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A New Method for Microcapsule Characterization

Use of Fluorogenic Genipin to Characterize Polymeric Microcapsule Membranes

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Abstract

Numerous microcapsule systems have been developed for a wide range of applications, including the sustained release of drugs, cell transplantation for therapy, cell immobilization, and other biotechnological applications. Despite the fact that microcapsule membrane is a dominant factor governing overall microcapsule performance, its characterization is challenging. We report a new method for characterizing microcapsule membranes, using the most common alginate-poly-L-lysine-alginate (APA) microcapsule as an example. Our data demonstrate that genipin, a naturally derived reagent extracted from gardenia fruits, interacts with poly-L-lysine (PLL) and generates fluorescence. This fluorescence allows clear visualization and easy analysis of the PLL membrane in the APA microcapsules using confocal laser scanning microscopy. The results also show that PLL binding correlates to the reaction variables during PLL coating such as PLL concentration and coating time. In addition, five other different microcapsule formulations consisting of PLL and/or chitosan membranes were examined, and the results imply that this method can be extended to characterize a variety of microcapsule membranes. These findings suggest that genipin can serve as a fluorogenic marker for rapid characterization of microcapsule membranes, a superior method that would have important implications for microcapsule research and potential in many other applications.

Index Entries: Microcapsule; poly-L-lysine; alginate-poly-L-lysine-alginate; genipin; chitosan; fluorescence; confocal laser scanning microscopy.

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Introduction

Microencapsulation of bioactive materials such as drugs, vaccines, antibodies, enzymes, and live cells is a promising delivery method for numerous applications including the treatment of a number of diseases *(1–9)*. Alginate-based microcapsules are one of the most widely used systems, owing to the superior biocompatibility and mild process suitable for both the host and the enclosed materials. For the preparation of microcapsules, generally, alginate droplets are gelled by multivalent ions (typically Ca^{2+}). Additional polymers are used to envelope the calcium-alginate beads, which create stable and semipermeable membranes for purposes of immunoisolation of live cells and sustained release of drugs. For example, positively charged polymers, also termed *polycations,* bind to the negatively charged alginate gel via electrostatic interactions and form strong polyelectrolyte complex membrane on the microcapsule surfaces *(10,11)*. The primary amine-containing poly-L-lysine (PLL) and chitosan, both simply named as polyamine in this article, are widely used polycations for the construction of microcapsule membranes. The preparation and properties of alginate-poly-L-lysine-alginate (APA) and alginate-chitosan (AC) microcapsules have been studied extensively *(12–17)*.

Previous research shows that microcapsule utility depends on its membrane properties. For example, membrane thickness is known to correlate with permeability, resistance, mechanical strength, drug release capacity, and biocompatibility *(12,13,15,17–20)*. A solid understanding of the structure-property of the microcapsule membranes, therefore, is essential for in vitro and in vivo applications. However, precise determination of the membrane thickness and polycation binding is challenging *(13),* owing to the small size, spherical shape, and hydrogel nature of microcapsules, along with the fragility of the membrane and other factors. Many techniques have been employed in previous studies; however, they have had many limitations. For example, regular light or stereoscopic microscopy, although simple, may limit the resolution in measurement *(17,20,21)*. Electron microscopic methods, such as scanning electron microscopy and transmission electron microscopy, are typically destructive *(22–25)* and require skilled knowledge for preparation of samples and interpretation of results. Gravimetric measurement, also destructive and cumbersome, cannot assess the distribution of the coating polymers *(15)*. Additionally, approaches using radiolabeled polymers and enzyme-linked sorption assays involve elaborate preparation of samples *(13,18)*, and the availability of the particular materials needed may also impede the process. Other methods for assessing thin films and small particles, such as ellipsometry *(25)* and surface plasmon resonance spectroscopy *(26)*, may not be suitable for hydrogel microcapsule membrane systems. In recent years, a nondestructive approach, confocal laser scanning microscopy (CLSM), has attracted much interest in microcapsule research *(14,27–30)*. In previous studies, the microcapsule core and/or membrane components had to be labeled with fluorescent markers, such as fluorescein isothiocyanate or

rhodamine B isothiocyanate prior to encapsulation *(12,14,24,29–33)*. This process, however, presents risks of blocking some of the functional groups in the involved polymers essential for polyelectrolyte complexation, leading to weak binding *(14,32)*. In addition, other issues pertaining to the stability and solubility of the labeled polymers, the control of labeling efficiency, the separation of free markers, as well as their influences on the encapsulation process still remain *(14,32)*. Therefore, there are persistent difficulties in characterizing microcapsule membranes.

