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A Design Full of Holes: Functional Nanofilm-Coated Microdomains in Alginate Hydrogels[†]

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Abstract

This study demonstrates the successful manufacture and functional characterization of alginate hydrogels containing a variety of encapsulates within polyelectrolyte multilayer-coated micropores. These microporous alginate (MPA) hydrogels are prepared *via* one-step internal ionotropic gelation of the alginate using polyelectrolyte multilayer-coated CaCO₃ microspheres along with the weak acid glucono- γ -lactone. Here, successful encapsulation of a model macromolecule and fluorescent nanoparticles within microcapsules—distributed throughout the larger alginate hydrogel—is confirmed with confocal microscopy, while the porous morphology of the MPA hydrogels is examined with scanning electron microscopy. Hydrogels constructed with uncoated CaCO₃ microspheres release their contents into the surrounding environment, while those constructed with polyelectrolyte multilayer-coated CaCO₃ microspheres retain the materials within the pores. MPA hydrogels containing the model enzyme glucose oxidase retained activity and are capable of reacting with small molecules from the external environment. The ability to encapsulate an assortment of functional materials within a moldable, biocompatible alginate matrix gives this approach great flexibility and potential in a wide variety of biomedical applications.

Introduction

The design and development of biocompatible matrix materials for use in biomedical applications is an area of primary focus in biomedical engineering. One important aspect of the development of such encapsulation matrices is the control of the functional aspects of the matrix.

Layer-by-layer (LbL) self-assembly has been used in a variety of applications to control diffusion of solutes and modify surface interfacial properties.^{1–6} LbL films can be deposited onto a substrate—ranging in size and geometry from large planar substrates to micro- or nanoparticles—*via* a repetitive process of electrostatic adsorption of oppositely-charged polyelectrolytes.⁷ The resulting polyelectrolyte multilayers (PEMs) can be manufactured in a controllable manner with respect to their physical and functional characteristics (*e.g.*, thickness, permeability, and interfacial properties), making them useful in biomedical

[†]The authors declare no competing financial interest. Electronic supplementary information (ESI) available: Confocal and SEM micrographs of microporated alginate hydrogel containing 100 nm or 200 nm FluoSpheres (Fig. S1), three-dimensional reconstruction of microporated alginate hydrogel containing 100 nm FluoSpheres (Video S1) and video demonstrating 200 nm FluoSpheres moving within microcavities of alginate hydrogel (Video S2). See DOI:

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applications such as bionanoreactors,⁸ nanofiltration,^{4, 5} drug delivery,^{9–11} and biosensors.^{12–15}

LbL has been widely used in conjunction with CaCO₃ microspheres to form microcapsules.^{16–24} PEMs are deposited onto the CaCO₃ surface, and once the desired number of layers has been deposited, the CaCO₃ core can be dissolved in a slightly acidic solution or with a calcium-chelating agent to form a capsule. By pre-loading the CaCO₃ microspheres, such microcapsules could contain a wide variety of materials including small molecules, macromolecules, and nanoparticles. Once again, a high degree of control over the physical and functional properties of the microcapsules can be achieved; capsule size, thickness, permeability, and interfacial interactions can all be *designed* by choosing the appropriately-sized CaCO₃ template and materials, as well as controlling the deposition conditions (*e.g.*, salt concentration, pH, *etc.*), number of layers, and the appropriate terminating layer. Usually, these nano-engineered constructs are used or studied as suspensions or, in some cases, immobilized onto other surfaces; however, little work has been done towards integrating these units into larger-scale bulk materials to form complex composites with added functionality.

One well-studied bulk encapsulation material is the biomaterial alginate, a linear polysaccharide consisting of the negatively-charged sugars α -D-mannuronate and α -L-guluronate.^{25, 26} These negatively charged moieties allow for alginate to form a hydrogel in the presence of divalent cations such as calcium, barium, strontium, *etc.*^{27, 28} Alginate hydrogels have many attractive physicochemical properties, such as relatively high mechanical strength, high water content, and gel formation under mild conditions.^{29–34} Additionally, the biocompatibility of purified alginate hydrogels has generally been shown to be very high, though there remains an ongoing investigation into the many factors affecting the material's biocompatibility (*e.g.*, purification process, mannuronic-guluronic acid ratio, *etc.*).^{35–40} Due to the numerous advantageous properties of alginate, there has been great interest in the material for a wide variety of biomedical applications.

