Genipin Cross-Linked Alginate-Chitosan Microcapsules: Membrane Characterization and Optimization of Cross-Linking Reaction

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The genipin cross-linked alginate-chitosan (GCAC) microcapsule, composed of an alginate core and a genipin cross-linked chitosan membrane, was recently proposed for live cell encapsulation and other delivery applications. This article for the first time describes the details of the microcapsule membrane characterization using a noninvasive and in situ method without any physical or chemical modifications on the samples. Results showed that the cross-linking reaction generated the fluorescent chitosan-genipin conjugates. The cross-linked chitosan membrane was clearly visualized by confocal laser scanning microscopy (CLSM). A straightforward assessment on the membrane thickness and relative intensity was successfully achieved. CLSM studies showed that the shell-like cross-linked chitosan membranes of approximately 37 μm in thickness were formed surrounding the microcapsule. The reaction variables, including cross-linking temperature and time significantly affected the fluorescence intensity of the membranes. Elevating the cross-linking temperature from 4 to 37 °C drastically intensified the membrane fluorescence, suggesting the attainment of a high degree of cross-linking on the chitosan membrane. Extended cross-linking time altered the cross-linked membranes in modulation. Although genipin concentration and cross-linking time had little effects on the membrane thickness, cross-linking at higher temperatures tended to form relatively thinner membranes.

Introduction

Microencapsulation has received increasing attention over the past two decades in various fields of both fundamental research and industrial applications.[1−4] Among others, cell encapsulation for therapy has generated considerable excitement as it enables the transplantation of live nonautologous cells in the absence of immuno-suppression by providing protection through a physical barrier. Potential applications include the treatment for enzyme deficiencies, diabetes, liver and kidney failure, cancers, and many other diseases[2,5−11]. In all of the applications, the effectiveness of the immuno-protection achieved by microencapsulation greatly depends on the integrity of the capsular membrane. The microcapsule membranes should exhibit sufficient structural stability to bear environmental constraints during processing, implantation, as well as both short-term and long-term in vivo utilization. The alginate-poly-L-lysine-alginate (APA) membrane[12] is widely investigated for cell encapsulation due to the gentle preparation process. These APA microcapsules have been used successfully to limit the major immuno-rejection problems related to the use of live cells and bacteria in some animal models.[7,9,13−15] However, problems regarding membrane instability arise over long-term in vivo conditions.[16−20] This mechanical insufficiency was associated with activation of the complement system, degradation of the poly-L-lysine coating, and destabilization of the alginate core matrix; accordingly, graft survival was usually limited.[18] Therefore, there is clearly a need for the development of stronger microcapsules that can protect the cells for a long time.

As an alternative to the APA system, we have proposed a novel alginate−chitosan complex microcapsule covalently cross-linked by naturally derived genipin.[21] Genipin is an aglucone of geniposide extracted from gardenia fruits.[22] It has been used as a traditional herbal medicine and natural colorant in the food and fabric industries.[23] Genipin has been reported to bind with biopolymers such as chitosan and gelatin, leading to covalent coupling.[24−26] Rather than the commonly used synthetic cross-linking reagents which have a recognized disadvantage of potential cytotoxic effects,[27−30] genipin is derived from herbal plant and has been reported 5000 to 10 000 times less cytotoxic than glutaraldehyde.[24] This encouraged the use of genipin in cell encapsulation. Results from our earlier study suggested the suitability of the novel genipin cross-linked alginate-chitosan (GCAC) microcapsule for the encapsulation of live engineered bacteria.[21] Recent research on the fluorogenic characteristics of genipin showed the usefulness of genipin on the characterization of microcapsule membranes.[31,32] The objective of this paper is to characterize the cross-linked chitosan membrane on the GCAC microcapsules and optimize the cross-linking reaction using a novel, noninvasive, and in situ method by confocal laser scanning microscopy (CLSM).

Experimental Section

Chemicals. Sodium alginate (low viscosity) was purchased from Sigma-Aldrich, USA. Chitosan (low viscosity, 73.5% degree of deacetylation and $M_v = 7.2 \times 10^4$) and genipin were obtained from Wako BioProducts, USA. All other reagents and solvents were of reagent grade and used as received without further purification.