Genipin is an aglucone of geniposide extracted from gardenia fruits *(34)* and has been used traditionally as an herbal medicine *(35)*. It is known that genipin reacts rapidly with amino acids to make blue pigments, which are currently used as a natural colorant in the food and fabric industries *(36)*. The present study introduces a new and simple method of using genipin to characterize microcapsule membrane by CLSM without complicated prior labeling and sample treatment. Specifically, we characterized the commonly used PLL membrane in APA microcapsules using this novel method and evaluated the feasibility of characterizing polyamine microcapsule membranes using five other different microcapsule formulations.

Materials and Methods

Chemicals

Sodium alginate (low viscosity), PLL (mol wt = 27,400), and pectin (25% degree of esterification) were purchased from Sigma-Aldrich. Polyethylene glycol (PEG) (mol wt = 20,000) was obtained from Fluka (Switzerland). Chitosan (low viscosity, 73.5% degree of deacetylation and M_v = 7.2×10^4) and genipin were obtained from Wako BioProducts. All other reagents and solvents were of reagent grade and used as received without further purification.

Fluorometric Study of Reaction Between Genipin and PLL

To investigate the fluorescent characteristics of the reaction between PLL and genipin, genipin and PLL were dissolved in physiological saline (PS) at a mass ratio of 2.5:1. The mixture was then incubated at 37°C for 4 h. The absorption and fluorescence spectra of the mixture were acquired using a spectrofluorometer (FluoroMax-2) with the slit width set at 3.5 nm and compared with those of the reactants.

Preparation of Microcapsules

Preparation of APA Microcapsules

Droplets of an Na-alginate solution (15 mg/mL) were generated by an encapsulator (Inotech) and gelled in a stirred $CaCl_2$ solution (11 mg/mL) for 15 min. The obtained Ca-alginate beads, with a diameter of 508.4 ± 10.7 μm (n = 10), were exposed to PLL solution (1 mg/mL) for 10 min to form AP beads; this was followed by washing with PS and a subsequent

coating by Na-alginate solution (1 mg/mL) for 10 min. The obtained APA microcapsules were then washed and collected. To investigate the effects of PLL concentration and reaction time on PLL binding in the APA microcapsule membrane, different microcapsules were prepared using PLL at a concentration of 0.5, 1.0, 2.5, or 5.0 mg/mL for varied incubation times (10, 60, and 120 min) during the coating process. To examine the influence of storage on the bound PLL layer, the APA microcapsules were made as just described using PLL at a concentration of 1.0 mg/mL for 10 min of coating and stored in PS for 0, 5, 14, and 20 d prior to genipin treatment and CLSM analysis.

Preparation of Other PLL-Based Microcapsules

PEG was incorporated into APA microcapsules to form alginate-poly-L-lysine-PEG-alginate (AP-PEG-A) microcapsules by immersing the aforementioned AP beads in a PEG solution (5 mg/mL) following PLL incubation. After washing with PS, a final layer of alginate was coated using 1 mg/mL of alginate solution for 10 min. Multilayer microcapsules composed of alginate-poly-L-lysine-pectin-poly-L-lysine-alginate (AP-PEC-PA) were also prepared by integrating pectin into the APA microcapsules. Briefly, the aforementioned AP microcapsules were incubated in a pectin solution (1 mg/mL), followed by another coating of PLL (1 mg/mL) and a final layer of alginate (1 mg/mL). All coating processes were carried out using the aforementioned method with an exposure time of 10 min and three PS washes after each coating step.

Preparation of Chitosan-Based Microcapsules

To prepare the AC microcapsules, the aforementioned Ca-alginate beads were coated for 30 min in a chitosan solution (5 mg/mL, in dilute acetic acid with a final pH of 5.4). Multilayer alginate-chitosan-PEG-alginate (AC-PEG-A) and alginate-chitosan-PEG-poly-L-lysine-alginate (AC-PEG-PA) microcapsules were prepared by immersing the AC microcapsules in the PEG solution (5 mg/mL) for 10 min and subsequently coating with either a layer of alginate (1 mg/mL) for 10 min, or a layer of PLL (1 mg/mL) followed by an alginate layer (1 mg/mL) for 10 min each. Three PS washes were applied after each coating to remove unbound polymers.

Genipin Treatment on Microcapsules

The microcapsules obtained as described in the previous preparation section were immersed in a genipin solution (2.5 mg/mL in PS) for 20 h at 37°C unless otherwise stated. The resulting microcapsules were washed and directly used for CLSM studies.