A great deal of attention has been given to methods that combine alginate gelation with material entrapment.^{29–31, 41–53} Furthermore, a substantial amount of work has been devoted to the development of in situ gelled hydrogels for a variety of biomedical applications.⁵⁴ Alginate, due to its ability to be ionotropically crosslinked under mild conditions, has been investigated for in situ gelation applications, resulting in multiple solutions.^{55, 56} In work by Kuo and Ma, it was shown that CaCO₃ could be used in combination with glucono- δ -lactone (GDL) to gel alginate through release of calcium internally (*i.e.*, internal ionotropic gelation), resulting in a stronger, more homogenous hydrogel.⁵⁵ This method provides a relatively slow gelation time of the alginate, which allows for molding into a variety of shapes.

In this work, we utilize CaCO₃ microspheres—coated with PEMs using LbL—in conjunction with GDL and alginate to form moldable hydrogels containing encapsulated materials, which are confined to micropores within the larger hydrogel. Entrapping material within such microporous hydrogel matrices in a controllable manner is appealing to a variety of biomedical applications including sensing, drug delivery and tissue engineering. This method benefits from the tunability of the PEMs, as well as the gentle ionotropic gelation of the biocompatible, biodegradable alginate matrix. To our knowledge, the manufacture of such functional microporous hydrogels using alginate and PEM-coated CaCO₃ microspheres has not been reported.

Experimental Procedures

Materials

Poly(sodium 4-styrenesulfonate) (PSS, average $M_w = 70,000$ Da), poly(allylamine hydrochloride) (PAH, average $M_w = 51,495$ Da), Rhodamine B isothiocyanate (RITC, mixed isomers), albumin from bovine serum (BSA, >99% by agarose gel electrophoresis, *ca.* 66 kDa), alginic acid sodium salt from brown algae (281 cps for a 2% aqueous solution at 25 °C), and peroxidase from *Amoracia rusticana* (type II, 188 U/mg solid) were obtained from Sigma and used without further purification. Glucose oxidase from *Aspergillus niger* (GOx, 257 U/mg solid, 71.1% protein by Lowry, *ca.* 160 kDa) was obtained from BBI Enzymes. FluoSpheres® (2% solids, carboxylate-modified microspheres, $r_{ex} = 505$ nm, $r_{em} = 515$ nm) were obtained from Invitrogen.

Entrapment of materials in CaCO_3

Loaded CaCO_3 microspheres were prepared by adding 8 mL CaCl_2 (0.25 M) to 8 mL Na_2CO_3 (0.25 M) containing 4 mg/mL PSS and the material to be encapsulated (*ca.* 1 mg/mL rhodamine-labeled BSA (RITC-BSA), 24 nm FluoSpheres at 0.01% solids, 100 nm FluoSpheres at 0.01% solids, or 200 nm FluoSpheres at 0.01% solids). For CaCO_3 microspheres containing GOx, the Na_2CO_3 solution consisted of 8 mg/mL GOx only (*i.e.*, no PSS). The solution was stirred vigorously for 30 s, after which time the agitation was removed and the particles were allowed to mature under static conditions for 12.5 min. The suspension was centrifuged and the supernatant was removed. The microspheres were rinsed three times with deionized water.

LbL deposition of PEMs

PEMs were deposited onto the loaded CaCO_3 microspheres using LbL self-assembly.⁵⁷ Briefly, microspheres were suspended in polyelectrolyte solutions (PSS or PAH at 2 mg/mL containing 0.5 M NaCl in deionized water) for 12 min, rinsing with deionized water between each deposition step to remove unbound polyelectrolyte. PSS was deposited initially, and then PAH and PSS were alternated until 10.5 bilayers of PSS/PAH were deposited.

Formation of alginate hydrogels

Based on the findings of Kuo and Ma,⁵⁵ the CaCO_3 :carboxylic acid (from alginate):GDL ratio was maintained at 1:0.27:2 for all alginate hydrogels. A 16.8 mg/mL solution of CaCO_3 microspheres (250 μL , PEM-coated or uncoated) was thoroughly mixed with 500 μL of 3% (w/v) alginate. To this solution, 250 μL of 29.8 mg/mL GDL was added. After further mixing, the solution was placed into the appropriate mold and allowed to gel for 24 h. Fig. 1 schematically illustrates the process of forming microporated alginate (MPA) hydrogels.

Physical characterization of microspheres, microcapsules, and hydrogels

Alginate hydrogels constructed with PEM-coated and uncoated CaCO_3 microspheres containing RITC-BSA or FluoSpheres were cast between two glass slides with 0.03" Teflon spacers for electron and optical microscopy. A benchtop scanning electron microscope (JEOL model JCM-5000 NeoScope) was used to image microcapsules, PEM-coated and uncoated CaCO_3 microspheres, and hydrogels constructed using CaCO_3 microspheres. Samples were placed on conductive tape, air-dried at room temperature for 24 hours, and sputter-coated with a thin layer of gold to prevent charging. Samples were held under high vacuum and an accelerating voltage of 10 kV was used when collecting images.

