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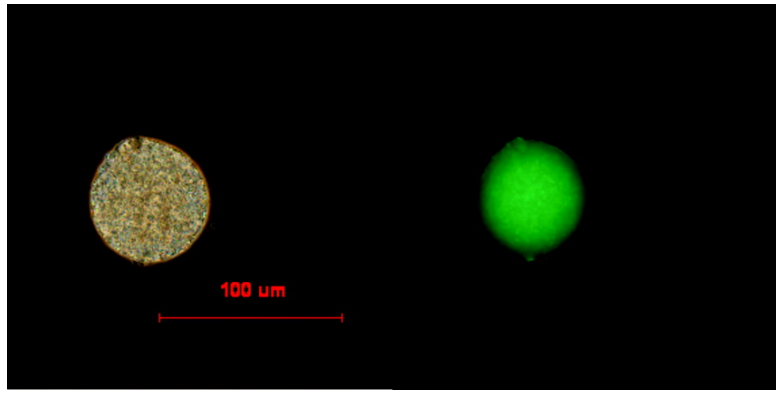

Matrix-Assisted Colloidosome Reverse-Phase Layer-by-Layer Encapsulating Biomolecules in Hydrogel Microcapsules with Extremely High Efficiency and Retention Stability

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The layer-by-layer (LbL) polyelectrolyte self-assembly encapsulation method has attracted much interest because of its versatility to use various polymers for capsule formation, ability to encapsulate different templates, and capability to control capsule permeability. Traditionally, the LbL method was performed in water as solvent and limited to poorly or non-water-soluble templates. Using the matrix-assisted LbL method, complex mixtures of water-soluble proteins or DNA could be encapsulated within agarose microbeads templates but leakage of biomolecules into the water phase during the LbL process results in low encapsulation efficiency. Recently, the reverse-phase LbL (RP-LbL) method was introduced to perform LbL and encapsulation of water-soluble templates in organic solvents, thus preventing the templates from dissolving and allowing high encapsulation efficiency. However, encapsulation of complex mixtures of biomolecules or other substances with quantitative encapsulation efficiency remained impossible. Here we present a new approach for encapsulation of biomolecules or complex mixtures thereof with almost 100% encapsulation efficiency. The ability of our method to achieve high encapsulation efficiency arises from the combination of two strategies. (1) Using microparticles as surface stabilizer to create stable biomolecule-loaded hydrogel microbeads, termed matrix-assisted colloidosome (MAC), that are able to disperse in oil and organic solvents. (2) Using the RP-LbL method to fabricate polymeric capsule "membranes", thereby preventing diffusion of the highly water-soluble biomolecules. Using an oil phase during emulsification and an organic solvent phase during encapsulation could completely prevent leakage of water-soluble biomolecules and almost 100% encapsulation efficiency is achieved. Microcapsules fabricated with our method retained nearly 100% of encapsulated proteins during a 7 day incubation period in water. The method was demonstrated on model proteins and may be extended to other biomolecules or mixtures. Our method is a valuable addition to the family of encapsulation techniques and can significantly contribute to the fields of bioreactors and bioanalytical microcapsules.

Introduction

Microcapsules encapsulating various biomolecules have been used in the areas of therapeutics,[1−3] microbioreactors,[4,5] biocatalysis,[6] bioremediation,[7,8] and bioanalytical applications.[9−11] Such microcapsules, with micrometer dimensions, possess a large surface-area-to-volume ratio that allows efficient diffusion of materials into or out of the microcapsules. Various methods have been developed to encapsulate biomolecules within microcapsules such as liposomes[12] and polymeric microcapsules fabricated from colloidosomes,[13] the sacrificial template method,[14−16] and the templated layer-by-layer (LbL) polyelectrolyte self-assembly technique.[17]

Liposomes, lipid-based microcapsules, have the advantage of providing high retention stability of biomolecules within their interior. However, the liposome wall has no permeability for polar molecules and incorporation of protein channels is necessary to allow diffusion of polar molecules through the wall.[18] Also, liposomes have fair mechanical stability, and fusion of liposomes can spontaneously occur.

Alternatively, biomolecules can be encapsulated within polymeric microcapsules. These microcapsules are mechanically more stable than liposomes, and the permeability of the microcapsule wall can be tailored. There are different strategies to encapsulate biomolecules within polymeric microcapsules. One strategy utilizes colloidosomes which are microcapsules

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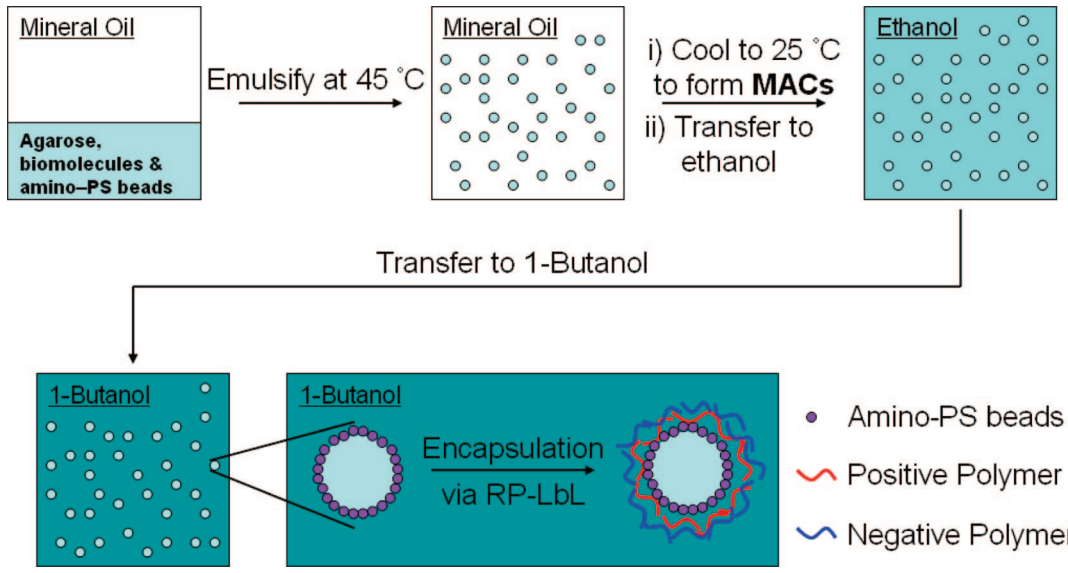