Characterization of Microcapsule Membrane by CLSM

The morphology and membrane structure/density of the microcapsules were examined 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,

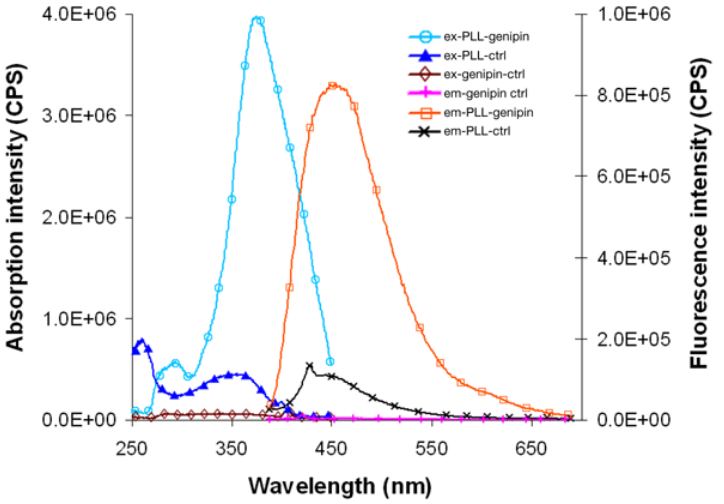

Fig. 1. Absorption and fluorescence spectra of PLL, genipin, and their reaction mixture. CPS, counts per second.

the microcapsules were directly placed in a chambered cover glass system (Lab-Tek). One channel of the confocal laser scanning microscope 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 and fluorescence intensity. An average of eight consecutive scans of a single field was taken. The thickness of the fluorescent membrane in the microcapsules was analyzed using the equipped LSM 510 software and given as the mean ± SD of at least 10 measurements. The fluorescence intensity profile corresponding to a line across the microcapsule membrane at the focal plane was acquired by computational profile analysis (LSM 510 software). The relative fluorescence intensity of the membrane representing the PLL density on the APA membrane was plotted as a function of PLL reaction conditions.

Results

PLL-Genipin Reaction and Fluorescent Characteristics of Its Products

The reaction between PLL and genipin occurred gradually after mixing the two solutions and could be observed by changes in the physical appearance of the mixture, from clear and colorless to a viscous and blue solution. To investigate the fluorogenic activity of this reaction, the fluorometric spectra of PLL, genipin, and the reaction mixture were studied. The results showed that two peaks appeared at wavelengths of approx 267 and 370 nm in the absorption spectra of the mixture, which were otherwise absent or very weak in the spectra of the controls (genipin and PLL individually) (Fig. 1). After the genipin-PLL reaction, there is a large increase

in fluorescence intensity of the emission spectrum with maximum emission at 453 nm. This increase reflects the chemical modifications of PLL and genipin. By contrast, other polymers used including alginate, pectin, and PEG did not show this fluorescence peak (data not shown).

Visualization of PLL Membrane in Genipin-Treated APA Microcapsules Under CLSM

The APA microcapsules and their PLL membrane were visualized using CLSM. It was found that under the regular transmission light channel, the microcapsules before and after genipin treatment looked similar (Fig. 2A,C). However, they differed when viewed under the fluorescent channel (Fig. 2B,D). The genipin-treated PLL layer in the microcapsule wall was clearly identified by the appearance of a bright circle circumscribing each microcapsule core. Fluorescence from the non-genipin-treated microcapsules, conversely, was barely detectable under the same CLSM settings. Moreover, Fig. 2E,F exemplifies the effects of the alginate/PLL interaction and the structural changes in the microcapsule membrane under varied reaction conditions. When a low concentration of PLL (0.5 mg/mL) and a short coating time (10 min) were used (Fig. 2E), the fluorescent signals of the microcapsule membrane were weak. Increasing PLL concentration and exposure resulted in stronger fluorescence intensities of the membrane (Fig. 2F,G). Figure 2H shows the fluorescence profile corresponding to the line across the optical and equatorial section of the microcapsules shown in Fig. 2G. It was clear that the intensity of the inner alginate cores was similarly low when compared to the background signals, whereas peaks corresponding to the fluorescence of the microcapsule membrane appeared, with the relative intensity attaining 150.

Characterization of APA Microcapsules Using Genipin

To evaluate the influence of PLL coating variables on the APA microcapsule morphology, PLL membrane thickness, and PLL binding density, APA microcapsules were prepared using different concentrations of PLL for varied periods of incubation time and examined by CLSM after genipin treatment. The results are described individually as follows for each characteristic of the APA microcapsules: swelling behavior, PLL membrane thickness, and PLL binding density.