Optical micrographs were obtained using a confocal microscope (Leica model TCS SP5) equipped with a 63x oil immersion objective. An Ar ion laser ($\lambda_{em} = 488$ nm) was used to

excite the FluoSpheres, while a HeNe laser ($\lambda_{em} = 543 \text{ nm}$) was used to excite the RITC-BSA; in all cases that hydrogels were imaged, the pinhole diameter was minimized to eliminate out-of-focus light from the scattering samples. A second confocal microscopy system (Leica model TCS SP5-RS) equipped with a high-speed resonant scanner and a 63x water immersion objective was used to collect a z-stack of an MPA hydrogel containing 100 nm FluoSpheres. ImageJ was used to produce a three-dimensional reconstruction of the images (Video S1). An epifluorescence microscope (Nikon model Eclipse TE2000-U) equipped with a monochromatic camera (Photometrics model CoolSNAP HQ²) was used to collect the video demonstrating the 200 nm FluoSpheres moving within the microcavities of the alginate hydrogel (Video S2).

Release study

Hydrogels made with PEM-coated and uncoated CaCO_3 microspheres loaded with RITC-BSA (100 μL each) were cast into individual wells of a 96-well microplate. After the initial gelation period of 24 h, an extreme excess of GDL (100 μL of 0.1 M) was added to each hydrogel-containing well to ensure full dissolution of the CaCO_3 . After 30 min, the GDL solution was removed and placed in an empty well, and 100 μL of release buffer (*i.e.*, 0.1 M HEPES, pH 7) was added to the alginate hydrogel-containing well. After the appropriate incubation time, release buffer from each sample was then removed and placed in an empty well, and fresh buffer was added to the wells containing the alginate hydrogels.

Fluorescence intensity ($\lambda_{ex} = 560 \text{ nm}$, $\lambda_{em} = 590 \text{ nm}$) was measured for each sample and timepoint (including the first GDL wash) using a microplate reader (Tecan model Infinite M200 PRO).

Enzymatic activity assay

MPA hydrogels made with PEM-coated CaCO_3 microspheres containing GOx (100 μL each) were cast into individual wells of a 96-well microplate. After 24 h gelation time, activity assays were performed using a colorimetric assay.⁵⁸ All measurements were performed at pH 5.1 and 35 °C. Briefly, 200 μL of the reaction cocktail (0.17 mM *o*-dianisidine, 1.72% (w/v) glucose, 2 units/mL peroxidase in 50 mM sodium acetate buffer, pH 5.1) were added to each well using the automated dispensing system on the microplate reader. Absorbance was then monitored at 500 nm for 10 min for each sample to determine its apparent activity.

Results and Discussion

Physical characterization of microspheres, microcapsules, and hydrogels

Alginate hydrogels were prepared using an adapted internal ionotropic gelation method.^[47] Initially, materials were loaded into CaCO_3 microspheres by co-precipitation, followed by LbL deposition of PEMs onto the loaded microspheres (Fig. 1, left). Three materials—Rhodamine B-labeled bovine serum albumin (RITC-BSA), FluoSpheres, and glucose oxidase (GOx)—were used to produce three different types of loaded microspheres representing incorporation of a model macromolecule, model nanoparticle, and model enzyme, respectively. Before manufacture of alginate hydrogels, microspheres containing RITC-BSA were exposed to GDL to confirm particle dissolution and demonstrate capsule integrity. Fig. 2 shows PEM-coated CaCO_3 microspheres containing RITC-BSA exposed to deionized water (Fig. 2A and B) or GDL (Fig. 2C and D). Complete dissolution of the CaCO_3 template by GDL is evident by electron microscopy (Fig. 2C); the microcapsules, which are composed of PEMs that remain after GDL treatment, appear collapsed due to dehydration. Fluorescence microscopy confirms that the microspheres contain RITC-BSA (Fig. 2B), and that the encapsulated material is retained within the microcapsules following GDL exposure (Fig. 2D). Fig. 3 illustrates the remarkable difference between alginate

