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Combined Physical and Chemical Immobilization of Glucose Oxidase in Alginate Microspheres Improves Stability of Encapsulation and Activity

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Abstract

Chemical sensors utilizing immobilized enzymes and proteins are important for monitoring chemical processes and biological systems. In this study, calcium-cross-linked alginate hydrogel microspheres were fabricated as enzyme carriers by an emulsification technique. Glucose oxidase (GOx) was encapsulated in alginate microspheres using three different methods: physical entrapment (emulsion), chemical conjugation (conjugation), and a combination of physical entrapment and chemical conjugation (emulsion–conjugation). Nano-organized coatings were applied on alginate/GOx microspheres using the layer-by-layer self-assembly technique in order to stabilize the hydrogel/enzyme system under biological environment. The encapsulation of GOx and formation of nanofilm coating on alginate microspheres were verified with FTIR spectral analysis, ζ -potential analysis, and confocal laser scanning microscopy. To compare both the immobilization properties of enzyme encapsulation techniques and the influence of nanofilms with uncoated microspheres, the relationship between enzyme loading, release, and effective GOx activity (enzyme activity per unit protein loading) were studied over a period of four weeks. The results produced four key findings: (1) the emulsion–conjugation technique improved the stability of GOx in alginate microspheres compared to the emulsion technique, reducing the GOx leaching from microsphere from 50% to 17%; (2) the polyelectrolyte nanofilm coatings increased the GOx stability over time, but also reduced the effective GOx activity; (3) the effective GOx activity for the emulsion–conjugation technique (about $3.5 \times 10^{-5} \text{ AU } \mu\text{g}^{-1} \text{ s}^{-1}$) was higher than that for other methods, and did not change significantly over four weeks; and (4) the GOx concentration, when compared after one week for microspheres with three bilayers of poly- (allylamine hydrochloride)/sodium poly(styrene sulfonate) ({PAH/PSS}) coating, was highest for the emulsion–conjugation technique. As a result, the comparison of these three techniques showed the emulsion–conjugation technique to be a potentially effective and practical way to fabricate alginate/GOx microspheres for implantable glucose biosensor application.

INTRODUCTION

Chemical sensors utilizing immobilized enzymes and proteins are important for monitoring of chemical processes and biological systems. They have been widely used for the processing of a variety of products from food to environmental control, and even for biomedical applications (1–3). The main goal behind enzyme immobilization is to entrap the protein in a semipermeable support material, which prevents the enzyme from leaching while allowing its substrate(s) to pass at an appropriate rate (4). Some essential features of the immobilization matrix are nondegradability and compatibility with the enzymes. Because of the three-dimensional structure of enzymes that determines their specific function, it is critical that the encapsulation matrix and procedures have minimal effect upon the structure and function of the proteins.

In search of suitable matrices for enzyme immobilization, polysaccharides such as alginate and chitosan (5–8) have been found to be among the top candidates due to their biocompatibility and processability. Ionically cross-linked alginate encapsulation has been reported as suitable for many biological components including cells (9–18), owing to the relatively inert aqueous environment within the matrix, the mild room temperature encapsulation process, and high gel porosity allowing high diffusion rates of macromolecules (19, 20). Chemical immobilization, in which the enzyme is covalently linked to the hydrogel matrix, has been employed to stably entrap the enzyme, but this approach often results in significant loss of enzymatic activity (21). In contrast, simple one-step coating procedures have been applied to decrease enzyme leaching (22–25).

The layer-by-layer (LbL) self-assembly technique enables polyelectrolytes of opposite charge to be deposited onto charged templates, forming a stable multilayer with each component layer being 1–10 nm thick (26, 27). These nanofilm coatings applied to negatively charged alginate matrix provide a protective shell for encapsulated biomolecules and prolong the life span of alginate microspheres in a biological environment. Multilayer coatings involving chitosan/alginate, poly(lysine)/alginate, and other polyelectrolyte coatings have been used as barrier membranes for alginate microspheres to slow release of the encapsulated macromolecule (28–33). Recent work in our lab has shown that the application of self-assembled ultrathin film coatings can reduce leaching of macromolecules from alginate microspheres (34, 35). Diffusion of the enzyme from the gel matrix depends upon porosity of the gel and concentration gradients, as well as the permeability of the thin-film coatings to the enzyme. The nanofilm coatings also slow down the decomposition of alginate microspheres in PBS buffer or biological environments relative to bare alginate microspheres without coatings, which make the structures useful for long-term biomedical applications. Coimmobilization of glucose oxidase and an oxygen-quenched fluorescent compound (such as ruthenium or porphyrin compound) within alginate hydrogel microspheres coated with specialized biocompatible ultrathin films has been proposed as an approach to realize optical glucose microsensor systems (3). The nanofilms provide stabilization of the hydrogel spheres in addition to a transport barrier. These ratiometric sensors respond to glucose transients via local oxygen changes, which can be transduced by the fluorescence intensity of the oxygen quenched fluorescent compound.