Effects of Concentration of PLL Solution and Coating Time on APA Microcapsule Swelling

The results showed that the diameters of the microcapsules increased inversely with the concentration of PLL solution and incubation time (Fig. 3A,C). When coated with PLL at a low concentration (0.5 mg/mL), the APA microcapsules swelled substantially, from 508.4 ± 10.7 μm of the original Ca-alginate beads to 783.3 ± 26.4 μm. For high PLL exposure (5.0 mg/mL, 10 min), shrunken and even collapsed microcapsules were observed (*see* Fig. 2G), with the diameter reduced to 482.1 ± 15.6 μm (Fig. 3A).

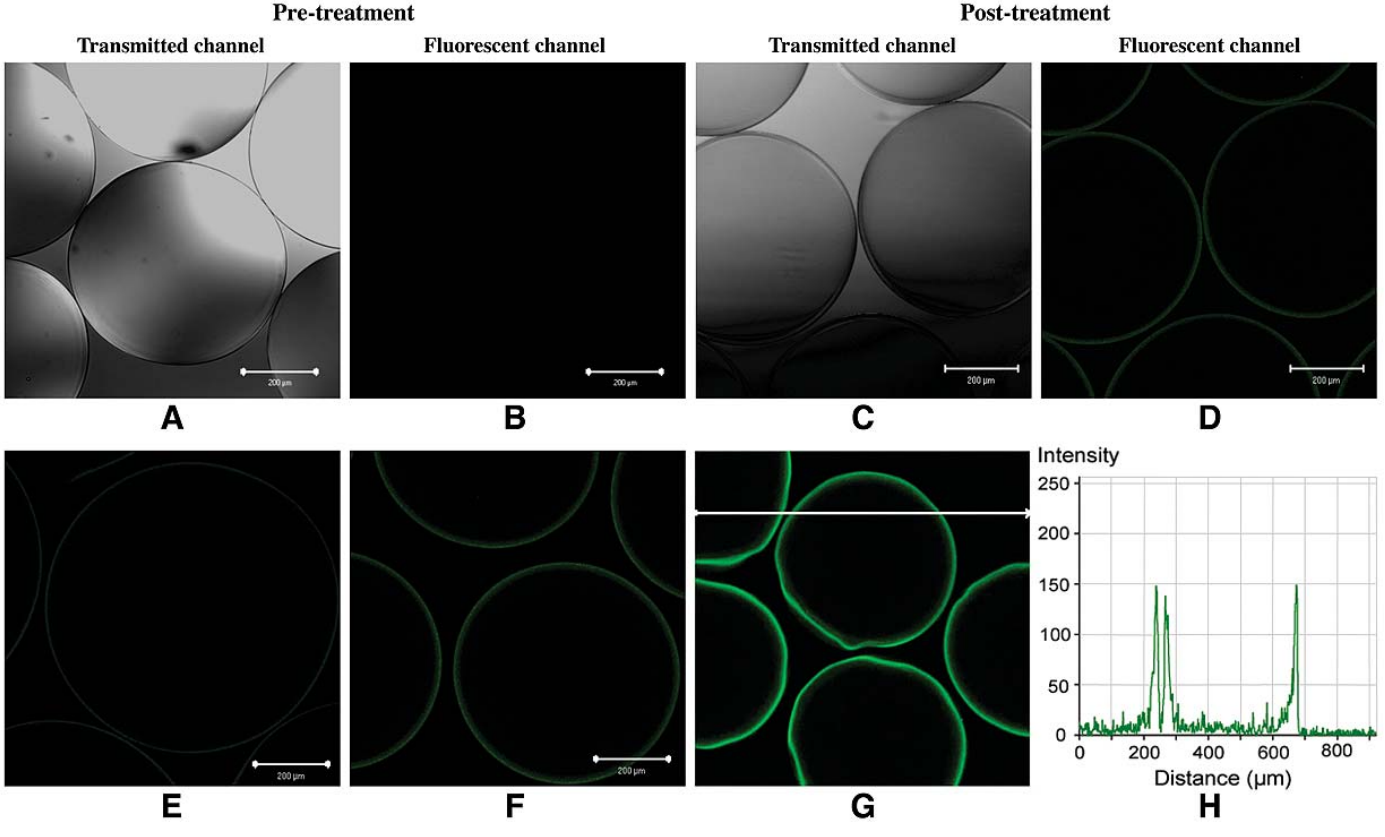

Fig. 2. CLSM images of APA microcapsules and representative fluorescence intensity profile: **(A–D)** microcapsules made by 2.5 mg/mL of PLL for 10 min pre- and post-genipin treatment viewed in transmitted channel and fluorescent channel; **(E–G)** microcapsules with varied PLL binding viewed in fluorescence channel after genipin treatment ([E] 0.5 mg/mL of PLL for 10 min; [F] 1.0 mg/mL of PLL for 60 min; [G] 5.0 mg/mL of PLL for 10 min); **(H)** fluorescence intensity profile corresponding to white line in **(G).** Note the presence of the bright circles around the alginate beads that were PLL membranes. Bars = 200 μm.