Figure 1. Schematic diagram illustrating the matrix-assisted colloidosome reverse-phase layer-by-layer (MAC RP-LbL) microcapsules fabrication process.

consisting of coagulated or partially fused microparticle shells fabricated through self-assembly of microparticles onto the interface of emulsion droplets.[19] In order to obtain mechanically stable colloidosomes, fixation of the microparticle shells using a chemical cross-linking reagent or sintering the shells at a temperature of 100 °C is required.[20,21] The requirement of such harsh conditions limits their application for encapsulation of biomolecules. Another strategy involves fabrication of hollow polymeric microcapsules through sacrificial templating methods[22,23] followed by loading of the microcapsules with the desired biomolecules.[24−26] Loading is carried out by immersing the hollow microcapsules into a solution containing the desired biomolecules and allowing the biomolecules to diffuse into the microcapsules. However, this method has certain limitations such as difficult control over the loading concentration, and only a small percentage of the initial biomolecules can usually be encapsulated. In addition, hollow microcapsules have the tendency to collapse and are difficult to handle.

A third approach to encapsulate biomolecules within polymeric microcapsules can be achieved via the templated LbL polyelectrolyte self-assembly technique. Using this technique, polymeric microcapsules consisting of porous microspheres or hydrogel matrix microspheres (as core templates) preloaded with the desired biomolecules have been fabricated.[27,28] However, the preloaded biomolecules tend to leach out from the microspheres before a sufficient number of LbL polyelectrolyte layers are coated onto the microsphere surface, thus resulting in a low encapsulation yield. An advanced strategy utilizing organic or protein microcrystals as templates followed by LbL encapsulation has been introduced by Caruso et al.[29,30] In traditional LbL techniques, polyelectrolyte depositions are conducted in an aqueous phase. Therefore, encapsulation of microcrystalline templates within polymeric microcapsules is limited to poorly or non-water-soluble materials. Recently, Beyer et al. reported the encapsulation of highly water-soluble microcrystalline materials using a reverse-phase LbL (RP-LbL) technique.[31] In brief, the RP-LbL technique is carried out in an organic phase such that highly water-soluble substances can retain their microcrystalline morphology. Next, nonionized polyelectrolytes (niPolyelectrolyte) (prepared by protonation of anionic polyelectrolytes and deprotonation of cationic polyelectrolytes) are coated onto the microcrystalline materials to form a polymeric capsule "membrane" around each microcrystal. Fabrication of polymeric microcapsules loaded with a very high concentration of water-soluble molecules was demonstrated with the RP-LbL method. However, preparation of microcapsules encapsulating mixtures of biomolecules by the RP-LbL technique is difficult as "hybrid" microcrystals consisting of a mixture of various biomolecules with defined concentration are difficult to prepare.

With the limitations and drawbacks of existing polymeric microcapsule encapsulation methods, a new method to encapsulate water-soluble biomolecules or mixtures thereof within polymeric microcapsules having quantified concentrations/ratios and high encapsulation efficiency is desired.

In this article, we report a novel encapsulation technology based on the matrix assisted colloidosome reverse-phase LbL (MAC RP-LbL) technique to prepare polymeric hydrogel microcapsules which can be loaded with biomolecules or mixtures thereof with an encapsulation efficiency of almost 100% of the initial starting material. Preparation of MAC RP-LbL microcapsules is illustrated in Figure 1. In brief, a mixture of hydrogel, biomolecules and microparticles is emulsified in oil at 45 °C to produce water-in-oil (w/o) emulsion droplets stabilized by the microparticles which assemble at the hydrogel/oil interface

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followed by cooling of the emulsion to solidify the droplets into microparticles stabilized hydrogel microbeads (matrix assisted colloidosomes or MACs). The biomolecule-loaded MACs are then harvested from the emulsion through washing with ethanol followed by dispersing in 1-butanol (organic phase). The MACs dispersed in the organic phase are subsequently coated with niPolyelectrolyte multilayers using the RP-LbL technique to form polymeric hydrogel microcapsules. Finally, the microcapsules are extracted from 1-butanol and redispersed in water.

The MAC RP-LbL encapsulation technology has several advantages. (1) The hydrogel cores of the MACs contain almost 98% water, thereby providing a favorable physiological environment for the encapsulated biomolecules. (2) The hydrogel matrix acts as a scaffold to provide mechanical support for maintaining the spherical shape of the microcapsules and to facilitate manipulation during the LbL coating process. (3) Fabrication of MACs using the w/o emulsion technique can ensure that all initial starting biomolecules are emulsified into microbeads, and the microparticles assembled on MAC surfaces help to stabilize and disperse the MACs in the organic phase for RP-LbL coating. (4) Thickness and permeability of the resulting polymeric capsule "membrane" can be controlled by the number of LbL layers. (5) The permeable polymeric capsule "membrane" can retain large encapsulated molecular weight proteins and allow small molecular weight molecules to diffuse through the "membrane". In the following, the feasibility of the MAC RP-LbL technique for encapsulation of biomolecules such as bovine serum albumin, glucose oxidase, and peroxidase is demonstrated. The encapsulation efficiency, retention efficiency, and biological activity of the biomolecules within the resultant microcapsules are also studied.