The work described here is a comparison of glucose oxidase encapsulation in alginate microspheres using three different encapsulation techniques, with the integration of the LbL self-assembly nanofilm coating to stabilize alginate/enzyme microspheres, with an effort to maximize the enzyme loading in alginate hydrogel microspheres while retaining its catalytic activity. In this work, alginate microspheres with a diameter of $<10\ \mu\text{m}$ were used, due to their intended application as implantable glucose sensors for diabetic monitoring (3). To compare the immobilization properties of enzyme encapsulation techniques and the influence of nanofilm thickness for coated and uncoated microspheres, the relationship between enzyme loading, release, and effective activity was studied over a period of four weeks. These fundamental results should provide useful information for further investigations, including testing of enzymatic sensors in *in vitro* and *in vivo* studies, toward practical implantable biosensors.

EXPERIMENTAL PROCEDURES

Materials

Sodium alginate (low viscosity, 250 cps, MW 12–80k) and anhydrous calcium chloride were obtained from Aldrich. Sorbitan trioleate (SPAN 85) and polyoxyethylene sorbitan trioleate (TWEEN 85) were purchased from Sigma. OmniSolv 2,2,4-trimethylpentane (isooctane) was purchased from EMD Chemicals Inc. 1,6-Diaminohexane, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), and *N*-hydroxysulfosuccinimide sodium salt (NHSS) were obtained from Fluka. Sodium poly(styrene sulfonate) (PSS, MW ~70 000), poly-(allylamine hydrochloride) (PAH, MW ~70 000) were obtained from Aldrich. Glucose oxidase (GOx, from *Aspergillus niger* type VII, lyophilized powder, 198 000 units/g solid (without added oxygen)) was obtained from Sigma. Peroxidase (Sigma), *o*-dianisidine (Aldrich), and β -D-glucose (Sigma) were used for the GOx activity test. Fluorescein isothiocyanate (FITC, isomer I) and rhodamine B isothiocyanate (RITC) were obtained from Sigma. For confocal fluorescence microscopy, PAH was labeled with FITC in pH 8.5 sodium bicarbonate buffer and precipitated in acetone. GOx was labeled with RITC or FITC in pH 8.5 sodium bicarbonate buffer and purified through a Sephadex G-25M column (Amersham Pharmacia Biotech AB). All chemicals were reagent grade and used as received.

Synthesis of FITC-Alginate Derivative

Sodium alginate (120 mg) was mixed with EDC/NHSS (50 mg/30 mg) for the activation of carbonyl groups on alginate in pH 5.0 sodium acetic buffer for 30 min, followed by addition of 1,6-diaminohexane (60 mg) for another 4 h. The mixture was precipitated in 2-propanol to remove unreacted diamine. The alginate-amine derivative was reacted with FITC (0.5 mg) in pH 8.5 sodium bicarbonate solution for 4 h and precipitated in acetone to remove unreacted FITC. The resulting FITC-alginate derivative was dissolved in DI water, and absorbance was measured with a UV-vis spectrometer (Lambda 45, PerkinElmer), and then the labeled alginate was mixed with 50 g of 3 wt % unlabeled sodium alginate solution and made into microspheres using the following process.