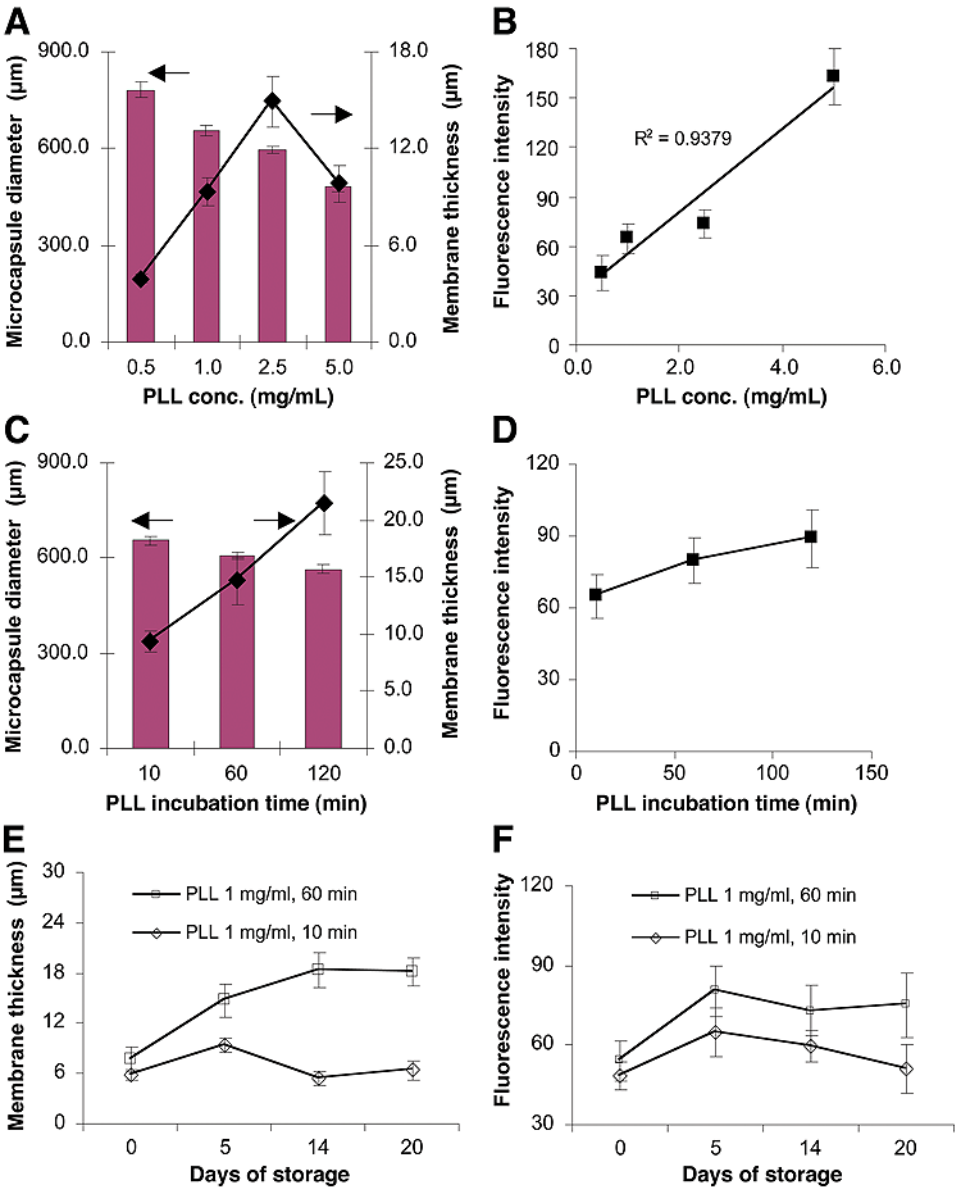

Fig. 3. Quantification of PLL binding to APA microcapsules: **(A,B)** Dependence of PLL concentration on (A) APA microcapsule diameter and PLL membrane thickness, and (B) fluorescence intensity; **(C,D)** dependence of PLL incubation time on (C) APA microcapsule diameter and PLL membrane thickness, and (D) fluorescence intensity; and **(E,F)** effects of storage of APA microcapsules in PS on PLL (E) membrane thickness and (F) fluorescence intensity. Data represent the mean ± SD (n = 10).

Increasing incubation time in PLL solution did not drastically alter the morphology of the microcapsules, although a slight decrease in diameter was found (Fig. 3C). These results implied that optimizing the PLL coating

variables could significantly constrain microcapsule swelling, which is indicative of the formation of dense alginate-PLL complex membrane *(19)*.