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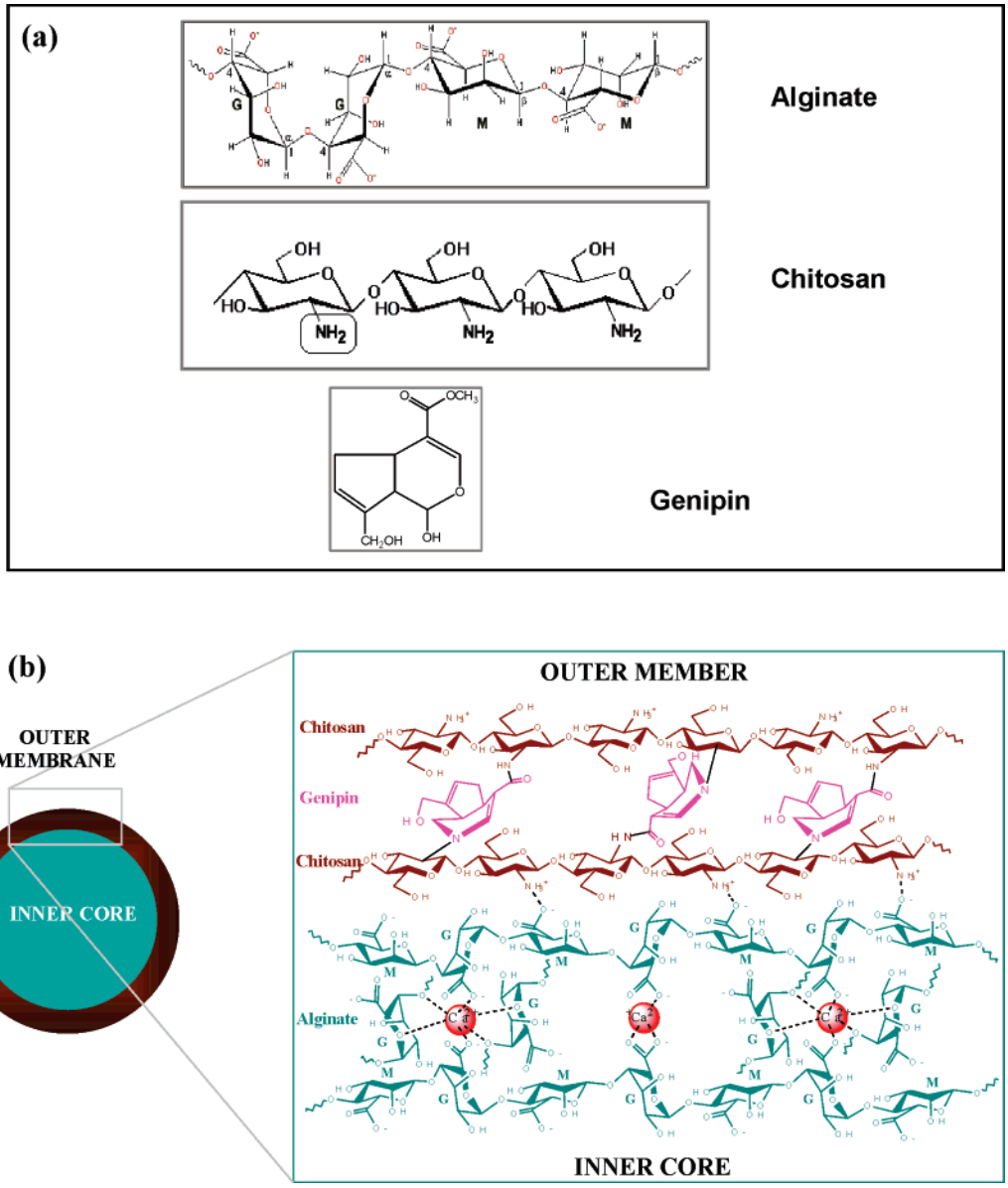

Figure 1. (a) Schematics of the chemical structures of alginate (top), chitosan (middle) and genipin (bottom) used in microcapsule preparation. (b) Schematic molecular structure of the genipin cross-linked alginate−chitosan (GCAC) microcapsules.

Table 1. Control Factors and Their Levels for the Cross-linking Reaction

	level				
factor	1	2	3	4	5
A. concentration (mg/mL)	1.0	2.5	5.0		
B. reaction temperature (°C)	4	20	37		
C. reaction time (h)	5	10	24	48	72

Preparation of Genipin Cross-Linked Alginate-Chitosan (GCAC) Microcapsules. The microcapsules were prepared as previously described.[21] Briefly, droplets of a sodium alginate solution (15 mg/mL) were generated by an encapsulator (Inotech. Corp.) and gelled in a stirred $CaCl_2$ solution (11 mg/mL). The Ca-alginate beads were then coated for 30 min in a chitosan solution of 10 mg/mL containing $CaCl_2$ (11 mg/mL), producing alginate-chitosan (AC) microcapsules, and cross-linked by immersing the AC microcapsules in an aqueous genipin solution. The resulting genipin cross-linked alginate-chitosan (GCAC) microcapsules were washed and collected. Ca−alginate beads with genipin treatment (AG) and AC microcapsules without genipin treatment were also prepared in a similar process and used as controls.

Characterization of Microcapsules by Confocal Laser Scanning Microscopy (CLSM). The morphology and internal structure of the microcapsules were investigated using a Zeiss LSM 510 laser scanning confocal imaging system (Carl Zeiss, Jena, Germany), equipped with a Zeiss Axiovert 100M microscope and an argon-ion laser. For image acquisition, the microcapsules in storage solution (deionized H_2O) were directly placed in a chambered coverglass system (Lab-Tek). One channel of the CLSM was used in the single green fluorescence mode at an excitation of 488 nm and with the filter block BP500−550IR. The other channel was set to the transmitted light detector. The focal planes were set at the equatorial sections of the microcapsules. All images were acquired at constant microscopic settings under computer control in order to obtain comparable images. The fluorescent intensity profile corresponding to a line across the focal plane of the microcapsules was acquired by computational profile analysis (LSM 510 software).

Atomic Force Microscopic (AFM) Observation. The internal structure of the GCAC microcapsules was characterized by AFM (Digital Instrument, Veeco metrology Group, USA). The samples were cross-linked by genipin at the concentration of 5.0 mg/mL for 72 h at room temperature. After dehydration by gradient acetone, the microcapsules were embedded in epoxy resin and cross-sectioned by ultra