hydrogels formed using uncoated (Fig. 3A–D) and PEM-coated (Fig. 3E–H) CaCO_3 microspheres containing RITC-BSA. When uncoated CaCO_3 microspheres were used in the manufacture of alginate hydrogels, no noticeable pores were observed in the electron micrographs (Fig. 3A–C). Instead, they exhibit a smooth appearance with some wrinkling due to dehydration. The nonporous nature of the hydrogels manufactured with uncoated microspheres is expected, as this is similar to the internal gelation mechanism employed by Kuo and Ma, which resulted in homogenous alginate hydrogels.^[47] When these hydrogels were inspected using confocal microscopy immediately after gelation, fluorescent material can be found in close proximity to where microspheres were dissolved (Fig. 3D). However, these patches have undefined boundaries due to the free diffusion of material in the alginate. It is noteworthy that after a period of hours, distinct patches of fluorescent material can no longer be found in hydrogels formed using uncoated CaCO_3 (data not shown). This absence of visible fluorescence further demonstrates the free diffusional nature of the macromolecule in the matrix, which eventually results in the complete loss of material from the bulk alginate into the surrounding solution. In contrast, when PEM-coated CaCO_3 microspheres were used in the manufacture of alginate hydrogels, a clearly distinguishable microporous structure was observed (Fig. 3E–G).

Upon dehydration of the MPA hydrogels in preparation for electron microscopy, they exhibited a wrinkled morphology with randomly distributed micropores. In confocal micrographs, the encapsulated RITC-BSA appears to occupy distinct regions of the hydrogel within defined boundaries (Fig. 3H), demonstrating confinement of the fluorescent macromolecules within the micropores. MPA hydrogels manufactured with PEM-coated CaCO_3 microspheres containing FluoSpheres had a comparable microporous structure to that of hydrogels containing RITC-BSA (Fig. S1); however, the PEM-coated pores did not fully collapse under vacuum as in the RITC-BSA-containing MPA gels, presumably due to the greater volume of encapsulated material. A three-dimensional reconstruction of an MPA hydrogel containing the FluoSpheres demonstrates the distribution of PEM-lined micropores within the alginate matrix and the confinement of the encapsulated material within those pores (Video S1). In addition to showing containment within the pores, free movement of the nanoparticles was confirmed by observation of their random motion within the micropores of the MPA hydrogels (Video S2). Aside from the morphological differences in the alginate hydrogels prepared using PEM-coated and uncoated CaCO_3 microspheres, it was observed that the MPA hydrogel gelation time was drastically increased, presumably due to decreases in small molecule transport (*e.g.*, GDL, Ca^{2+} , *etc.*) through the PEMs.

Release and retention of encapsulated macromolecules

To evaluate the release of encapsulated macromolecules from the alginate hydrogels and determine the effects of PEM coatings on this behavior, hydrogels prepared with PEM-coated and uncoated CaCO_3 microspheres loaded with RITC-BSA were cast into wells of a microplate. Release of the labeled macromolecule into the surrounding media was then monitored over a period of 55 h. Alginate hydrogels formed using PEM-coated CaCO_3 microspheres loaded with RITC-BSA showed significantly improved retention of the encapsulated material as compared to hydrogels formed using uncoated microspheres (Fig. 4, left). The similar shape of the individually normalized curves (Fig. 4, right) suggests similar transport behavior (*i.e.*, similar diffusional characteristics of the RITC-BSA through the bulk alginate) in both types of hydrogels; however, RITC-BSA release from hydrogels formed using uncoated microspheres was approximately 50 times higher than the release observed from hydrogels formed using PEM-coated microspheres. In the ideal case, in which PEM coatings are completely devoid of defects, no release of the encapsulated macromolecules from the microcapsules is expected. Therefore, the release of a minuscule amount of material in the hydrogels formed using PEM-coated microspheres is likely due to

defects in a small fraction of the microcapsules (*e.g.*, incomplete film coverage, tearing of the microcapsule wall, *etc.*), and the release behavior of these MPA hydrogels is influenced primarily by the bulk alginate matrix.

Catalytic function of encapsulated enzyme

The preservation of an encapsulated enzyme's activity is paramount to the utility of the system in catalytic and/or biosensing functions. Therefore, hydrogels prepared with PEM-coated CaCO_3 microspheres loaded with GOx were cast into wells of a microplate and assayed for relative enzymatic activity to determine whether catalytic function is preserved during fabrication of an MPA hydrogel. As depicted in Fig. 5, a substrate cocktail (*i.e.*, glucose, peroxidase, and the chromophore *o*-dianisidine) was added on top of the GOx-containing MPA hydrogel, resulting in an observable absorbance change within the hydrogel and in the surrounding media. This absorbance change results from coupled enzymatic reactions catalyzed by GOx confined within the microcavities of the hydrogels, as well as peroxidase within the media. It is important to notice that the rate of absorbance increase represents the *apparent* activity, and is affected by the concentration and reaction rate of both enzymes, as well as diffusional limitations of the various substrates imposed by the alginate matrix and the PEMs. These data confirm that the encapsulated GOx retains enzymatic activity throughout the entire MPA hydrogel formation process (*i.e.*, from entrapment within the CaCO_3 microspheres to dissolution of the template by GDL). Moreover, it demonstrates that small molecules can quickly permeate the alginate matrix and diffuse into and out of the micropores, while confining the larger, enzymatically-active material within the micropores.