Evaluation of Effects of PLL Solution Concentration and Coating Time on Thickness of PLL Layer

Membrane thickness, a reflection of the distribution of PLL molecules along the microcapsule surface and their diffusion into the microcapsule cores, was found to be heavily dependent on the PLL concentration and complexation time. The thickness of the PLL layer increased linearly with the concentration of PLL solution used (Fig. 3A), except for the highest level of PLL (5.0 mg/mL), at which the APA membrane became wrinkled and the APA microcapsules tended to collapse. Extended time of interaction with PLL (1.0 mg/mL) from 10 to 120 min considerably increased membrane thickness (9.4 vs 21.4 μm) (Fig. 3C). A similar trend with respect to the influence of the alginate-PLL complex was previously reported by several groups using a fluorescence labeling method, although the reported thickness of the similarly prepared PLL membrane was higher in the literature (>20 μm) *(12,14,33)*.

PLL Solution Concentration, Coating Time, and Density of PLL Binding in Membrane

We evaluated the effects of PLL solution concentration and coating time on PLL binding density in the membrane. The results show that the fluorescence intensity of the membrane, representing the density of PLL deposition on the microcapsule wall, is directly proportional to the concentration of PLL solution used for coating ($R^2 = 0.938$) (Fig. 3B). Weak fluorescent signals were detected (43.6 ± 10.2) for low PLL exposure (0.5 mg/mL, 10 min). When coated with concentrated PLL (5.0 mg/mL), the membrane exhibited strong fluorescence (163.0 ± 17.0), demonstrating denser PLL deposition in the microcapsule membrane. On the other hand, Fig. 3D shows that the membrane fluorescence intensified, however only moderately, under extended exposure to PLL solution, from 64.8 ± 9.0 in 10 min to 89.0 ± 12.0 in 120 min. These results corroborated with those of earlier investigations using labeled PLL *(12,14)*.

Effects of Storage on PLL Layer in APA Microcapsules

Using genipin we investigated the fate of the bound PLL in microcapsules during storage. For this, APA microcapsules were stored in physiologic saline up to 3 wk prior to genipin treatment and CLSM observation. It was found that the membrane thickness of the microcapsules made by a 10-min incubation in PLL solution (1.0 mg/mL) faintly changed over the 3-wk storage (Fig. 3E). For APA microcapsules coated for 60 min, the PLL membrane doubled in thickness during the initial 5-d storage and further expanded to approx 18 μm in the subsequent 2 wk but did not increase further for the remainder of the experiment. Despite these shifts, the overall thickness of the PLL layer remained less than 20 μm (Fig. 3E), which was not

consistent with previous reports *(12,33)*. Furthermore, the relative fluorescence intensity of the PLL membrane slightly fluctuated during storage (Fig. 3F). An increase in the intensity occurred during the first 5 d of storage, after which the membrane intensity gradually declined in the subsequent 2 wk.

Evaluation of Polyamine Membrane in Other Microcapsules

To assess the feasibility of using genipin to characterize other microcapsule membranes, different kinds of polyamine-based microcapsules were prepared. The subsequent genipin treatment and CLSM studies were performed using the method described in "Materials and Methods." Figure 4 exemplifies the fluorescence intensity of the polyamine membrane in different microcapsule formulations after genipin treatment, in which the profiles correspond to the microcapsules shown in the insets. Similar to the APA microcapsules, fluorescent and shell-like membranes were distinguished in the AP-PEC-PA, AP-PEG-A, AC, AC-PEG-A, and AC-PEG-PA microcapsules. A sharp exterior delimitation and a slight inward spreading of the fluorescent membrane were observed. Table 1 summarizes the fluorescence intensity and the thickness of the polyamine membranes in six different microcapsule formulations. In contrast to the thin membrane in the PLL-based microcapsules (<9 μm), chitosan formed a relatively thicker complex with alginate (>11 μm), and the fluorescence intensity of the membrane was higher, attaining 246.9 ± 10.8, 167.6 ± 12.0, and 253.9 ± 2.3 for the AC, AC-PEG-A, and AC-PEG-PA microcapsules, respectively.

Discussion

Genipin is known to react rapidly with primary amines *(37–39)*. Although visibly evident, the detailed mechanism of the polyamine-genipin reaction remains under investigation *(40,41)*. It has been suggested that the amino groups in the polycation molecules initiated a nucleophilic attack at C-3 of genipin, resulting in opening of the dihydropyran ring and formation of a nitrogen-iridoid as well as aromatic intermediates after dehydration. Radical-induced polymerization occurred in the subsequent steps, creating highly conjugated heterocyclic genipin derivatives *(41)*, which may thus explain the fluorescent characteristics exhibited after the PLL-genipin reaction.