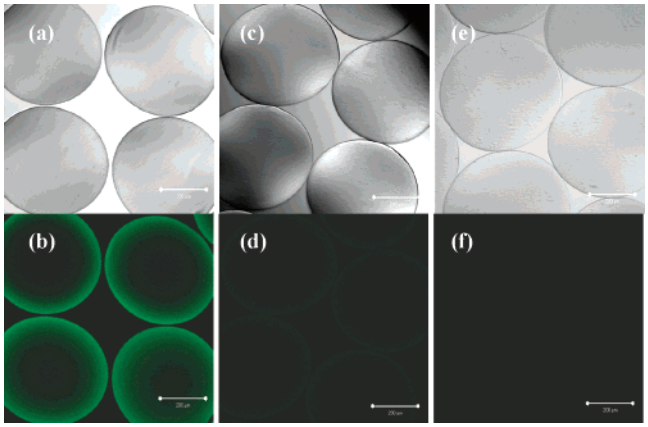

Figure 2. Photomicrographs of the GCAC (a and b), the control alginate-chitosan (c and d) and the control alginate-genipin (e and f) microcapsules viewed from the transmitted light channel (upper row) and the fluorescence channel (lower row) of CLSM. Genipin treatment (1.0 mg/mL) on microcapsules was performed at 37 °C for 24 h. Bars = 200 μm.

microtome. The AFM topographic images were obtained by scanning the cross-sectioned microcapsules using a sharpened tip in contact mode at a rate of 1.0 Hz. The roughness profiles and the membrane thickness were analyzed using the equipped NanoScope Image software.

Effects of Reaction Variables and Optimization of Cross-linking Process. To evaluate the influences of cross-linking conditions on the microcapsule membrane, three control factors including the genipin concentration, cross-linking temperature, and time were selected to vary. For each factor, at least three levels were chosen to cover a wide range of variation. The factors and their levels were listed in Table 1. The microcapsules were prepared accordingly, and at least 10 beads per batch were assessed by CLSM. The relative fluorescence intensities along the microcapsule membranes (500 μm in length) were analyzed, and the membrane thickness was measured using LSM 510 software. A statistical analysis using range tests[33] was performed to determine the relative magnitude of the control factors and estimate the optimum levels with regard to generating microcapsule membranes with highest cross-linking degree. The degree of confidence was set at 95%.

Results

Formation of the GCAC Microcapsules and Cross-Linking of Chitosan by Genipin. The preparation of the GCAC microcapsules involved a three-step procedure, all under mild and aqueous conditions. The cross-linking was achieved by the interaction of genipin with the chitosan bound on the alginate beads, and the chitosan−genipin conjugates were formed within the membrane. Figure 1 displays the schematic diagrams for the structures of the materials used in microcapsule preparation and the predicted molecular structure of the GCAC microcapsules. It was found that the cross-linking treatment did not noticeably affect the morphology of the microcapsules. They remained intact, spherical in shape, and similar in size (471.9 ± 9.3 μm) and had high homogeneity (Figure 2a) though an apparent color change was observed. In particular, the GCAC microcapsules turned from white to dark blue in color if cross-linked at 37 °C, to light blue at 20 °C, and to faintly yellow−blue at 4 °C.

Characterization of GCAC Microcapsule Membranes by CLSM. To visualize the microcapsules and their membranes in the same imaging field, CLSM was employed with one channel set to the single green fluorescence mode and the other to the transmitted light detector. Figure 2 depicts the CLSM images of the GCAC microcapsules in comparison to the control

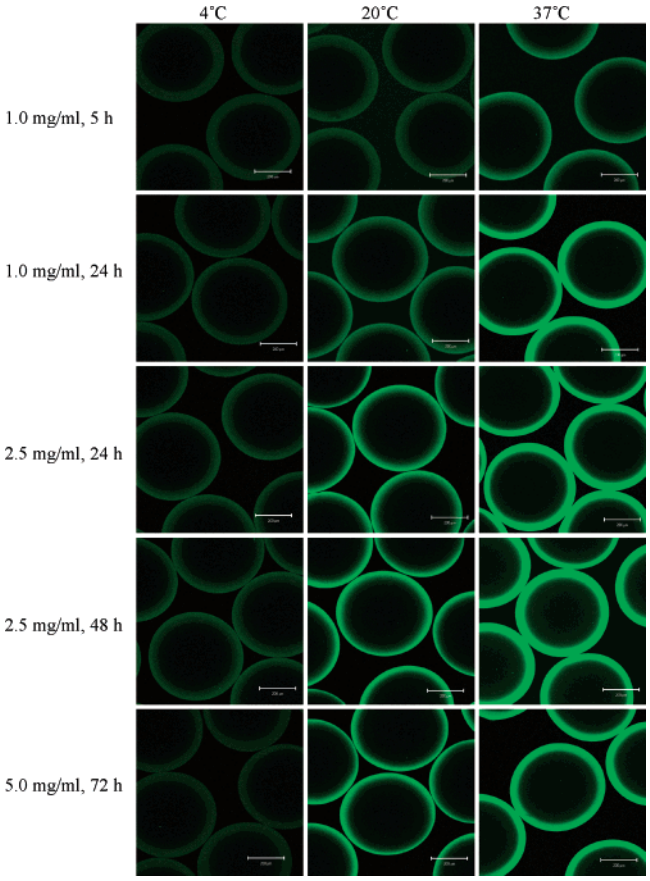

Figure 3. CLSM images of the GCAC microcapsules. The microcapsule membrane was cross-linked at varied genipin concentrations (1.0, 2.5, or 5.0 mg/mL) and temperatures (4, 20, or 37 °C) for different reaction time (5, 24, 48, or 72 h). Bars = 200 μm.

AC and AG beads. Under the regular transmission light channel, microcapsules looked similar regardless of genipin cross-linking (Figure 2, upper row), with the exception of considerable swelling of the AG beads (Figure 2e). When viewed under the fluorescent channel, the alginate cores were shown as the black interior of the microcapsules, whereas the genipin cross-linked chitosan coating was clearly identified by the appearance of distinguishing bright circles circumscribing the alginate cores (Figure 2b). In contrast, neither the control AC microcapsules without genipin cross-linking (Figure 2d) nor the control AG beads without chitosan coating (Figure 2f) fluoresced under the same microscope settings. It was clear that the fluorescent signals were induced by the chitosan−genipin reaction.

