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Stabilization of Glucose Oxidase in Alginate Microspheres With Photoreactive Diazo-resin Nanofilm Coatings

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Abstract

The nanoassembly and photo-crosslinking of diazo-resin (DAR) coatings on small alginate microspheres for stable enzyme entrapment is described. Multilayer nanofilms of DAR with poly(styrene sulfonate) (PSS) were used in an effort to stabilize the encapsulation of glucose oxidase enzyme for biosensor applications. The activity and physical encapsulation of the trapped enzyme were measured over 24 weeks to compare the effectiveness of nanofilm coatings and crosslinking for stabilization. Uncoated spheres exhibited rapid loss of activity, retaining only 20% of initial activity after one week, and a dramatic reduction in effective activity over 24 weeks, whereas the uncrosslinked and crosslinked {DAR/PSS}-coated spheres retained more than 50% of their initial activity after 4 weeks, which remained stable even after 24 weeks for the two and three bilayer films. Nanofilms comprising more polyelectrolyte layers maintained higher overall activity compared to films of the same composition but fewer layers, and crosslinking the films increased retention of activity over uncrosslinked films after 24 weeks. These findings demonstrate that enzyme immobilization and stabilization can be achieved by using simple modifications to the layer-by-layer self-assembly technique.

Keywords

alginate; emulsification; glucose-oxidase; diazo-resin; biosensors

INTRODUCTION

Immobilized enzymes and proteins have been widely used for the processing of a variety of products from food to environmental control, and even for biomedical applications (Brown et al., 2004; Liang et al., 2000; McShane, 2002). The main principle behind enzyme immobilization is to entrap the protein in a semipermeable support material. This prevents the enzyme from leaching, while allowing its substrates to pass at an appropriate rate (Amiji and Taqieddin, 2004). 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 has minimal effect upon the structure and function of the proteins.

In search of suitable matrices for enzyme immobilization, ionically crosslinked hydrogels, such as alginate and chitosan (Gregor et al., 1996; Heng and Chan, 2002; Heng et al., 2002a,b), have been thoroughly investigated. Alginate encapsulation has been reported as suitable for many biological components including cells (Blandino et al., 2000, 2001; Bregni et al., 2000; DeGroot and Neufeld, 2001; Goosen et al., 1999; Hussain et al., 1985, Kierstan and Bucke, 1977; Lanza and Chick, 1997; Neufeld and Quong, 1998; Skjak-Braek and Smidsrod, 1990), 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 (Okano et al., 1997; Skjak-Braek et al., 2002). 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 loss of significant enzymatic activity (Ghanem and Ghaly, 2004). In contrast, simple one-step coating procedures have been applied to decrease enzyme leaching (Amsden and Turner, 1999; Liu et al., 1997; Skjak-Braek et al., 1996, 1999).

The layer-by-layer (LbL) self-assembly technique enables polyelectrolytes of opposite charge to be deposited onto charged templates, forming stable multilayers with each component layer being 1–10 nm thick (Decher et al., 1992; Mohwald et al., 1998). These nanofilm coatings applied to an alginate matrix help to physically immobilize the enzyme within the matrix. Recent work in our lab has shown that the application of self-assembled ultrathin film coatings can reduce leaching of macromolecules from alginate microspheres. Diffusion of 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. In an effort to identify thin-film coating with increased resistance to enzyme leaching, photo-crosslinkable coatings were investigated.

It has been shown that polyelectrolyte complexes formed using diazoresins as the cationic polyelectrolyte can be made to convert their linkage from a weak ionic interaction to a covalent bond upon irradiation with ultraviolet (UV) light (Cao et al., 2002a). This can be accomplished with a variety of materials alternated with the diazoresins, including those containing sulfonate groups, carboxylic acid groups, or phenol groups (Cao et al., 2002b; Caruso et al., 2001; Shen et al., 2001; Zhang et al., 2003). Crosslinked diazo-resin films have been shown to be more stable at higher pH values, to exhibit increased resistance to solvent etching (Bruening et al., 1999, 2001), and to result in denser, more rigid films (Picart et al., 2004).