Fabrication of Alginate Hydrogel Microspheres

Alginate hydrogel microspheres were prepared by an emulsification method (36–38). Briefly, 50 g of 3 wt % sodium alginate aqueous solution was dispersed in 75 g of isooctane containing 1.7 g of SPAN 85 using an ultrasonicator (Cole Palmer, CPX-750) at 60% power for 5 min. A solution containing 0.9 g of TWEEN 85 in 5 g of isooctane was then added to the emulsion and ultrasonicated at the same power for another 5 min to achieve stable water/oil emulsion droplets. After this, 20 mL of aqueous solution containing 10 wt % of calcium chloride was added to allow formation of ionic cross-links while stirring for 20 min. The microspheres were then rinsed three times with DI water by successive centrifugation cycles and stored in DI water at room temperature. FITC-alginate derivative was added into alginate solution to facilitate the confocal microscopy (Leica TCS SP2) investigation. The number and size distribution of alginate microspheres were measured with a Coulter counter (Beckman Coulter Z Series).

Conjugation of GOx to Alginate Microspheres

GOx was first mixed with alginate solution followed by alginate cross-linking by calcium ions, as described above, in which case the GOx was physically entrapped in alginate microspheres. This GOx encapsulation method is named the “emulsion technique” in this study and is a commonly used technique for materials encapsulation in alginate hydrogel microspheres. However, there is severe limitation for this technique, as the entrapped molecules can easily leach out from the hydrogel microspheres after fabrication, causing substantial loss of the enzyme from the matrix.

Two conjugation methods were used to improve the stability of GOx in alginate microspheres. For the first method, alginate microspheres (200 μ L, without enzyme) were incubated in 1 mL of pH 5.0 buffer for 30 min, and then 2.0 mg of EDC and 1.2 mg of NHSS were added to activate the alginate microspheres for another 30 min. The activated alginate microspheres were centrifuged and rinsed with DI water to remove unreacted EDC and NHSS. Next, 1 mL of GOx solution (5 mg/mL in DI water) was added to react with alginate microspheres for another 4 h. The microspheres were centrifuged and triple rinsed with DI water to remove unreacted GOx, followed by slowly adding 200 μ L of 1 M CaCl_2 to strengthen the alginate hydrogel microspheres and avoid de-cross-linking during the following LbL assembly procedure. In this case, GOx will only react with activated alginate. This GOx encapsulation method is named the “conjugation technique” in this study. For the second method, alginate microspheres already containing GOx (from the emulsion step) were also reacted with EDC/NHSS in pH 5.0 buffer for 4 h, which is named the “emulsion–conjugation” technique. In this case, GOx should react with not only alginate but also the enzyme itself, since enzyme contains both amine groups and carboxylic acid groups. Fourier transform infrared spectroscopy (Nexus 470 FTIR) was used to analyze the unmodified alginate microspheres and alginate/GOx microspheres. Confocal micrographs were taken with a Leica TCS SP2 equipped with a 63 $\times$ oil-immersion objective, using the 488/514 nm lines of an Ar/Kr laser for FITC/RITC excitation, respectively.

Layer-by-Layer Assembly of PAH/PSS Nanofilm Coating on Alginate Microspheres

The PSS and PAH solutions used for alternating adsorption of {PAH/PSS} multilayers on alginate (or alginate/GOx) microspheres were prepared at a concentration of 2 mg/mL in DI water containing 0.1 M CaCl₂. For deposition of each coating, 1 mL of polyelectrolyte solution was added to a microcentrifuge tube containing 100 μ L of microsphere suspension. Adsorption was allowed to proceed for 20 min, after which the suspension was centrifuged at 3000 rpm to separate the spheres from remaining unadsorbed polyelectrolyte. The microspheres were then triple-rinsed with DI water by successive centrifugation cycles. The process was repeated for the oppositely charged polyelectrolyte, and then alternated until a total of three bilayers of {PAH/PSS} films were obtained. The assembly of polyelectrolyte layers on alginate microspheres was monitored by electrophoretic mobility measurements (ZetaPlus ζ -potential analyzer, Brookhaven Instrument Corp.).

Enzyme Stability in Alginate/GOx Microspheres

For wet storage loss experiments, alginate/FITC-GOx microspheres prepared by the emulsion, conjugation, and emulsion–conjugation techniques, with zero (uncoated alginate) and three bilayers of {PAH/PSS} coatings, were suspended in individual cuvettes containing 1.5 mL of 0.01 M PBS buffer at pH 7.4, which were stored at room temperature, covered and in the dark. Leaching analysis was performed by removing the supernatant by centrifugation and recording the fluorescence from the supernatant with a scanning spectrometer (Photon Technologies Inc, QM-1), allowing determination of the relative supernatant enzyme concentration by comparison to a standard FITC-dextran emission (7 μ M in 2 mL of PBS buffer) at each point of time. This supernatant was then placed back with the sphere suspension. The relative fluorescence resulting from leaching through different coatings was then used to compare final total enzyme lost from the microspheres with each coating.