Figure 3 shows representative CLSM images of the GCAC microcapsules viewed in the fluorescence channel. Despite signals being weak or strong, the fluorescent cross-linked chitosan membranes were successfully imaged for all of the GCAC microcapsule samples prepared in this study. Deposited homogeneously around the microcapsules, the cross-linked chitosan formed a shell-like membrane near the surface of the microcapsule, with stronger fluorescence intensity at the external border of the membrane (Figure 4). Also clearly evidenced, the relative fluorescence intensity of the microcapsule membranes was correlated with the cross-linking conditions. For example, the fluorescence intensity of the membrane was high (~ 250) if cross-linked at 37 °C (Figure 4a); it decreased by roughly half (~ 120) when reacted at 20 °C (Figure 4b). If the cross-linking temperature dropped to 4 °C, the intensity was significantly lower (~ 45) (Figure 4c). The 3-D diagrams shown in Figure 5 further illustrate this trend. The intensity of the interior

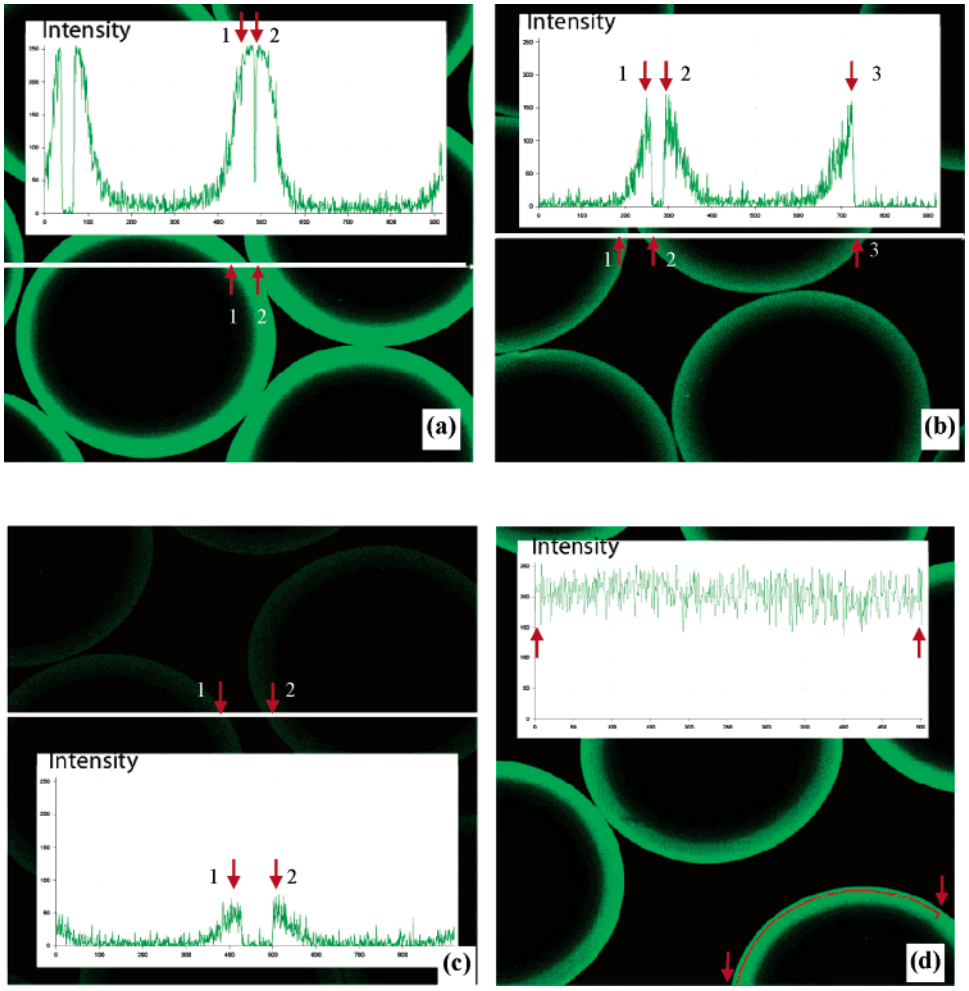

Figure 4. Fluorescence intensity profiles corresponding to the lines drawn across the focal plane of the GCAC microcapsules cross-linked by genipin at a concentration of 2.5 mg/mL for 24 h at (a) 37, (b) 20, and (c) 4 °C. (d) shows the fluorescence intensity of the membrane corresponding to a red line of 500 um in length indicating homogeneous distribution of the cross-linked chitosan in the microcapsule membrane.

alginate cores, shown in dark blue, was as low as the background signals (<10), while the fluorescence of the membrane was strikingly higher. The higher the cross-linking temperature and the longer the reaction time, the greater level of the intensity exhibited by the microcapsule membranes, denoting stronger fluorescence (Figure 5).

Effects of Reaction Parameters on the Fluorescence Intensity of the Cross-linked Chitosan Membranes on the GCAC Microcapsules

To assess the effects of the cross-linking process on the microcapsule membranes, three control factors including genipin concentration, cross-linking temperature and time were selected to vary (Table 1). The corresponding fluorescence intensities of the microcapsule membranes were analyzed semiquantitatively and plotted in Figure 6. It appeared that when cross-linked at 4 °C, the membranes displayed low fluorescence (<50) which was hardly altered by the extended reaction time and the use of concentrated genipin (Figure 6a). At 20 °C, extending the reaction time led to steady increase in the fluorescence intensity of the membranes, indicating more cross-linking points were formed after longer reaction time (Figure 6b). In addition, the membrane fluorescence increased rapidly at 37 °C and attained a saturated level after 24 h of cross-linking (Figure 6c).