Experimental Section

Materials. Poly(allylamine hydrochloride) (PAH) M_w 70 000 Da, poly(acrylic acid) (PAA) M_w 15 000 Da, and poly(sodium 4-styrenesulfonate) (PSS) M_w 70 000 Da were purchased from Aldrich. Glucose oxidase (GOx) from *Aspergillus niger* (156 U/mg), horseradish peroxidase (HRP) (181 U/mg), D-(+)-glucose, 98% sulfuric acid, 1-butanol anhydrous 99.8%, bovine serum albumin (BSA), mineral oil, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), and BSA-fluorescein isothiocyanate (BSA-FITC) were purchased from Sigma. Span 80, Rhodamine 123, *N*-hydroxysuccinimide (NHS), Ampliflu Red, and sodium hydroxide (NaOH) were purchased from Fluka. Amino-polystyrene (amino-PS) microparticles with a diameter of 1.0 μm were purchased from Polysciences Inc. Low-melting agarose was purchased from Promega. Ethanol was purchased from Fisher Scientific. Dimethyl sulfoxide (DMSO) was purchased from MP Biomedicals, Inc. PBS was purchased from BASE. All materials were used as received. Double-distilled water (dd H_2O) was distilled using a Fistreem Cyclone machine.

Preparation of Matrix-Assisted Colloidosomes (MACs). An aliquot of 500 μL of amino-PS microparticles from the stock solution was concentrated by centrifugation (4000 rpm, 6 min), and the supernatant was subsequently removed. The amino-PS microparticles were redispersed in ethanol and washed under sonication for 30 min followed by centrifugation (2000 rpm, 3 min) and removal of the supernatant. This washing procedure was repeated 3 times. The microparticles were then dried, weighted, and redispersed with dd H_2O to prepare a 20% w/v suspension. The microparticles were subsequently used for fabrication of MACs. For preparation of biomolecule-loaded MACs, amino-PS microparticles were blocked with 1% w/v BSA overnight to reduce nonspecific adsorption of biomolecules onto the surface of the amino-PS microparticle. All necessary reagents were prewarmed and kept at a temperature of 45 °C. A solution of 4% w/v low-melting agarose was prepared and kept molten at a temperature of 45 °C. The molten agarose solution was then mixed with the amino-PS microparticles (20% w/v) and desired biomolecule solutions (e.g., BSA-FITC, GOx, and HRP) to prepare a mixture with a final concentration of 2% w/v amino-PS microparticles in 2% w/v agarose containing the desired concentration of biomolecules. The agarose/amino-PS/biomolecules mixture was added to prewarmed mineral oil at 45 °C containing 0.1% Span 80 and stirred vigorously for 15 min to form water-in-oil (w/o) emulsion droplets stabilized by the microparticles assembled at the hydrogel/oil interface. The emulsion droplets were then cooled to 25 °C while stirring for another 10 min to allow solidification of the molten agarose core and forming of MACS. The MACs were further stabilized by placing at −20 °C for 5 min.

Coating of MACs by Reverse-Phase LbL (RP-LbL). Nonionized deprotonated PAH (niPAH) solution was prepared by dissolving 1 g of PAH in 10 mL of dd H_2O, addition of 5 mL of 10 M NaOH solution followed by vortexing with 1-butanol for 2 h. Subsequently, the mixture was centrifuged (4000 rpm, 10 min), and the 1-butanol containing the niPAH was extracted. An aliquot of 1 mL of 1-butanol extract was dried and weighed, and the amount of deprotonated PAH was calculated. The extract was then diluted with 1-butanol to prepare a 5 mg mL^{-1} niPAH solution. Nonionized protonated PAA (niPAA) solution was prepared by mixing 20 mL of 35% w/v PAA with 5 mL of 98% H_2SO_4 followed by vortexing with 1-butanol for 2 h. Subsequently, the mixture was centrifuged (4000 rpm, 10 min) and the 1-butanol containing the niPAA extracted. The niPAA concentration was determined as described above for niPAH, and the extract was diluted with 1-butanol to prepare a 25 mg mL^{-1} niPAA solution.

The RP-LbL coating process was entirely performed in 1-butanol with deprotonated PAH and protonated PAA as niPolyelectrolytes. To transfer the MACs from mineral oil to 1-butanol an equal amount of ethanol was added to the suspension and mixed vigorously followed by centrifugation (1000 rpm, 2 min). The mineral oil and ethanol were then discarded, and the pellet containing the MACs was washed twice with 1-butanol by centrifugation (1000 rpm, 2 min) and redispersion cycles. The resulting MACs were incubated with 1.5 mL of niPAH for 15 min under gentle vortexing followed by removal of the excess niPolyelectrolyte by three washing, centrifugation (1000 rpm, 2 min), and redispersion cycles with 1-butanol. The niPAH-coated MACs were overcoated with a second layer of niPAA using similar incubation, washing, and redispersion procedures. Alternate deposition of niPAH and niPAA was performed until the desired number of layers was achieved. The resulting RP-LbL-coated MACs were washed twice with ethanol using similar centrifugation and redispersion procedures and finally redispersed in dd H_2O or buffer for storage.