Enzyme Activity in Alginate/GOx Microspheres

Glucose oxidase activity was monitored through a colorimetric assay, based on the oxidation of *o*-dianisidine through a peroxidase-coupled system.

The GOx activity assay comprised 2.4 mL of *o*-dianisidine solution (0.21 mM in 0.01 M PBS buffer pH 7.4), 0.5 mL of β -D(+)-glucose solution (100 mg/mL in 0.01 M PBS buffer), and 0.1 mL of peroxidase solution (60 purpurogallin units/mL in DI water). During continuous stirring with a magnetic bar at 25 °C (Peltier temperature controlled cell holder, Quantum Northwest), a 100 μ L aliquot of GOx-loaded alginate microspheres suspended in DI water (approximately 10⁶ spheres) was added to the assay and the absorbance at 500 nm was monitored as a function of time, resulting in a catalytic profile of the encapsulated GOx.

The experiment was repeated for the different alginate/GOx microspheres (different preparation techniques, bare and three bilayer coating spheres) after a period of 0, 1, and 4 weeks, and the results were compared by calculating the slopes of the activity curves. In each case, the experiments were performed in triplicate, and results were normalized to the mass of glucose oxidase present in the spheres, as determined using the Lowry assay. The effective GOx activity ($\text{AU } \mu\text{g}^{-1} \text{ s}^{-1}$, enzyme activity per unit protein loading) is expressed as total GOx activity (absorbance units/second) divided by the mass of GOx (micrograms) encapsulated in alginate microspheres.

RESULTS AND DISCUSSION

Synthesis of FITC-Alginate Derivative

Figure 1 contains the UV–vis absorbance spectrum of unmodified alginate and FITC-labeled alginate. The unmodified alginate material does not display any characteristic absorbance between 300 and 700 nm. However, the FITC-alginate derivative possessed strong absorbance at 488 nm, which corresponds to the absorbance of FITC and verifies the successful synthesis of the FITC-alginate derivative. For quantitative analysis of FITC-alginate derivative, a series of solutions of known concentration of FITC were measured with UV–vis spectroscopy, which gives the relationship between FITC absorbance and concentration. The FITC content was calculated to be 1.7 wt % in FITC-alginate derivative. The FITC-labeled alginate was therefore mixed with alginate and made into microspheres for confocal imaging.

Characterization of Alginate Microspheres

The emulsion technique used for fabrication of alginate hydrogel microspheres is illustrated in Figure 2a. Calcium ions were used to cross-link the alginate microspheres while in the emulsion. By adjusting the ultrasonicator power, ratio of surfactant, or concentration of alginate solution, different sizes (1–200 μm) of alginate microspheres can be obtained. Figure 2b is an image of typical alginate microspheres obtained by confocal microscopy, with FITC-labeled alginate showing green fluorescence. Coulter counter measurements revealed that the process resulted in a typical alginate microsphere mean diameter of $4.25 \pm 0.49 \mu\text{m}$ ($N > 5000$ spheres counted). The microspheres were found to be stable in DI water after removal of the surfactant, and the microspheres exhibited the expected negative surface charge, which therefore could be used for polyelectrolyte LbL assembly to provide protective shells. It was also found, as illustrated in Figure 2c, that the FITC-labeled GOx was uniformly distributed in the alginate hydrogel microspheres made by the emulsion technique.

Conjugation of Enzyme to Alginate Microspheres

Alginates are linear chains of 1,4'-linked residues of β -D-mannuronic acid (β -DMA) and α -L-guluronic acid (α -LGA). A key property of sodium alginate is the formation of ionically cross-linked gel in the presence of multivalent cations. Thus, when sodium alginate is exposed to CaCl_2 solution, the carboxylic acid groups on polyguluronate units from different alginate molecules form a cross-linked structure with calcium ions called an “egg-box” junction with interstices in which the cations may pack and be coordinated. Because

the carboxylic acid groups will not be depleted during the ionic-cross-linking procedure, the residual carboxylates in alginate microspheres could be activated by EDC/NHSS and bind to amine-containing molecules such as enzymes. Under biological conditions, the calcium-cross-linked structure may be replaced and de-cross-linked slowly by monovalent cations such as sodium ions and therefore are not stable for long-term application (39); however, polyelectrolyte coatings can provide protective shells and improve the stability of the alginate microspheres to some extent. A schematic diagram of enzyme conjugated to alginate microspheres and subsequent coating with polyelectrolytes is shown in Scheme 1.