The work described here is the integration of the LbL self-assembly technique with the immobilization of macromolecules in alginate microspheres, specifically using photo-crosslinkable materials for coatings. In this work, alginate microspheres of diameter <10 μm were used, due to their intended application as implantable glucose sensors for diabetic monitoring (Brown et al., 2004; McShane, 2002). To compare the immobilization properties of crosslinked and uncrosslinked coatings of different thickness with uncoated spheres, the relationship between enzyme loading, release, and effective activity was studied over a

period of 6 months. The simple modifications to the layer-by-layer self-assembly technique described here may enable development of approaches to entrap and stabilize biomolecules for a wide range of applications.

MATERIALS AND METHODS

Diazo-resin (Diazo-10, 4-diazodiphenylamine/formaldehyde condensate hydrogen sulfate-zinc salt) was purchased from PC Associates (Livingston, NJ). Sodium alginate (low viscosity; 250 cps, 3%), glucose oxidase from *Aspergillus niger* (Catalog No. G2133, Type VII lyophilized powder 100,000–200,000 units/g solid), lipophilic surfactant, sorbitan trioleate (SPAN 85), and hydrophilic surfactant, polyoxyethylene sorbitan trioleate (TWEEN 85), the microsphere gelling agent, calcium chloride (96% purity), sodium poly (styrene sulfonate) (PSS, MW 1000,000), peroxidase, *o*-dianisidine, β -D(+)-glucose, and phosphate buffered saline (PBS) were purchased from Sigma (St. Louis, MO). The Lowry reagent comprised sodium hydroxide, copper sulfate, sodium potassium tartarate, sodium carbonate, and the Folin-Ciocalteu reagent, all of which were purchased from Sigma. OmniSolv[®] 2,2,4-trimethylpentane (iso-octane) was purchased from EMD Chemicals Inc. (Gibbstown, NJ). Fluorescein isothiocyanate (FITC, MW 389.4) and FITC-dextran (MW 2 MDa) were purchased from Sigma. For confocal fluorescence microscopy and fluorescence spectroscopy, glucose oxidase was covalently attached with FITC using an amine labeling procedure (Haugland, 2003) in pH 9.0 sodium bicarbonate buffer and purified through a Sephadex G-25M column (Amersham Pharmacia Biotech AB, Piscataway, NJ). All chemicals were reagent grade and used as received.

An ultrasonicator (CPX-750, 750W total power; Cole-Palmer, Vernon Hills, IL) was used to disperse water droplets in the oil. Separation of the microspheres from the emulsion was achieved by centrifugation (Eppendorf 5804R). A 100 W long wave UV lamp (Blak-ray Model B 100P; Entela, Grand Rapids, MI) was used to irradiate the microspheres to crosslink the {DAR/PSS} nanofilms. A ζ -potential analyzer (ZetaPlus; Brookhaven Instruments Corp., Holtsville, NY) was used to measure the surface charge of the microspheres. A laser scanning confocal microscope (TCS SP2; Leica Microsystems, Mannheim, Germany) was used to image the microspheres. Sizes and particle counts of alginate microspheres were obtained with a Beckman Coulter counter (Z2; Beckman Instruments, Fullerton, CA/Palo Alto, CA) using a 100 μ m aperture. A fluorescence spectrometer (QM-4; Photon Technology International, Inc., Lawrenceville, NJ) was used to measure the fluorescence intensities of suspensions of microspheres and their supernatants. Enzyme activity assays were performed at constant temperature using a Perkin-Elmer Lambda 45 UV-Vis spectrometer. A 96-well plate reader (Tecan Systems, Inc., San Jose, CA) was used to measure absorbance for the Lowry assay.

Preparation of Alginate Microspheres

Alginate gel microspheres were prepared by a method adapted from previous reports (Wan et al., 1992, 1993, 1994, 2003) for encapsulation of macromolecules as illustrated in Figure 1a. The preparation of the alginate microspheres using an ultrasonicator is to obtain smaller microspheres (typically <10 μ m). This is possible due to the formation of a water-in-oil type