Preparation of Agarose Microbeads. Agarose microbeads were prepared with similar procedures as mentioned in the preparation of MACs except no amino-PS microparticles were used in the fabrication step.

Coating of MACs and Agarose Microbeads by Aqueous-Phase LbL. MACs or agarose microbeads were transferred from mineral oil to PBS buffer by centrifugation (1000 rpm, 2 min). Subsequent coating of the MACs or agarose microbeads was performed using PAH (5 mg mL^{-1} in 0.01× PBS) and PSS (5 mg mL^{-1} in 1.5× PBS) and washing, centrifugation, and redispersion cycles with 0.01× PBS buffer as described above for RP-LbL coating.

Characterization of Nonionized Polyelectrolyte (niPolyelectrolyte) Multilayer Coating. A PAA-Rhodamine 123 conjugate was synthesized based on EDC/NHS chemistry. The carboxyl group of PAA was activated by addition of excess EDC and NHS for 15 min. Rhodamine 123 dissolved in DMSO was mixed with the activated PAA at a molar ratio of 1:10 (Rhodamine 123 molecule: PAA monomer) and incubated for 12 h at 25 °C. The PAA−Rhodamine 123 conjugate was purified by dialysis (molecular weight cutoff of 3.5 kDa) and dried. niPAA−Rhodamine 123 was prepared with similar procedures as mentioned for the preparation of niPAA. For demonstration of multilayer deposition, niPAH and a mixture of niPAA and niPAA−Rhodamine 123 (9:1) were used to coat the MACs. The resulting MAC RP-LbL microcapsules were examined by fluorescence microscopy.

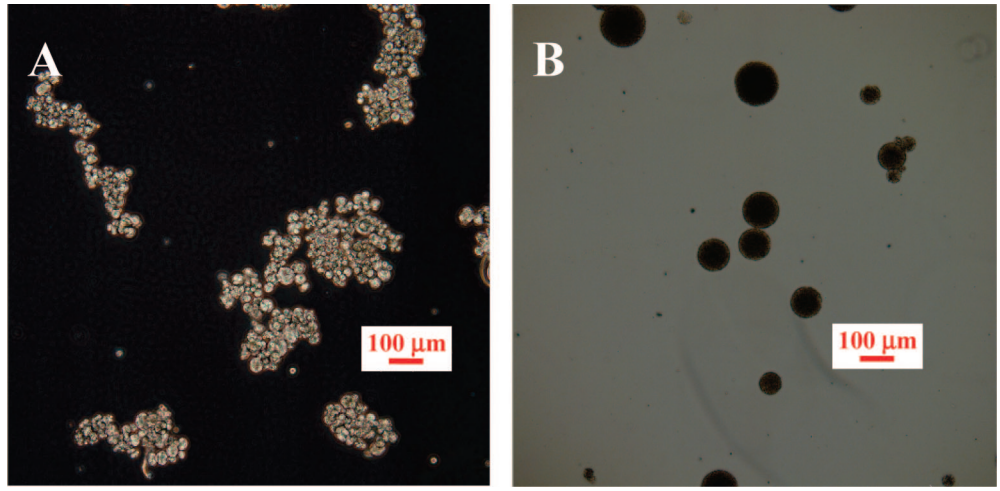

Figure 2. Phase contrast micrographs of (A) agarose microbeads aggregated in 1-butanol and (B) MACs dispersed in 1-butanol with good colloidal stability.

Biological Activity Study. Seven layers of niPolyelectrolyte-coated MAC RP-LbL microcapsules that contain 2.34 U GOx and 2.715 U HRP as the initial enzyme loading concentrations were fabricated with similar procedures as mentioned above. The resulting microcapsules were incubated with 0.5× PBS buffer (pH 7.4) and allowed to stand for 90 min for stabilization. The enzymatic activity of the encapsulated enzymes within the MAC RP-LbL microcapsules was compared with a bulk-phase enzyme solution containing the same amount of enzymes. A solution mixture containing 2.84 mL of 1× PBS (pH 7.4), 1 mL of 5 mg mL^{-1} glucose in 1× PBS, and 10 μL of 5 mg mL^{-1} Ampliflu Red in DMSO was continuously shaken with either 150 μL of MAC RP-LbL microcapsules or an enzyme solution that contains the same enzyme concentrations. The enzymatic reaction rates of the reaction mixtures were determined by measuring the absorbance of the reaction mixture at 571 nm with a UV−vis spectrophotometer, UV-2450 (Shimadzu Corp., Japan).

Optical and Fluorescence Microscopy. Phase contrast and fluorescence microscopic images were recorded using a CCD color digital camera, Retiga 4000R (QImaging, Canada), connected to a system microscope (Olympus BX41) with a mercury arc (Olympus HBO103W/2) excitation source. Bandpass filters with an excitation wavelength of 488 nm and emission wavelength of 520 nm were used for FITC and Rhodamine 123 detection. Images were captured with QCapture Pro software (Version 5.1.1.14, QImaging, Canada) and analyzed by QCapture Pro software or ImageJ software (Scion Corp., USA). Stereomicroscopic images were acquired using a CCD color camera, Evolution MP (MediaCybernatics, USA), connected to a stereomicroscope, Axiostar plus (Carl Zeiss GmbH, Germany). Confocal fluorescence microscopic images were captured using a laser scanning confocal microscope, Leica TCS SP2 (Leica Microsystem Heidelberg GmbH, Germany).