Since alginate and GOx ($pI = 4$) exhibit the same negative charge around neutral pH, it is difficult for GOx to be stable within alginate microspheres when only physical entanglement between alginate and GOx exists. Similarly, it is also difficult for free GOx to diffuse into alginate microspheres and interact with alginate. To improve the stability of GOx in alginate microspheres for long-term applications, two different conjugation techniques were applied, which are called the “conjugation technique” and the “emulsion–conjugation technique”. To verify conjugation-made alginate/GOx microspheres, alginate microspheres were mixed with GOx solution for 4 h, without adding EDC/NHSS. Both treated alginate microspheres and bare microspheres were triple rinsed with DI water before FT-IR measurement (Figure 3). The spectrum of alginate microspheres mixed with GOx solution was exactly the same as that of alginate microspheres, with strong absorbance at 1600 cm^{-1} contributed by carbonyl groups. This result shows that GOx cannot become entrapped in alginate microspheres by simply mixing GOx with the spheres after fabrication. In contrast, the spectrum for conjugation-made alginate/GOx microspheres possesses two shoulders beside the 1600 cm^{-1} carbonyl peak, related to amide I (1750 cm^{-1}) and amide II (1550 cm^{-1}) bonds. The amide signal may be contributed by amide bonds on the enzyme or amide bonds formed between carboxylic acid groups on alginate and amine groups on enzyme; in either case, this result demonstrates that the GOx could be chemically conjugated to alginate microspheres by using EDC/NHSS activation process.

The LbL assembly of polyelectrolyte layers on alginate (or alginate/GOx) microspheres was monitored by electrophoretic mobility measurements. Three bilayers of {PAH/PSS} were deposited on alginate microspheres and GOx-encapsulated alginate microspheres. It was found that there was no significant difference between alginate cores and alginate cores with encapsulated GOx. The surface potential of the microspheres was observed to change regularly from -25 mV for alginate (or alginate/GOx) and PSS to $+40\text{ mV}$ for PAH (Figure 4), indicating the formation of the desired {PAH/PSS}₃ wall architecture. This result shows that the alginate microspheres still have strong negative surface charge after calcium gelation, which makes polyelectrolyte nanofilm deposition onto alginate microspheres straightforward. The polyelectrolyte multilayer wall may serve multiple purposes, including providing a protective shell for encapsulated biomolecules and stabilizing alginate microspheres against dissolution in a biological environment, as well as a necessary barrier to control substrate mass transport in the development of enzymatic glucose sensors (3, 40).

Confocal microscopy was used to image conjugated alginate/GOx microspheres, and coating of {PAH/PSS} on microspheres (Figure 5), which clearly shows that the GOx was uniformly distributed in the alginate microspheres, no matter whether the conjugation

(Figure 5a) or the emulsion–conjugation (Figure 5b) technique was used. RITC-labeled GOx solution was also mixed with bare alginate microspheres, however they exhibit no RITC fluorescence under confocal microscopy after rinsing with DI water. Three bilayers of {FITC-PAH/PSS} were coated on emulsion–conjugation-made alginate/GOx microspheres, as illustrated in Figure 5c, which shows that the GOx was still uniformly distributed in alginate microspheres after coating with polyelectrolytes. It should be noted that the GOx cannot be conjugated into alginate microspheres once they have been coated with polyelectrolytes, although GOx is able to penetrate through the {PAH/PSS} multilayer wall (data not shown); this is because most of the GOx becomes entrapped in the polyelectrolyte wall, rather than diffusing into the sphere and reacting with alginate.