To further evaluate the effects of the cross-linking variables, statistical range tests [33] were performed to determine the relative magnitude of each control factor. Table 2 shows that the ranges of the cross-linking temperature and reaction time are higher than the corresponding confidence limits, suggesting that these two selected factors significantly affected the fluorescence intensity of the membranes ($p < 0.05$); whereas, the effect of genipin concentration was irrelevant (range < confidence limit). Results also showed that the cross-linking temperature, with the highest range of 161, was the dominant factor affecting the extent of the reaction (Table 2). As can be seen in Figure 7a, elevating the cross-linking temperature from 4 °C to 37 °C intensified the membrane fluorescence at an exponential rate ($r = 0.998$). The factor of reaction time changed the cross-linked membrane in modulation (Figure 7b). The fluorescence intensity increased quickly during the initial 24 h of cross-linking and slowed thereafter until the end of the experiment, the trend of which well fit with a logarithmic correlation at the confidence degree of 95% ($r = 0.946$). On the other hand, varying the genipin concentration within the experimental span had little effect on the fluorescence of the cross-linked membranes, though slightly stronger fluorescence could be attained by using 2.5 mg/mL genipin (Figure 7c).

Membrane Thickness of the GCAC Microcapsules

Experiments were also designed to investigate the membrane thickness of the GCAC microcapsules and the results were summarized in Table 3. It was found that despite variations, the GCAC microcapsule membrane had an overall mean thickness of 37.3 μm. Results from statistical range tests (Table

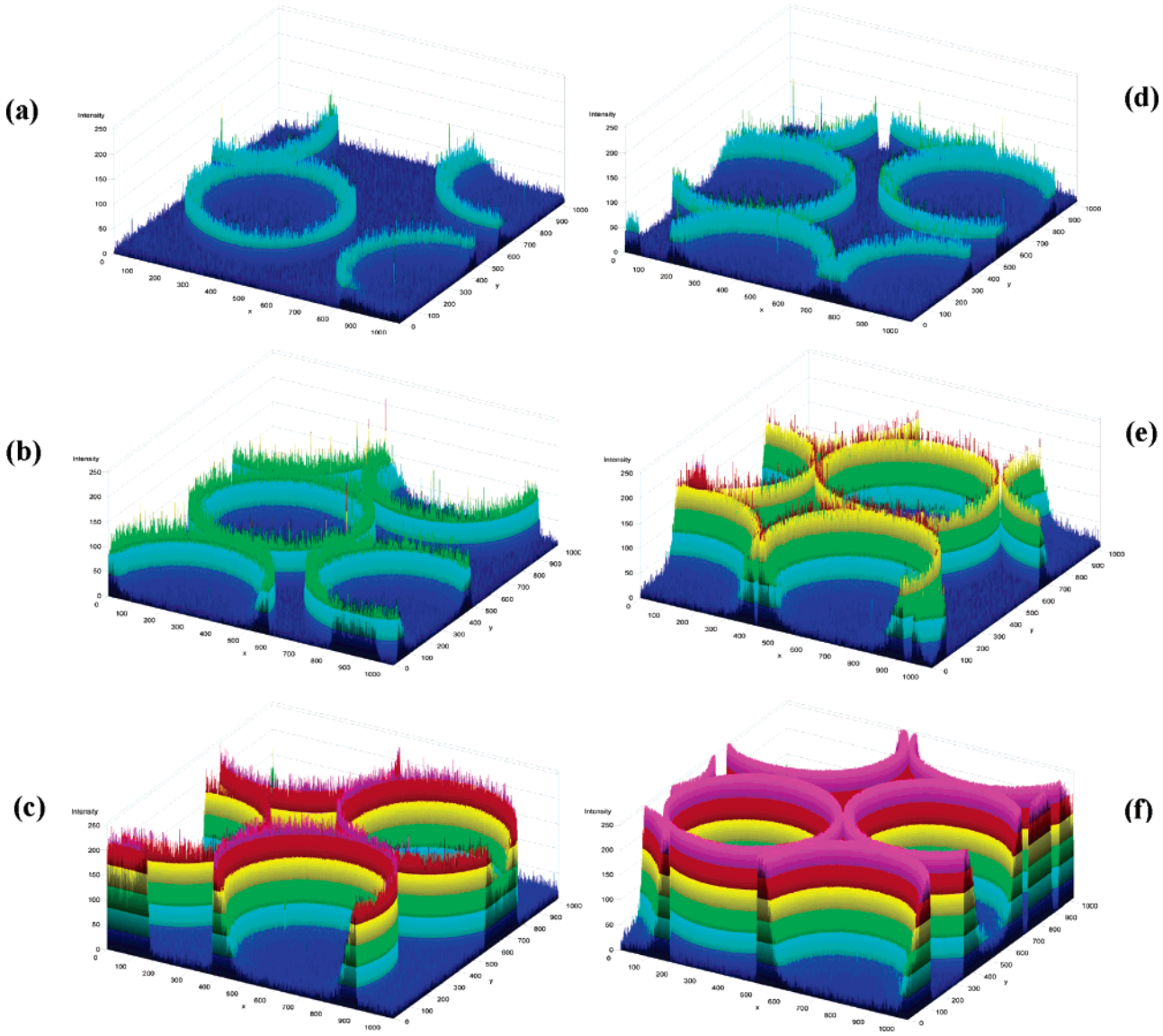

Figure 5. 3-D diagrams representing the intensity distribution over the scan areas and the relative fluorescence intensity of the GCAC microcapsule membranes. The GCAC microcapsules were cross-linked by genipin (2.5 mg/mL) at (a) 4 °C, 10 h; (b) 20 °C, 10 h; (c) 37 °C, 10 h; (d) 4 °C, 24 h; (e) 20 °C, 24 h; and (f) 37 °C, 24 h.