Results and Discussion

Morphology and Stability of Matrix-Assisted Colloidosomes (MACs) in the Organic Phase. Agarose is hydrophilic and a good matrix material to entrap biomolecules. However, dispersion of hydrophilic agarose microbeads in a hydrophobic organic phase (1-butanol) is difficult as agarose microbeads in 1-butanol aggregate severely with a reduction in diameter (Figure 2A). It is believed that aggregation is caused by a polar−polar interaction between the agarose microbeads so as to minimize the interfacial surface area exposed to 1-butanol, and the reduction in diameter is a result of dehydration by anhydrous 1-butanol.

In order to stabilize the agarose microbeads in the organic phase, microparticles were introduced into the agarose microbead fabrication process to produce MACs. During fabrication of MACs through an emulsification process, the microparticles would migrate and self-assemble at the interfaces of the w/o emulsion droplets (agarose droplet surfaces) so as to minimize the total interfacial energy.[32,33] The assembled microparticles form a shell around the surface of each agarose microbead and reduce the interfacial surface area between the hydrophilic agarose core and the 1-butanol. This reduction in direct surface area exposure of the agarose beads to 1-butanol is believed to facilitate the stable dispersion of MACs in 1-butanol and to slow down the process of dehydration.

The resulting MACs were transferred and dispersed in 1-butanol where the morphology and stability of the MACs were examined with confocal and stereomicroscopy. The phase contrast micrograph of Figure 2B shows MACs in 1-butanol having a perfect spherical shape and exhibiting no significant aggregation. The confocal micrograph of Figure 3A depicts MACs fabricated with BSA-FITC-tagged microparticles having a fluorescent ring around each MAC, indicating a large population of microparticles lining up on the surface of each MAC to form a shell. A high-magnification stereomicrograph shows assembled microparticles distributed on the surface of an agarose microbead (Figure 3B). Besides morphology, the size distribution of MACs dispersed in the organic phase (1-butanol) and aqueous phase (dd H_2O) were compared to determine the effects of dehydration. Figure 4A shows no significant changes in the size distribution of MACs from the same preparation batch in either dispersant. Assuming MACs in aqueous phase to possess the highest degree of hydration, the insignificant changes in size distribution indicates that there was insignificant dehydration of MACs when dispersed in 1-butanol. On the contrary, agarose microbeads from the same preparation batch show a significant shift in size distribution to a smaller diameter range when dispersed in 1-butanol (Figure 4B). Both size distributions of MACs and agarose microbeads were obtained immediately after dispersing in their respective solvents. These results indicate that microparticles are important in stabilizing the agarose core and MACs are stable in the organic phase for subsequent RP-LbL coating.

Importance of an Organic Phase To Disperse MACs Loaded with Biomolecules. In order to achieve a high encapsulation efficiency of biomolecules, the preloaded biomolecules have to be prevented from leaching out of the hydrogel core during formation of the polymeric capsule "membrane".

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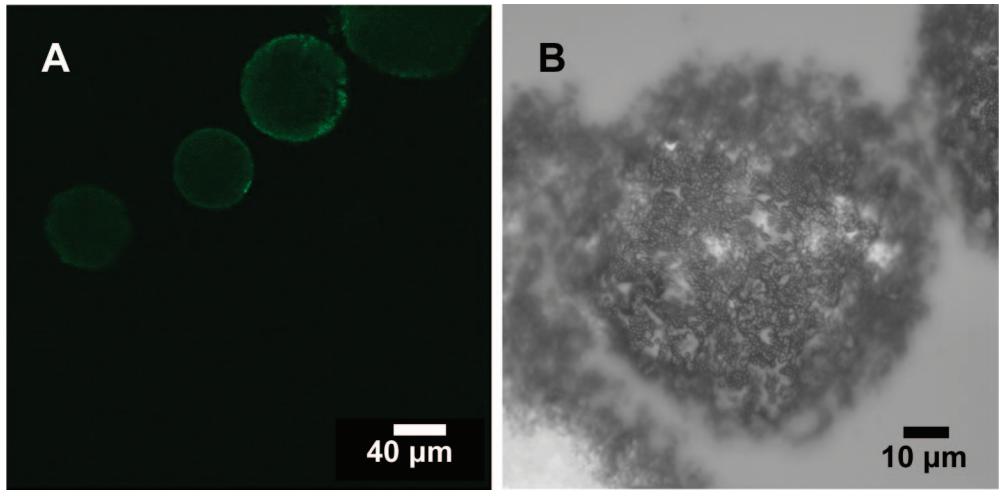

Figure 3. (A) Confocal micrograph of MACs fabricated with BSA-FITC tagged amino-PS microparticles. A fluorescence ring is observed on the MACs surface. (B) High magnification stereomicrograph of MACs showing the assembled microparticles distributed on the surface of an agarose microbead.

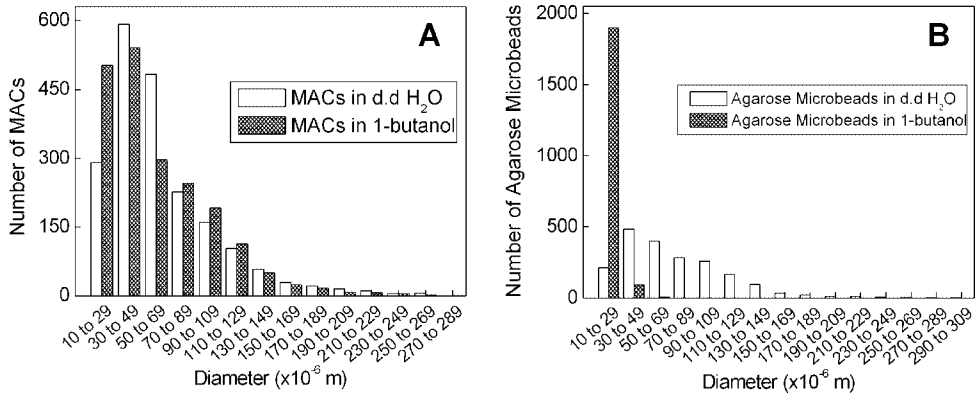

Figure 4. (A) Size distribution curve of MACs ($n = 2000$ for either dispersant) and (B) agarose microbeads ($n = 2000$ for either dispersant) dispersed in 1-butanol (shaded column) and dd H_2O (blank column) ($p = 1$ for a two-tailed t test based on size range). MACs dispersed in 1-butanol and dd H_2O show a similar size distribution, while agarose microbeads show a significant shift in size distribution to a smaller diameter range when dispersed in 1-butanol.