Enzyme Stability in Alginate/GOx Microspheres

Fluorescence spectroscopy was employed to investigate and compare the stability of GOx in alginate microspheres made by the emulsion, conjugation, and emulsion–conjugation techniques, with 0 and 3 bilayers of {PAH/PSS} nanofilm coating. The result of the leaching study (Figure 6) showed the percentage of GOx leached out from alginate microspheres over one week. It can be seen that the emulsion-made alginate/GOx microspheres leach approximately 50% of the standard concentration in 1 week. This may be attributed to relatively large pores in alginate hydrogel microspheres, which allow large and small molecules to pass through, and charge repulsion between alginate and GOx in pH 7.4 PBS buffer. As expected, three bilayers {PAH/PSS} of nanofilm decreased the GOx leaching substantially, from 50.0% to 17.8%.

Unexpectedly, the GOx in conjugation-made alginate/GOx microspheres was found to be unstable in PBS buffer (48.5% leaching), although GOx has been verified to chemically conjugate to alginate. The reason for this may be that the cross-linked alginate hydrogel was decomposed via ion-exchange since PBS buffer contains many monovalent cations (Na^+ , K^+) and polyvalent anions (PO_4^{3-}). The calcium ions on alginate hydrogel microspheres may be replaced by those monovalent cations and form insoluble calcium phosphate. Alginate–GOx conjugate, rather than GOx, was therefore leached from the alginate microspheres. For bare alginate microspheres without polyelectrolyte coating, the microspheres will be decomposed and eventually disappear over 2–3 months in PBS buffer. The polyelectrolyte nanofilm coatings were proved to prevent the alginate hydrogels from decomposing substantially, as seen from the leaching result of conjugation-made alginate/GOx microspheres with three bilayers of {PAH/PSS} (3.5% leaching).

The emulsion–conjugation technique also improved the stability of GOx in alginate microspheres. The leaching data for uncoated and three bilayers of {PAH/PSS} alginate microspheres indicated 16.9% and 17.6% loss, respectively. Compared with the conjugation technique, the emulsion–conjugation technique increased the GOx stability for uncoated microspheres, which is especially important since most of the enzyme leaching occurs in the first few hours after the fabrication of alginate microspheres, and substantial loss even occurs during the polyelectrolyte coating process.

Enzyme Activity in Alginate/GOx Microspheres

Since the primary function of enzymes is catalysis, it is ideal to preserve as much activity as possible when encapsulated in alginate microspheres. To determine the effect, if any, of different encapsulation techniques and nanofilm assembly on activity of encapsulated enzyme, the activity of the alginate/GOx microspheres was studied over four weeks of wet storage (0.01 M PBS buffer, room temperature). Figure 7 contains a summary of the experimental results to determine the effect of three different encapsulation techniques and nanofilms on the effective catalytic activity per unit mass of encapsulated GOx (determined by Lowry assay).

For the emulsion technique (Figure 7a), the results show that uncoated alginate microspheres exhibited an 80% reduction in initial activity after one week, with no further notable reduction in activity after four weeks. Coating of {PAH/PSS} nanofilm was observed to reduce the GOx activity from 3.5×10^{-5} to 2.7×10^{-5} AU $\mu\text{g}^{-1} \text{s}^{-1}$; however, reduction of enzyme activity over time for nanofilm coated microspheres was decreased substantially. As seen from Figure 7a, the GOx activity for uncoated microspheres at 4 weeks is about 0.75×10^{-5} AU $\mu\text{g}^{-1} \text{s}^{-1}$, while the GOx activity for the three bilayers of {PAH/PSS} coated microspheres at 4 weeks remains 2.0×10^{-5} AU $\mu\text{g}^{-1} \text{s}^{-1}$.

For the conjugation technique (Figure 7b), the enzyme activity test results show that increasing nanofilm thickness leads to increased enzyme activity after one week, with the enzyme activity also increasing over time. These unexpected results may arise from two possible explanations: The first is that the total enzyme activity before normalization (division by enzyme mass) is much lower (about 60 times lower than uncoated microspheres at 0 week) than the activity from the emulsion technique. According to our observations, low activity due to low enzyme concentration will still result in relatively high normalized enzyme activity, with larger standard deviation, using the peroxidase assay. A second reason for increasing enzyme activity may be the decomposition of alginate microspheres, in which case the GOx or alginate-GOx conjugates should regain activity as the steric barrier from the cross-linked hydrogel is reduced, providing greater access of substrate to enzyme. A combination of these reasons may cause the result shown in Figure 7b, which needs further studies in the future.