Genipin treatment was performed by simply immersing the microcapsules in a genipin solution under mild conditions. The small genipin molecules can freely penetrate into the microcapsule membranes and interact with polyamines bound to the alginate gels. This treatment did not noticeably affect the morphology of the microcapsules (Fig. 2); they remained intact and spherical in shape, although gradual development of blue color in the microcapsules was observed.

Results from CLSM examination confirm the formation of new fluorescent conjugates and demonstrate the polyamine distribution within the microcapsule membrane. The thin PLL layer revealed that the PLL

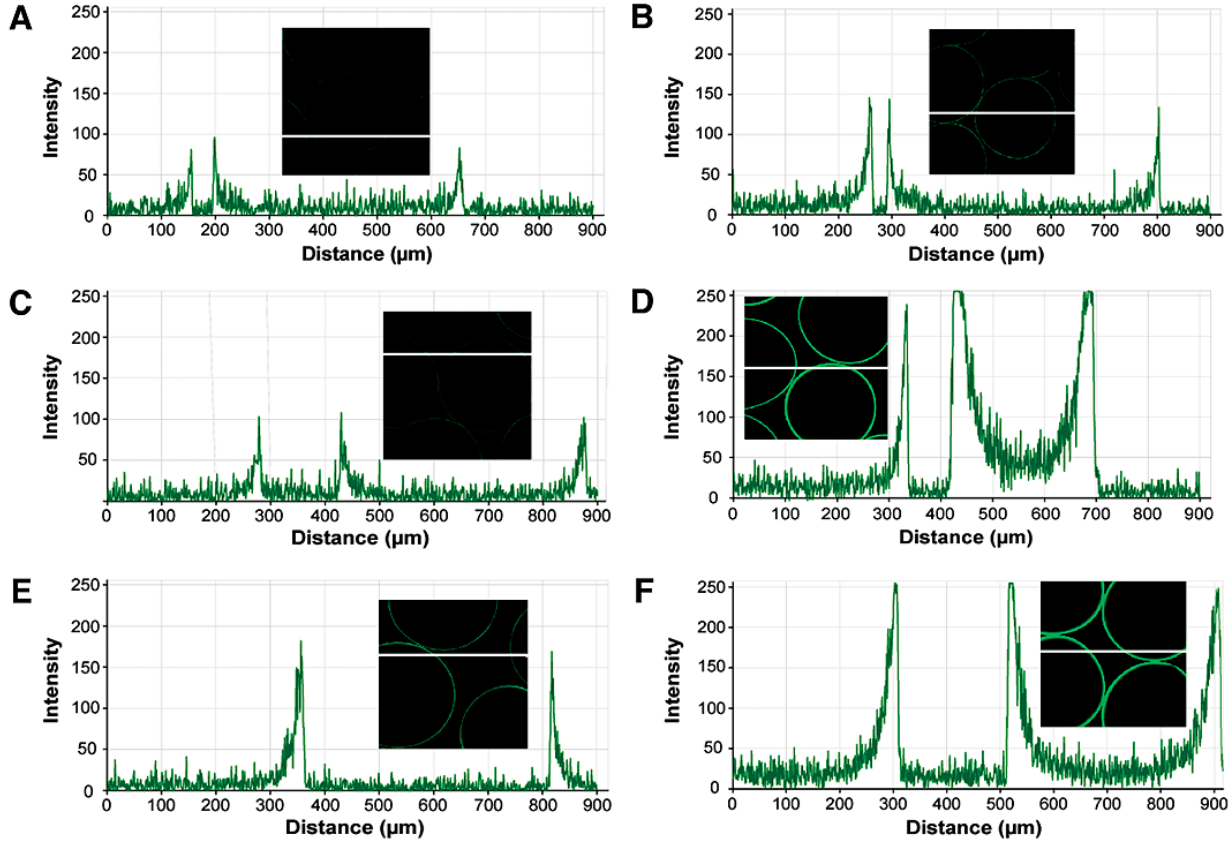

Fig. 4. Fluorescence intensity profiles corresponding to a line across optical equatorial sections of various polyamine-based microcapsules after genipin treatment: **(A)** APA microcapsules; **(B)** AP-PEC-PA microcapsules; **(C)** AP-PEG-A microcapsules; **(D)** AC microcapsules; **(E)** AC-PEG-A microcapsules; and **(F)** AC-PEG-PA microcapsules.