No significant leaching of biomolecules can occur during the w/o emulsion step as most biomolecules are poorly soluble in oil. However, leaching of biomolecules can occur during dispersion of MACs in the aqueous phase for traditional LbL coating. Therefore, selection of a suitable dispersant/solvent for dispersion of MACs and subsequent RP-LbL coating that prevents leaching of water-soluble biomolecules from MACs is necessary.

1-Butanol is an organic solvent immiscible with water due to its considerably long carbon chain. Also, it is less toxic than other organic solvents such as chloroform, toluene, and acetonitrile. These properties make 1-butanol a suitable organic dispersant for the MACs. The applicability of 1-butanol as dispersant to prevent leaching of water-soluble biomolecules from MACs was studied using BSA-FITC as a model material. The fluorescence intensity of BSA-FITC retained within individual MACs compared to the initial fluorescence intensity represents the relative entrapment efficiency. The initial fluorescence intensity of MACs in 1-butanol at zeroth day was normalized to 100% for data comparison. MACs loaded with 0.125 mg mL^{-1} BSA-FITC were fabricated and transferred to either 1-butanol or dd H_2O. The resulting MACs were incubated for a period of 5 days, and the fluorescence content within the MACs was measured. Figure 5A shows that no significant leakage of BSA-FITC was observed when the MACs were dispersed in 1-butanol during the entire incubation period, while almost 100% of BSA-FITC leached out immediately from the MACs when dispersed in dd H_2O. The entrapment efficiency of BSA-FITC-loaded MACs in 1-butanol was statistically about 97%. Figure 5B shows the phase contrast and corresponding fluorescence micrographs of MACs after 5 days incubation in 1-butanol or dd H_2O. Fluorescence signal emitted from BSA-FITC was observed in MACs incubated in 1-butanol, while no fluorescence signal was observed from MACs incubated in dd H_2O. This result clearly demonstrates that 1-butanol is a suitable organic dispersant for MACs as it allows a high amount of water-soluble biomolecules to be retained inside the MACs.

Demonstration of Nonionized Polyelectrolyte (niPolyelectrolyte) Multilayer Coating onto MACs Using RP-LbL. The coating of polyelectrolyte multilayers onto MACs by RP-LbL using 1-butanol was studied with deposition of a fluorescent-labeled niPolyelectrolyte (niPAA-Rhodamine 123). The fluorescence intensity of MACs after each bilayer coating [niPAH/niPAA-Rhodamine 123] was measured by analyzing the fluorescence micrographs of MACs based on densitometry and represented by pixel values. The fluorescence intensity of MACs as a function of the number of bilayers is plotted in Figure 6, and it clearly shows the increment in fluorescence intensity of MACs when the number of bilayer coatings increases. The increase in fluorescence intensity is due to an accumulation of niPAA−Rhodamine 123 onto the MACs after each bilayer coating. This result demonstrates that stepwise RP-LbL self-

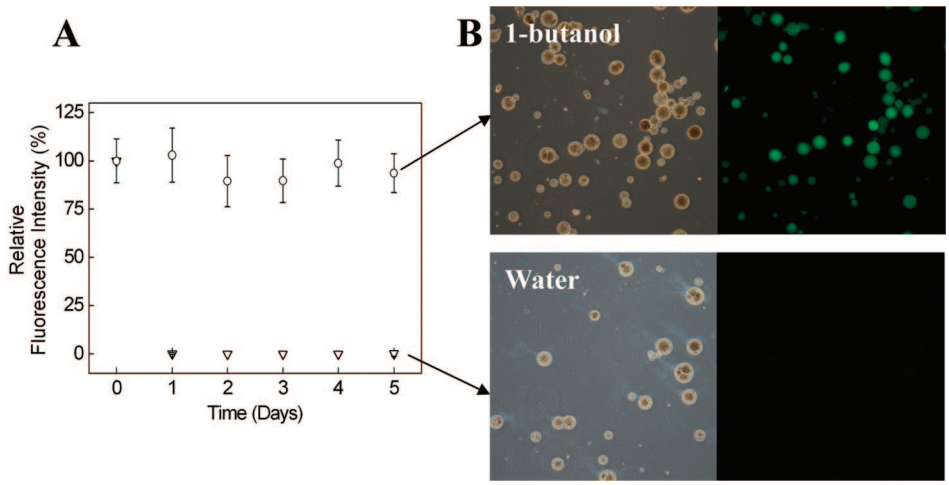

Figure 5. (A) Relative fluorescence intensity of MACs loaded with BSA-FITC dispersed in 1-butanol (circle) and dd H_2O (triangle) over a 5 day period. The 0th day represents the freshly fabricated MACs dispersed in 1-butanol. (B) Phase contrast and corresponding fluorescence micrographs of MACs after 5 days incubation in 1-butanol or dd H_2O.