4) showed that the range for the factor of cross-linking temperature was higher than the experimental confidence limit (14.4 vs 2.2), suggesting a significant effect of reaction temperature on the membrane thickness. At a low temperature (4 °C), the microcapsules tended to form relatively thicker cross-linked membranes ($\sim$ 45 μm); whereas slightly thinner GCAC membranes were formed at higher temperatures. Conversely, allowing for random experimental errors the other two factors including genipin concentration and reaction time did not significantly affect the membrane thickness (Table 4).

AFM Observations on the GCAC microcapsules

AFM studies were carried out to further characterize the inner structure of the GCAC microcapsules. The topography of the cross-sectioned GCAC microcapsule is shown in Figure 8. It was clear that the structure of the genipin-cross-linked chitosan membrane was significantly different from the alginate core and the outer epoxy resin used for embedment; a distinctly rough structure was seen in the area of the microcapsule membrane, and an exceedingly smooth pattern was shown in the microcapsule core where the pores of the Ca-alginate gel were filled with epoxy. The thickness of the chitosan coating measured by AFM was 32.1 $\pm$ 5.0 μm (n=3).

Discussion

As described earlier, the success of live cell encapsulation and delivery is chiefly dependent on the ability of microcapsules to protect the enclosed cells. The microcapsule membranes are of importance for addressing the complex problems associated with in vitro encapsulation and in vivo delivery for therapy. Earlier research showed that stronger microcapsules survived longer in vivo, which in turn would lead to a prolonged delivery of the therapeutic molecules and a greater efficiency of the cell encapsulation strategy.[20] Aiming to produce microcapsules with improved stability, covalent cross-linking was employed to strengthen the chitosan membrane using a naturally-derived cross-linker, genipin.[21] As shown in Figure 1-b, the hypothetical structure of this microcapsule includes the calcium-alginate core formed by ionotropic gelation, chitosan coating through complex coaervation, as well as the covalent cross-linking of chitosan by genipin. In the cross-linking reaction, genipin, a small molecule, can freely diffuse through the alginate-chitosan complex membrane and interact with the chitosan bound to the alginate gel. Specifically, the ester groups in genipin interact with the amino groups in chitosan leading to the formation of

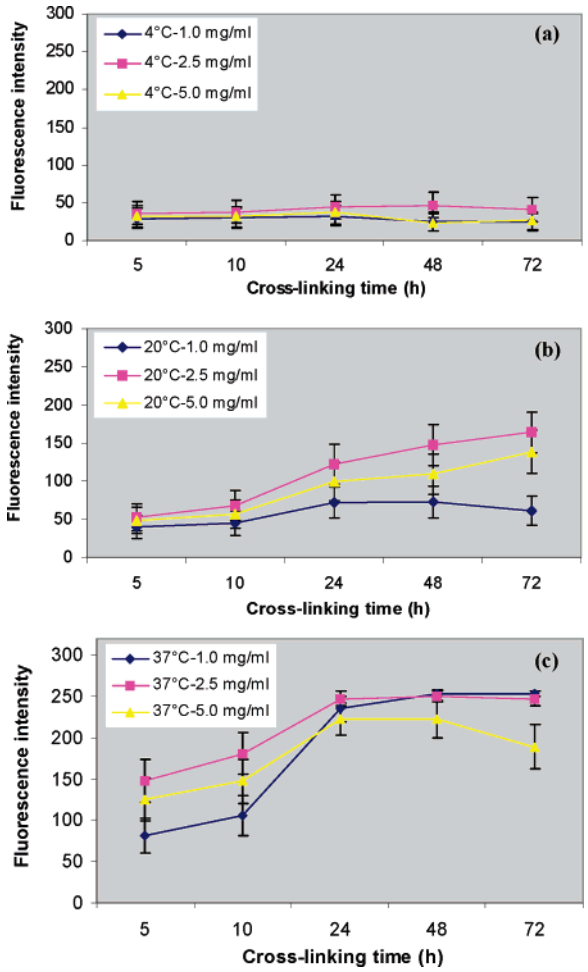

Figure 6. Fluorescence intensity of the GCAC microcapsules as a function of cross-linking time. The reaction temperatures used were (a) 4, (b) 20, and (c) 37 °C.

Table 2. Range Tests on the Fluorescence Intensity of the GCAC Microcapsule Membranes

	fluorescence intensity[a]						
	level						confidence
factor	1	2	3	4	5	range[b]	limit[c]
A. concentration	91	122	100	–	–	31	41
B. temperature	33	86	194	–	–	161	22
C. reaction time	66	78	123	128	127	62	52

[a] Mean of 15 (for factors A and B) or 9 (for factor C) fluorescence intensity data for microcapsules prepared under the same level of each factor. [b] Range = Maximum−Minimum. [c] Degree of confidence was set at 95%.

secondary amide linkages.[25] Additionally, the amino groups of chitosan initiate nucleophilic attacks at genipin, resulting in the opening of the dihydropyran ring followed by a number of reaction steps including the formation of nitrogen-iridoid, aromatic intermediates, as well as highly conjugated heterocyclic genipin-chitosan derivatives.[34] Eventually a three-dimensional network structure is created within the microcapsule membranes.