For the emulsion–conjugation technique (Figure 7c), increasing the nanofilm thickness generally decreased the enzyme activity. Furthermore, the enzyme activity also decreased over time. These results are similar with those of the emulsion technique. However, the overall effect on effective activity of nanofilm thickness and time is slight, relative to the emulsion technique. As a result, the enzyme activity of alginate/GOx with three bilayers of {PAH/PSS} made by the emulsion–conjugation technique is almost double that made by the emulsion technique.

All the above results showed the effective GOx activity (GOx activity per unit mass of encapsulated enzyme). It is also important to know the total GOx amount left in alginate microspheres, which is related with enzyme stability. The total enzyme activity could therefore be calculated from the effective GOx activity and amount in the alginate microsphere, which is responsible for the function of glucose biosensor. Table 1 presents the

enzyme concentration (determined by Lowry assay) in the alginate/GOx microspheres over 1 week. The GOx is found to be stable in conjugation-made alginate/GOx microspheres, although the enzyme concentration is very low; therefore, the conjugation technique may not be practically useful for large enzymes such as GOx to be encapsulated for biosensors. However, it should be useful to encapsulate small molecules with amine groups (such as peptides) in microspheres where other techniques are difficult to achieve and only a small amount of molecules is needed for encapsulation in this case. The GOx is also stable in the microspheres coated with three bilayers of nanofilm, however, most of the GOx leaching took place before the microspheres were coated with polyelectrolytes. The “emulsion–conjugation” technique greatly improved the GOx stability right after the alginate microsphere fabrication. High concentration of GOx and enzyme activity were retained after nanofilm assembly on microspheres. Among the three techniques investigated in our study, emulsion–conjugation was shown to be the most efficient and practical way for GOx encapsulation in alginate microspheres.

CONCLUSIONS

Glucose oxidase (GOx) was successfully encapsulated into calcium-cross-linked alginate hydrogel microspheres via three different techniques, the emulsion, conjugation, and emulsion–conjugation techniques, to be used as implantable glucose biosensors. FTIR, ζ -potential analyzer, and confocal microscopy were used to verify the encapsulation of GOx and formation of nanofilm coating on alginate microspheres. The stability and effective activity of GOx were also investigated to compare these three techniques, with and without polyelectrolyte multilayer nanofilms over 4 weeks. GOx concentration in alginate microspheres was also given to compare the total enzyme activity over time for these three techniques. Of these, the emulsion–conjugation technique was shown to be a practical and effective way to fabricate GOx-encapsulating alginate microspheres for future use as implantable glucose biosensors.

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Figure 1.
UV-vis absorbance spectrum of alginate and FITC-alginate derivative.

Figure 2.

(a) Emulsion technique for the production of alginate microspheres. (b) Confocal images of alginate microspheres, alginate labeled with FITC. (c) Confocal images of alginate microspheres with physically embedded GOx, where GOx was labeled with RITC.

Scheme 1.

Schematic Diagram of Enzyme Conjugated to Alginate Microspheres and Then Coated with Polyelectrolyte Multilayer Nanofilm

Figure 3.
FTIR spectrum of (a) alginate microspheres and (b) conjugation-made alginate/GOx microspheres.

Figure 4. ζ -Potential measurements for deposition of {PAH/PSS}₃ on (a) alginate microspheres and (b) alginate/GOx microspheres. The first measurement (layer 0) is the surface potential of uncoated alginate microspheres.

Figure 5.

Confocal images of alginate/GOx microspheres. (a) Conjugation-made alginate/GOx microspheres, GOx labeled with RITC. (b) Emulsion-conjugation-made alginate/GOx microspheres, GOx labeled with FITC. (c) Emulsion-conjugation-made alginate/GOx microspheres with 3 bilayers of {PAH/PSS}, GOx labeled with FITC and PAH labeled with RITC.

Figure 6.
GOx stability in alginate-templated microspheres one week after fabrication.

Figure 7. Enzyme activity of alginate/GOx microspheres over time. (a) Emulsion technique. (b) Conjugation technique. (c) Emulsion-conjugation technique.

Table 1

Enzyme Concentration in Alginate/GOx Microspheres

	<u>uncoated spheres (mM)</u>		<u>{PAH/PSS}₃-coated spheres (mM)</u>	
	0 week	1 week	0 week	1 week
emulsion	66.7	33.4	61.5	50.4
conjugation	3.38	1.69	0.45	0.43
emulsion–conjugation	86.0	68.8	70.1	58.2