emulsion, whose droplets are stabilized by the use of surfactants. This is in contrast to the preparation of alginate microspheres using an aqueous solution of alginate that slowly dropped into calcium chloride will yield large size microspheres (typically >200 μm). Briefly, 50 g of aqueous solution containing 3 wt% sodium alginate and 40 mg of GOx labeled to FITC were dispersed in 75 g of iso-octane containing 1.696 g of SPAN 85 using the ultrasonicator at 60% power for 10 min. A solution containing 0.904 g of TWEEN 85 in 5 g of iso-octane was then added to the emulsion and stirred at the same speed for 5 min to achieve stable water-in-oil emulsion droplets. After this, 20 g of aqueous solution containing 10 wt% of calcium chloride was added and allowed to react for 20 min while stirring at 250 rpm to allow ionotropic gelation of the particles. Before removal of the microspheres, the emulsion (180 mL) was diluted with 500 mL of distilled water. This dilution of the alginate emulsion outside the spheres reduces the possibility of spheres sticking together when they are in close contact; it also slows the gelation process. The spheres were left to settle from the solution using a separatory funnel, and they were then separated from solution (about 2 mL total volume of spheres) to be placed into microcentrifuge tubes. The microspheres were rinsed three times with DI water by successive centrifugation cycles, then small aliquots were pipetted onto microscope cover slides for imaging. The spheres with encapsulated macromolecules were used for all of the leaching and activity studies. After preparation, the microspheres were sized and counted using the Coulter counter (100 μm aperture), and the total amount of encapsulated enzyme per sample was determined using the Lowry assay. The alginate microspheres were completely dissolved in 0.025M EDTA solution before carrying out the Lowry assay. This ensures that the enzyme is free from the alginate microspheres and is available to bind to the Biuret reagent used in the Lowry assay. Also, a separate experiment was done with empty alginate microspheres, as well as alginate in solution, to assess the effect on Lowry assay. No difference in absorbance from the control (water) was observed for the different dilutions used.

Layer-by-Layer Self-Assembly of Ultrathin Films on Alginate Microspheres—

The diazoresin (DAR) was used as the cationic polyelectrolyte in the ultrathin film coatings, while PSS was the anionic polyion. For deposition of each coating, 1.5 mL of polyelectrolyte (2 mg/mL of DAR in 0.25M CaCl_2 or 2 mg/mL PSS in 0.25M CaCl_2 aqueous solution) was added to a microcentrifuge tube containing 200 μL of calcium alginate microsphere suspension (approximately 10^6 spheres). Adsorption was allowed to proceed for 20 min, after which time the suspension was centrifuged 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 alternated until three bilayer polyelectrolyte films were realized. The microsphere samples with one, two, and three bilayers of {DAR/PSS} were then irradiated under UV lamp for 5 min to crosslink the multilayer walls, followed by a final rinse in DI water.

Determination of Glucose Oxidase Leaching From Alginate Microspheres—

For wet storage loss experiments, microspheres with zero (uncoated alginate), one, two, and three bilayer uncrosslinked and crosslinked coatings were suspended in individual cuvettes containing 1.5 mL of 0.01M 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, allowing determination of the supernatant enzyme concentration by comparison to a standard FITC-dextran emission (7 μ M in 2 mL PBS) 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.

Activity of Glucose Oxidase in Alginate 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 0.01M PBS pH 7.4), 0.5 mL of β -D(+)-glucose solution (100 mg/mL in 0.01M PBS), and 0.1 mL of peroxidase solution (60 purpurogallin units/mL in DI water). During continuous stirring with a magnetic bar at 25°C, a 100 μ L aliquot of GOx-loaded alginate microspheres suspended in DI water (approximately 10^6 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 polyelectrolyte-coated microspheres after a period of one month and the results 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. Both the leaching and the activity results were analyzed using the two-tailed student's *t*-test.

RESULTS AND DISCUSSION

A confocal micrograph of microspheres with encapsulated FITC-GOx formed with the emulsification technique is presented in Figure 1b. The spheres appeared bright green, with uniform intensity distribution. The average size from three measurements was found to be $3.2 \pm 0.8 \mu$ m for a sample size of ≈ 12000 spheres. Following fabrication and size characterization of the alginate microspheres, deposition of multilayer nanofilms on the microspheres was performed. The surface charge of the polyelectrolyte-coated microspheres was measured using the ζ -potential analyzer, which verified reversal of the surface charge of the microspheres at each step due to polyelectrolyte adsorption as shown in Figure 2. The zeta potential values are averages of three readings. Thus, formation of DAR/PSS multilayers was verified.