The CLSM images of the GCAC microcapsules convincingly supported this hypothetic structure. Since genipin only interacts with primary amines[35] and alginate was not the target for genipin cross-linking (Figure 2), the presence of bright rings validated the formation of new fluorescent chitosan-genipin conjugates and visually demonstrated the shell-like cross-linked membranes surrounding the microcapsule cores. The relatively stronger

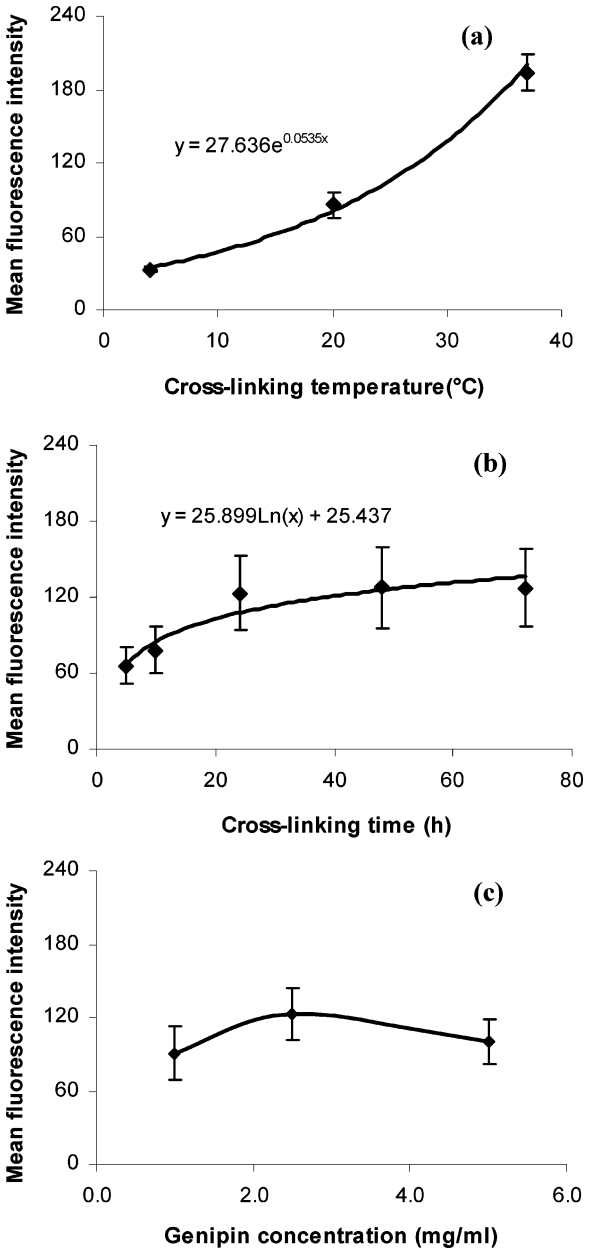

Figure 7. Effects of control factors on the fluorescence intensity of the GCAC microcapsule membranes. Control factors: (a) cross-linking temperature; (b) cross-linking time; and (c) genipin concentration. Error bars indicate the pooled s.d. of the mean fluorescence at each level.

fluorescent signals at the external border of the membrane may be induced by (1) restricted further diffusion of chitosan into the alginate core blocked by initial chitosan binding;[36,37] (2) a higher degree of cross-linking at the external border of the membrane where more chitosan was deposited; and (3) different structure and optical characteristics of the chitosan−genipin derivatives formed at the surface of the microcapsules. The generation of fluorescence in the cross-linked products allowed for easy evaluation of the cross-linking reaction and the membrane distribution in the microcapsules.

The fluorescence intensity generated by the genipin−chitosan reaction was in correlation with and indicative of the extent of cross-linking. The stronger the fluorescence, the more genipin−chitosan conjugates were formed, and thus, the higher cross-linking degree on the chitosan coating was attained. As a result of this, stronger microcapsules were likely generated. In the experiments, we investigated the influences of the cross-linking

Table 3. Membrane Thickness of GCAC Microcapsules Cross-Linked under Varied Conditions

genipin conc. (mg/mL)	reaction time (h)	membrane thickness (μm)[a] 4 °C[b]	20 °C[b]	37 °C[b]
1.0	5	48.7 ± 7.6	45.9 ± 8.1	34.2 ± 7.0
	10	44.6 ± 3.9	37.1 ± 7.2	28.0 ± 7.3
	24	44.2 ± 7.0	33.8 ± 3.7	34.7 ± 4.2
	48	40.1 ± 8.6	28.8 ± 6.7	38.0 ± 7.7
	72	49.1 ± 5.5	29.2 ± 7.3	41.3 ± 7.9
2.5	5	43.7 ± 8.1	30.1 ± 3.2	35.0 ± 9.7
	10	47.2 ± 2.7	29.4 ± 4.7	33.1 ± 7.3
	24	46.8 ± 5.4	31.1 ± 5.5	35.7 ± 5.4
	48	45.4 ± 3.8	30.2 ± 4.6	38.8 ± 9.4
	72	37.0 ± 9.3	27.6 ± 6.3	43.0 ± 5.7
5.0	5	46 ± 10	24.1 ± 4.6	35.1 ± 6.8
	10	46.1 ± 9.2	25.2 ± 4.7	37.1 ± 7.5
	24	44.6 ± 4.5	27.2 ± 5.1	37.9 ± 8.6
	48	43.5 ± 6.4	27.3 ± 6.3	38.6 ± 5.4
	72	44.3 ± 3.6	29.3 ± 5.9	41.1 ± 4.8
overall mean			37.3	

[a] Expressed as mean ± s.d ($n = 10$). [b] Temperature at which the cross-linking reaction was performed.