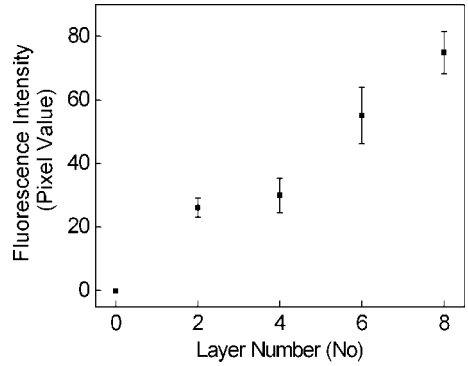

Figure 6. Fluorescence intensity (pixel value) of MACs coated with ni(PAH/PAA−Rhodamine 123) as a function of layer number.

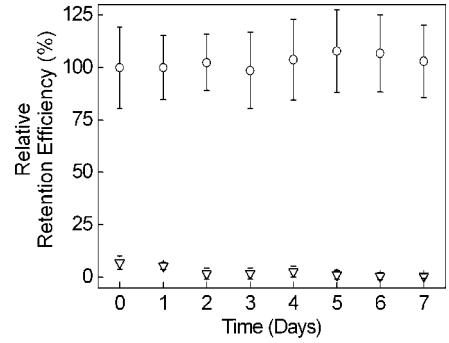

Figure 7. Relative retention efficiency of MAC RP-LbL microcapsules with 7 layers of niPolyelectrolytes (circle) and MACs with no layers (triangle) incubated in dd H_2O over a period of 7 days. The 0th day represents fluorescence intensity measured immediately after transferring the samples from 1-butanol to dd H_2O, and MAC RP-LbL microcapsules at 0th day were taken as 100%.

assembly of niPolyelectrolyte layers onto MACs for polymeric capsule "membrane" formation in 1-butanol is possible.

Retention of Biomolecules within MAC RP-LbL Hydrogel Microcapsules. As most biochemical reactions take place in an aqueous environment, it is therefore necessary to determine whether the resulting MAC RP-LbL microcapsules can retain their biomolecule contents when dispersed in an aqueous environment. Retention of biomolecules within MAC RP-LbL microcapsules was studied using BSA-FITC filled microcapsules. MACs containing 0.125 mg mL^{-1} BSA-FITC were coated with 7 niPolyelectrolyte layers and transferred from 1-butanol to dd H_2O followed by an incubation of 7 days. In order to demonstrate the importance of the polymeric capsule "membrane" for retention of biomolecules within MAC RP-LbL microcapsules, MACs containing 0.125 mg mL^{-1} BSA-FITC without LbL coating (i.e., no "membrane") were used as a control experiment.

The retention efficiency of BSA-FITC as a function of time is shown in Figure 7. The zeroth day represents the fluorescence intensity measured immediately after transferring the samples from 1-butanol to dd H_2O, and the fluorescence intensity of MACs at zeroth day is normalized to 100% retention efficiency. The fluorescence intensity measured within individual microcapsules after dispersion in water compared to the fluorescence intensity of MACs at zeroth day reflects the relative retention efficiency. MAC RP-LbL microcapsules retained statistically ~100% of BSA-FITC after 7 days of incubation, while MACs without LbL coating immediately released more than 90% of the BSA-FITC after dispersion in dd H_2O. This result demonstrates that retention of biomolecules depends on formation of the polymeric capsule "membrane" with sufficiently small pore size (i.e., the pore size of the "membrane" is small enough to prevent out diffusion of the large molecular weight biomolecules from the hydrogel core to the outer environment).

Significance of MACs and RP-LbL To Achieve High Encapsulation Efficiency. The ability of the MAC RP-LbL encapsulation technique to achieve an extremely high encapsulation efficiency arises from two strategies. First, using microparticles as surface stabilizer to create MACs that allow stable dispersion of agarose microbeads in organic phase. Second, using RP-LbL to perform niPolyelectrolyte coating in organic phase, thereby preventing out diffusion of the highly water-soluble materials from the core.

In order to emphasis on the unique advantages of the MAC RP-LbL technology, the encapsulation efficiency of MAC RP-LbL microcapsules was compared with those of microcapsules fabricated with traditional aqueous LbL (Aq-LbL) using PAH/PSS as polyelectrolytes and MACs or agarose microbeads as template. MACs containing 0.125 mg mL^{-1} BSA-FITC were coated with 7 layers of niPolyelectrolyte using RP-LbL, while MACs or agarose microbeads containing 0.125 mg mL^{-1} BSA-FITC were coated with 7 layers of polyelectrolyte using Aq-LbL. MACs containing 0.125 mg mL^{-1} BSA-FITC without polyelectrolyte coating were used as a control. Figure 8 shows

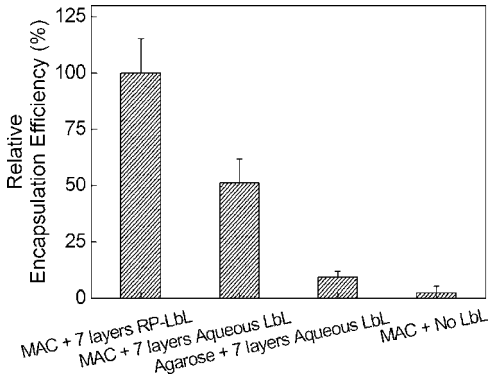

Figure 8. Relative encapsulation efficiency of microcapsules prepared from MACs with 7 layers of RP-LbL coating, MACs with 7 layers of Aq-LbL coating, agarose microbeads with 7 layers of Aq-LbL coating, and MACs without any LbL coating (control).