Conclusions

This study demonstrates the successful manufacture and characterization of MPA hydrogels containing a variety of encapsulates. Electron microscopy and confocal images confirm that alginate hydrogels can be created with uncoated or PEM-coated CaCO_3 microspheres; however, it is only when PEM-coated microspheres are used that stable microcavities are formed. In this case, material entrapped within the CaCO_3 microspheres is confined to the microcavities formed by GDL-induced microsphere dissolution. Though the PEM lining of these microcavities prevents the escape of macromolecules and nanoparticles, it still allows for the diffusion of small molecules such as glucose and H_2O_2 into and out of the microcavity. Moreover, encapsulated materials are free to move within the microcavities. In this way, functional materials can be confined to the interior cavities of the MPA hydrogel, while retaining the desirable characteristics of the bulk alginate material. Alternatively, the PEMs lining the microcavities of the MPA hydrogels could be designed to allow for controlled or stimulated release of encapsulated materials.^[49, 50] As we have shown with a limited set of model materials, a variety of functional materials may be encapsulated within hydrogels of this type, including sensing elements, bioactive agents, and various types of nanoparticles. Furthermore, this approach could be used to encapsulate *multiple* types of materials within the same hydrogel by simply using a mixture of different PEM-coated CaCO_3 microspheres containing the desired materials. This could be applied to create, for example, multianalyte sensors or combination systems including both sensing and drug delivery components. Ultimately, the flexibility to choose the encapsulated materials and tune the PEMs, combined with the gentle gelation mechanism and moldable nature of the hydrogel, endow this technology with the potential to be useful in a wide variety of applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BSA	Bovine serum albumin
GDL	glucono- γ -lactone
GOx	glucose oxidase
LbL	layer-by-layer
MPA	microporated alginate
PAH	poly(allylamine hydrochloride)
PEMs	polyelectrolyte multilayers
PSS	poly(styrene sulfonate)
RITC	Rhodamine B isothiocyanate

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Fig. 1.

Cartoon illustrating the synthesis of the loaded PEM-coated CaCO_3 microspheres and the creation of microcavities within the alginate hydrogel upon gelation. Material is co-precipitated with CaCO_3 to form loaded microspheres, which are coated with PEMs using LbL deposition (left). Loaded PEM-coated microspheres are mixed with alginate and exposed to GDL, leading to the dissolution of the CaCO_3 , release of calcium into the matrix, and the internal ionotropic gelation of the surrounding alginate (right).

Fig. 2.

Electron (a, c) and fluorescence (b, d) micrographs of coated CaCO_3 microspheres containing RITC-BSA exposed to deionized water only (a, b) or GDL (c, d). Electron micrographs of uncoated CaCO_3 microspheres were indistinguishable from coated CaCO_3 microspheres exposed to deionized water only, and resulted in the absence of microspheres when exposed to GDL.

Fig. 3. Electron (a–c, e–g) and confocal (d, h) micrographs of alginate hydrogels formed by internal ionotropic gelation with CaCO_3 microspheres. Hydrogels made with uncoated CaCO_3 containing RITC-BSA (a–d). Hydrogels made with PEM-coated CaCO_3 loaded with RITC-BSA (e–h).

Fig. 4.

Release of RITC-BSA from alginate hydrogels fabricated with uncoated CaCO_3 microspheres (circles) and PEM-coated CaCO_3 microspheres (squares). Values are normalized to the maximum fluorescence intensity value in the uncoated sample (a). Values are normalized to the maximum fluorescence intensity value for each sample type to emphasize the shape of the release curve (b). Error bars indicate 95% confidence intervals (uncoated $n = 7$; coated $n = 4$).

Fig. 5. Change in indicator absorbance due to the enzymatic activity of GOx within the MPA hydrogels. The solid line represents the baseline-subtracted average absorbance change of the system as a function of time for MPA hydrogels, while the lighter shaded area represents the associated 95% confidence interval ($n = 5$).