Table 1
Thickness and Relative Fluorescence Intensity
of Polyamine Coating on Genipin-Treated Microcapsules[a]

	APA[b]	AP-PEC-PA[b]	AP-PEG-A	AC	AC-PEG-A	AC-PEG-PA
Thickness (μm)	4.5 ± 0.6	6.3 ± 0.6	8.6 ± 1.4	11.6 ± 1.7	15.3 ± 1.4	16.5 ± 1.1
Fluorescence intensity	111.4 ± 15.5	160.3 ± 17.4	110.6 ± 11.8	246.9 ± 10.8	167.6 ± 12.0	253.9 ± 2.3[c]

[a]Data represent the mean ± SD (n = 10).
[b]Genipin treatment was carried out at room temperature.
[c]Maximum detectable intensity attained.

molecules were bound to the periphery of the alginate cores and that the diffusion of PLL was restricted to a small penetration depth (~10 μm) (Fig. 3 and Table 1). This was in accordance with investigations by Ma et al. *(15)* and Ross and Chang *(42)*. Furthermore, using this method we found that chitosan penetrated into the alginate gel to a larger extent and formed a relatively thicker membrane, which was consistent with previous reports using radiolabeling and indirect chromatographic methods *(17,18)*. Vandenbossche and colleagues proposed that the PLL membrane in the APA microcapsules would perpetually rearrange itself with time *(12,33)*, owing to diffusion of the PLL molecules, leading to a thicker membrane (~20 μm on d 1 to ~119 μm on d 7 of storage) *(33)*. Our results showed that this is not the case; the rearrangement of the bound PLL was extremely limited (Fig. 3E). In the earlier method, PLL was fluorescence labeled prior to coating *(33)*. This may reduce the functionalities ($-NH_2$) of PLL *(14)*, leading to weak binding to alginate gels. Furthermore, there may be possible migration of nonfirmly bound fluorescence markers liberating from PLL molecules *(31,32)*. In the present study, genipin, which is essentially nonfluorescent in its free form, was used to couple covalently and selectively with the PLL molecules already bound to the alginate beads. As such, the use of genipin is highly likely to overcome the limitations found in the aforementioned method.

Although the membrane thickness and coating density substantially affect the properties and performance of the microcapsules, these parameters are difficult to quantify. Discrepant results have been reported; for example, Ma et al. *(15)* and Ross and Chang *(42)* found that APA microcapsules had a wall thickness of 11–13 μm, whereas others reported a membrane thickness of 40–120 μm *(12,14,33)* using prior labeling methods. Other destructive methods such as cross-sectioning or mass measurement are time- and labor- consuming *(15,23)*. Previous attempts to visualize and assess microcapsule membranes have, therefore, had many shortcomings.

To overcome these limitations, our new method uses fluorogenic genipin treatment without prior labeling of polymers. It enables the visualization and quantification of polyamine microcapsule membrane rapidly, easily, and effectively. Because genipin is highly selective for coupling with primary amines *(37,38)*, in the evaluated microcapsule systems, only PLL and chitosan bound to the alginate beads are likely candidates for this reaction. Specifically, the covalently coupled genipin-polyamine conjugates formed *in situ* within the microcapsule wall is the only fluorescent material in the system. Free genipin and other –OH- and/or –COOH-containing polymers used in our study, such as alginate, PEG, and pectin, did not contribute to the fluorescent emission. Hence, the detected fluorescence revealed solely the distribution of these polyamines in the microcapsules and can be assessed without interference from other materials in the system. The dependence of the generated fluorescence on the polyamines presented provides a nondestructive and quantitative means of characterizing the microcapsule membranes. Furthermore, using this

approach only little sample treatment is required; it is thus possible to determine quickly the membrane thickness and the density of polyamine membrane during the process of development and optimization on a routine basis, so as to facilitate the understanding and improvement of microcapsule performance.

The approach described herein may be used for a variety of other microcapsule formulations and biomaterials. For example, gelatin, a partially denatured protein containing primary amines, has been reported to interact with genipin *(43)*, which makes it a promising candidate for our approach in many applications in forms of capsules for sustained release *(44)*, scaffold for tissue repairing *(45)*, and nanoparticles for tumor-targeted gene delivery *(46)*. Other polyamine candidates for the presented method include polyamido amide (PAMAM) dendrimers, which have recently become a subject of intense interdisciplinary research efforts as a new targeted drug delivery system *(47)*. Assuming that the $-NH_2$ terminals at the branches of PAMAM dendrimers interact with genipin, this fluorescence generation strategy could provide a valuable template for drug-targeting purposes.

In brief, the results of our research demonstrate a simple, sensitive, and robust method of using fluorogenic genipin for the characterization of microcapsule membranes, a superior approach that overcomes the challenges of previous methods and has potential to be used for a variety of applications.

Acknowledgments

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