the relative encapsulation efficiencies (calculated from fluorescence intensities of BSA-FITC) of microcapsules fabricated with MAC RP-LbL, MAC Aq-LbL, agarose Aq-LbL, and control MAC without LbL coating were 100%, 51.2%, 9.4%, and 2.5%, respectively. This result demonstrates that the MAC RP-LbL microcapsules have an encapsulation efficiency that is 10 times (i.e., achieved 100% encapsulation efficiency) better than the encapsulation efficiency of agarose Aq-LbL microcapsules. Interestingly, MAC Aq-LbL microcapsules demonstrated an encapsulation efficiency that is 5 times higher than that of agarose Aq-LbL microcapsules. This 5-fold increment in encapsulation efficiency can be attributed to the microparticles assembled on the surface of agarose microbeads. By assembling microparticles onto the surface of each agarose microbead, the microparticle shell reduces the interfacial surface area between each agarose microbead and the aqueous environment and effectively hinders the out diffusion of biomolecules from the core. This result demonstrates that the MAC RP-LbL method is an ideal method for encapsulating water-soluble biomolecules with extremely high encapsulation efficiency.

Biological Activity of Encapsulated Biomolecules within MAC RP-LbL Hydrogel Microcapsules. Besides the encapsulation and retention efficiency, the feasibility of the MAC RP-LbL method to encapsulate a mixture of biomolecules and preserve their biological activity was studied. A bienzyme system using GOx and HRP was used as a model. GOx converts glucose in the presence of oxygen into glucono-lactone and hydrogen peroxide, and HRP utilizes the generated hydrogen peroxide to oxidize Ampliflu Red into a red colored product with an absorbance at 571 nm. MAC RP-LbL microcapsules coated with 7 niPolyelectrolyte multilayers and using 15.6 mU μL^{-1} GOx and 18.1 mU μL^{-1} HRP as initial starting materials were prepared. The enzymatic activity of the enzymes encapsulated within the MAC RP-LbL microcapsules was compared with a bulk-phase enzyme solution containing the same amount of enzymes. Figure 9 shows the normalized absorption at 571 nm as a function of time. The slope of each data plot was calculated via linear regression analysis and represents the rate of the enzymatic reaction. The result shows that the reaction rates of the encapsulated enzymes within the MAC RP-LbL microcapsules and those of the free enzymes in bulk phase were 0.023 and 3.756, respectively.

The significant decrease in enzymatic reaction rate of the MAC RP-LbL microcapsules can be attributed to the different types of diffusion barriers created by the amino-PS microparticle shell, the agarose core matrix, and the polymeric capsule "membrane", where the microparticle shell barrier is the most likely cause for

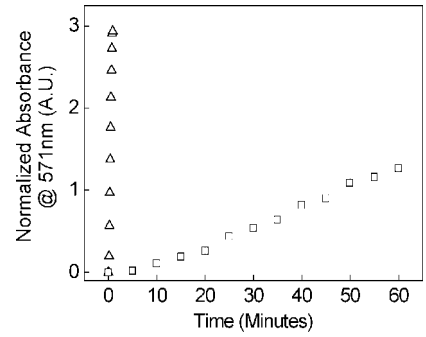

Figure 9. Enzymatic reaction rate of free enzymes in solution (triangle) and enzymes encapsulated within MAC RP-LbL microcapsules (square).

the reduced enzymatic reaction rate. In the bienzyme system the large molecular weight GOx (~160 kDa) and HRP (~44 kDa) remain encapsulated inside the MAC RP-LbL microcapsules, whereas the small molecular weight glucose (180 g mol^{-1}) and Ampliflu Red (257.2 g mol^{-1}) are required to diffuse through the diffusion barriers for the colorimetric assay to take place. Among all three diffusion barriers, the agarose core matrix and polyelectrolyte "membrane" remain permeable to the small molecular weight substrate molecules. In contrast, the amino-PS microparticles are impermeable to the substrate molecules and diffusion is only allowed through the gaps between microparticles within the microparticle shell. Since the amino-PS microparticles cover a high percentage of the total effective surface area of the MAC RP-LbL microcapsules, it is suggested that the slow enzymatic reaction rate observed is mainly due to the diffusion barrier created by the amino-PS microparticle shells.

Conclusion

We have demonstrated the feasibility of the MAC RP-LbL encapsulation technology to prepare biomolecule-loaded hydrogel microcapsules with high encapsulation efficiency and excellent retention stability. BSA-FITC with a molecular weight of ~65 kDa was used as a model protein for the encapsulation study. In the MAC RP-LbL encapsulation process, the biomolecules were first loaded into MACs through emulsification before the polymeric capsule "membrane" was fabricated in organic phase to prevent leaching of biomolecules. A high encapsulation efficiency of statistically almost 100% of the initial loaded biomolecules was achieved using the MAC RP-LbL technology. The ability of MAC RP-LbL to achieve high encapsulation efficiency is a result of the combination of two strategies. (1) Dispersing hydrogel microbeads in organic phase by creating MACs with microparticles as surface stabilizer. This reduces exposure of the hydrogel surface to the organic phase and thus prevents the hydrogel microbeads from aggregation and dehydration. (2) Using RP-LbL to fabricate polymeric capsule "membranes", thereby preventing out diffusion of the preloaded highly water-soluble biomolecules from the hydrogel microbeads into the organic phase dispersant. The high encapsulation efficiency of the MAC RP-LbL technology is especially useful for encapsulation of scarce, valuable, and expensive proteins. Limitations of this method are the loading density (which is limited by the solubility of the biomolecules in agarose) and retention of low molecular weight molecules. We believe that the MAC RP-LbL technology can significantly contribute to the development of microcapsule-based bioreactors and bioanalytical systems.

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