan/alginate microcapsules templated on CaCO$_3$(CMC) particles. (a) non-cross-linked and (b) cross-linked by 1% GA for 12 h. This figure is published in colour on http://www.ingenta.com
subjected to TEM (Fig. 5) and SFM (Fig. 6) characterizations. The hollow structures of the non-cross-linked and the cross-linked microcapsules are again confirmed in Fig. 5a and Fig. 5b, respectively, despite the aggregation of these microcapsules...
Figure 6. SFM images and their cross-section profiles to show the dried (chitosan/alginate)$_5$ microcapsule before (a) and after (b) GA cross-linking. This figure is published in colour on http://www.ingenta.com.
As a result of core removal. In SFM images, both kinds of the capsules exhibit hollow and intact morphology. Creases and folds can be observed in the uncross-linked microcapsule (Fig. 6a, also see Fig. 5a), while the cross-linked microcapsule exhibits less collapse (Fig. 6b, also see Fig. 5b). Moreover, derived from the line profiles by SFM, a relatively thicker wall was found for the cross-linked capsules. All these results would mean that the capsule walls may become harder after cross-linking [77], thus may lead to incomplete collapse of the capsule walls.

Glutaraldehyde is frequently used as a cross-linking reagent for chitosan. Detailed information can be found from the reviews [78, 79]. Two hydroxyl groups and one amino group exist in one glucosamine ring of chitosan. All of them can react with glutaraldehyde, but the amino group react much faster [80]. The characteristic peaks at 1157, 1085 and 1030 cm\(^{-1}\) (\(\nu_{\text{C-O-C}}\) vibration of glucose ether, contributed by both of the chitosan and alginate [81, 82] in the IR spectrum before cross-linking (Fig. 7a) shifted to 1154, 1071 and 1033 cm\(^{-1}\) after cross-linking (Fig. 7b). The broad peak centered at 1628 cm\(^{-1}\) in the spectrum of the uncross-linked microcapsules should be the superimposition of the following peaks: C-2 amine of glucosamine (1597 cm\(^{-1}\)) [83–85], N-acetyl amide I (1655 cm\(^{-1}\)) and amide II (1560 cm\(^{-1}\)) [47, 48, 81] from chitosan, and the carboxylate (1615 cm\(^{-1}\)) of alginate [82]. After cross-linking, a new weak peak at 1645 cm\(^{-1}\) appeared, which could be assigned to the \(\nu_{\text{C=\text{N}}}\) stretching mode of imine [83–85], confirming the reaction between aldehydes and amino groups and the occurrence of a Schiff base [83]. Due to the weakening of the disturbance of the amino groups, the absorbance of carboxylate of alginate at 1615 cm\(^{-1}\) is more clearly identified.

**Figure 7.** FT-IR spectra of (chitosan/alginate)\(_5\) microcapsules before (a) and after (b) GA cross-linking.

[Graph showing FT-IR spectra with peaks labeled 1628, 1654, 1154, 1071, 1033, 1645, 1615, and 1409 cm\(^{-1}\).]
(Fig. 7b). Moreover, although shifted from 1409 (Fig. 7a) to 1412 cm$^{-1}$ (Fig. 7b) [82, 86], the symmetric ($\nu_{\text{sym}}$) stretching of the carboxylate of alginate is still present after cross-linking. All these results suggest that the reaction between chitosan and GA has occurred, and alginate molecules still exist in the capsule walls after cross-linking. This is consistent with the zeta potential result.

After cross-linking, the stability of the resulting capsules against extreme pH treatments was substantially improved (Fig. 8). Before cross-linking, the chitosan/alginate capsules were readily dissolved in 0.1 M NaOH or 0.1 M HCl solution in few seconds, because of decharge of chitosan and alginate at high and low pH values, respectively. In contrast, the cross-linked microcapsules were stable under these conditions for at least 24 h.

Due to incorporation of the negatively charged CMC in the capsules, the as-prepared microcapsules have strong affinity to positively charged molecules, such as rhodamine 6G (Rd6G) (Fig. 9a). The strong fluorescence from the capsule interiors demonstrates that Rd6G had been spontaneously loaded into the capsules. However, no spontaneous accumulation effect was observed when the capsules were similarly incubated in a solution of negatively charged fluorescein (Fig. 9b). These results confirm that the driving force for Rd6G loading should be mainly the electrostatic interaction [87]. Thus, one can conclude that the biocompatible chitosan/alginate microcapsules are envisaged to be suitable candidates for drug carriers.

The biocompatibility of the as prepared microcapsules was preliminary assessed by in vitro culture of human fibroblasts in the existence of the (chitosan/alginate)$_5$ capsules (Fig. 10). Through all the culture period, no significant difference in cytoviability between the control (cells only) and the samples supplemented with cross-linked capsules, and cross-linked and lysine-treated capsules. This result demonstrates that the cross-linked capsules have no detectable cytotoxicity; hence, they possess good biocompatibility which is important for medical applications.

**CONCLUSIONS**

Chitosan and alginate multilayers are alternately assembled onto CMC-incorporated CaCO$_3$ microparticles to produce CMC-preloaded microcapsules after core removal. The CaCO$_3$ microparticles with spherical shape and narrow size distribution (2–4 $\mu$m) are synthesized from Ca(NO$_3$)$_2$ and Na$_2$CO$_3$ solutions under the mediation of CMC. The regular oscillation of zeta-potential between negative and positive values confirms that the layer growth of chitosan and alginate is surely in a LbL manner. Intact microcapsules are obtained after dissolution of the CaCO$_3$ cores by EDTA. GA cross-linking of the chitosan component can change both the capsule morphology and stability against extreme pH treatments. The GA cross-linked capsules in dry state present less folds and creases and relatively thicker walls. Chemical change after GA treatment is verified by IR spectroscopy. The cross-linked capsules are stable in 0.1 M NaOH or 0.1 M HCl solution for at least 24 h, while those untreated are dissolved within few seconds. Furthermore, the
Figure 8. CLSM images of (chitosan/alginate)₄/Rd-chitosan/alginate microcapsules after cross-linked by 1% GA for 12 h. (a) After incubation in 0.1 M HCl solution and (b) in 0.1 M NaOH solution for 24 h. This figure is published in colour on http://www.ingenta.com
Figure 9. CLSM images of GA cross-linked (chitosan/alginate)₃ microcapsules (a) mixed with 0.02 M Rd6G and (b) mixed with 0.02 M fluorescein. This figure is published in colour on http://www.ingenta.com
cross-linked capsules can spontaneously load positively charged substances such as Rd6G. Together with their better biocompatibility, one can thus conclude that these capsules are promising candidates as drug-delivery carriers.

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