Table 4. Range Tests on Membrane Thickness of the GCAC Microcapsule Membranes

	membrane thickness (μm)[a] level						
factor	1	2	3	4	5	range[b]	confidence limit[c]
A. concentration	38.5	36.9	36.5			2.0	3.8
B. temperature	44.8	30.4	36.8			14.4	2.2
C. reaction time	38.1	36.4	37.3	36.8	38.0	1.7	5.0

[a] Mean of 15 (for factors A and B) or 9 (for factor C) thickness data for microcapsules prepared under the same level of each factor. [b] Range = maximum − minimum. [c] Degree of confidence was set at 95%.

variables on the membrane fluorescence intensity and optimized the genipin reaction process with regard to highest fluorescent intensity generated. Results showed that the control factors of cross-linking temperature and reaction time significantly impacted the fluorescence intensity of the microcapsule membranes; the order of the effect's magnitude was cross-linking temperature > cross-linking time > genipin concentration. The remarkable impact of temperature, also evidenced by the physical observation on the color change of the microcapsules, may be ascribed to the different levels of molecular collision during reaction. Sparse cross-linking at low temperature could be explained by the restricted molecular movement. At higher temperature, drastic molecular collision accelerated the reaction leading to denser cross-linking and rapid increase in membrane intensity. The decrease in fluorescence intensity of the membranes cross-linked by concentrated genipin at 37 °C may probably be due to collisional quenching and further complex formation.[38] On the whole, it could be inferred that the construction of the genipin cross-linked chitosan membranes could be varied by manipulation of the reaction conditions. The optimal reaction conditions for generating the microcapsules with most cross-linking included 2.5 mg/mL of genipin concentration, at 37 °C for 24 h. As well, the cross-linking reaction should be under careful temperature control to ensure consistent results.

Additionally, membrane thickness is a very important parameter controlling microcapsule property.[39,40] This paper explored a new and easy method to study the distribution of

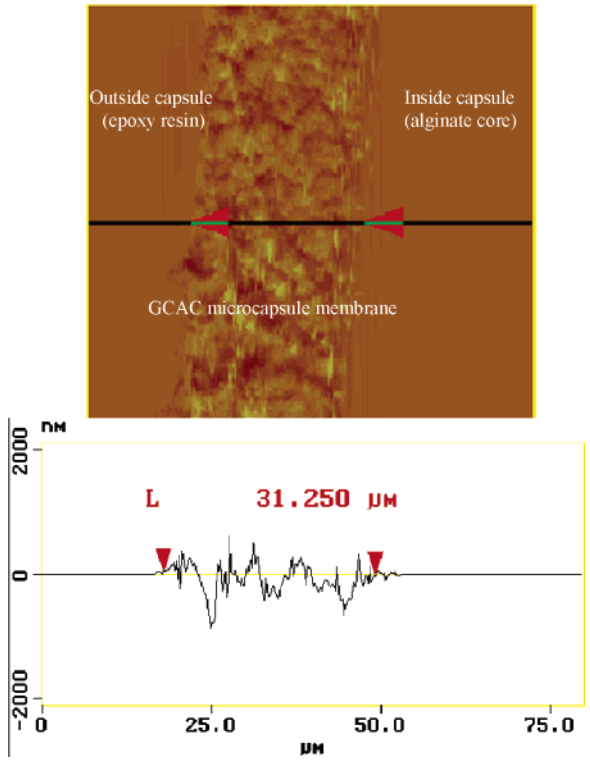

Figure 8. Topography of the cross-sectioned GCAC microcapsule membrane obtained by AFM.

microcapsule membranes in original intact samples. By CLSM, the GCAC microcapsule membrane can be distinguished from the interior core and the image background. This enabled the noninvasive and in situ assessment of the microcapsule membrane including thickness measurement without any extraction, dehydration, or chemical modifications on the samples, which was otherwise difficult or impossible using other methods.[40−42] The effectiveness of this new method was validated by the atomic force microscopic (AFM) observations, from which the results on the membrane thickness of the GCAC microcapsules were in close agreement with the CLSM measurements (32.1 vs 29.3 μm).

Previous research on alginate−chitosan complex by radiolabeling indicated that chitosan penetrated into the porous alginate gel matrix to a great extent [36,37] and the alginate−chitosan complex coaervation occurred not only at the surface of the capsule but also in the matrix.[36] Because genipin only interacted with the chitosan already bound to the alginate beads, the membrane thickness of the GCAC microcapsules would be mainly governed by the diffusion of chitosan and complexation with alginate. Our results corroborated with this hypothesis in that the GCAC microcapsules had relatively thick membranes, approximately 37 μm (Table 3), which were largely independent of the cross-linking conditions. One exception was that at higher cross-linking temperatures, slightly thinner membranes were formed, which could be attributed to the establishment of a denser membrane structure induced by the higher degree of cross-linking. Aside from this, it may be difficult to tailor the membrane thickness by varying the cross-linking conditions.

Conclusion

In summary, the present work characterizes the genipin cross-linked alginate−chitosan microcapsule membranes using a novel CLSM method. Results showed that the construction of the

genipin cross-linked chitosan membranes could be varied by manipulation of the genipin cross-linking conditions. The reaction factors of cross-linking temperature and reaction time significantly affected the fluorescence intensity of the membranes within the given ranges, whereas the tested genipin concentrations had little impacts. Cross-linking by genipin at 37 °C for 24 h may optimally yield the GCAC microcapsule membrane with strongest fluorescence and highest degree of cross-linking. These results will be useful in the future exploitation of the GCAC microcapsules for therapy.

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