UV-Vis absorbance spectroscopy was also used to observe the deposition of 1–3 bilayers of {DAR/PSS} on alginate microspheres. The absorbance at 380 nm attributed to the π - π^* transition of the diazonium group exhibited a linear increase with the number of {DAR/PSS} bilayers on alginate microspheres. The diazonium group is very active and may be decomposed easily under UV-irradiation or heat (Shen et al., 2000). Alginate microspheres coated with {DAR/PSS}₃ were irradiated with a UV lamp for different times to identify the conditions necessary for complete conversion. As shown in Figure 3a, the diazonium groups gradually decompose under UV irradiation, with a decrease in absorbance at 380 nm and the appearance of an isosbestic point at 340 nm, which is in accordance with results published previously (Cao et al., 2002a; 2002b). From this study, it was determined that irradiation for

30 s would be sufficient to completely crosslink the DAR/PSS in the nanofilms. The procedure for coating the microspheres with DAR/PSS and subsequently crosslinking them under UV irradiation is illustrated in Figure 3b.

Leaching of FITC-Glucose Oxidase From Alginate Microspheres

Loss of FITC-GOx from the alginate microspheres was studied using fluorescence spectroscopy, where release of the encapsulated FITC-GOx from the microspheres was observed as an increase in the fluorescence intensity of the supernatant. The leaching results for the alginate microspheres containing the enzyme were compared for the uncrosslinked {DAR/PSS} vs. the crosslinked {DAR/PSS} multilayers. Results from the experiment are given in Figure 4a, where the leached amount for FITC-GOx is plotted vs. number of coatings of {DAR/PSS}_n ($n = 0-3$; uncrosslinked case) and compared to the crosslinked {DAR/PSS}_n ($n = 1-3$) multilayers. The results show that bare microspheres leach approximately 50% of the standard concentration in 4 days. This suggests that bare alginate microspheres have relatively large pores, which allow large and small molecules to pass through. Because diffusion-driven leaching would result in equilibrium between the compartments, it is believed that eventually all of the encapsulated material is lost. Swelling of the alginate matrix could also be attributed for the fast release of the encapsulant. Release behavior of encapsulants from alginate beads have also been reported to be influenced by the ionic nature of the encapsulant, i.e., cationic, anionic, and non-ionic (You et al., 2001). Glucose oxidase being anionic at pH 7.4 will repel the negatively charged alginate matrix leading to faster release.

Conversely, the release profiles for microspheres coated with DAR/PSS multilayer nanofilms show that the application of these coatings does significantly reduce the loss of encapsulated material, which reduces to less than 20% of standard concentration over the same time span. However, there are negligible differences in the total leaching of the enzyme from alginate microspheres coated with more than one bilayer of polyelectrolytes. Crosslinking of the nanofilm coatings using UV irradiation also resulted in reduced leaching from alginate microspheres, although no significant differences were observed for each additional coating. When the leaching rate was compared for the different coatings, as shown in Figure 4b, a dramatic difference was seen for the comparison between the uncrosslinked films and the crosslinked films. The leaching rate for uncrosslinked films was observed to be higher than the leaching rate for the crosslinked films, with the results of the *t*-tests being significantly different between the uncrosslinked and crosslinked films in all cases. The same cannot be said for the comparison between bare and coated microspheres (uncrosslinked), in which case, the results were insignificant. These results suggest that the crosslinking of the coatings substantially reduces the pore sizes in the applied multilayers, as expected, enabling better retention of the enzyme. As a consequence of crosslinking, the rigidity and density of the films may be increased (Picart et al., 2004), which explains the reduced leaching rate through crosslinked multilayers. In addition, it has been shown that multilayers of crosslinked DAR/PSS result in a bilayer thickness of 14 nm (Zhu and McShane, 2005), which is substantially thicker than the bilayer thickness of 2–5nm reported for standard PAH/PSS films (Sukhorukov et al., 1998). In summary of these findings, the experimental results show that the application of {DAR/PSS} multilayer thin films to the

alginate microspheres was effective in reducing the loss of the encapsulated material from the microspheres. The leaching rate was also seen to decrease with nanofilm crosslinking.

Activity of Encapsulated Glucose Oxidase in Alginate Microspheres

To determine the effect, if any, of thin-film assembly on activity of encapsulated enzyme, the activity of the coated microspheres was studied over 6 months of wet storage (0.01M PBS, room temperature). Figure 5 summarizes the results of the experiments to determine the effect of uncrosslinked and crosslinked {DAR/PSS} films on the effective catalytic activity per unit mass of encapsulated GOx (determined for each sample using Lowry assay). The results show that uncoated alginate microspheres exhibited an 80% reduction in initial activity after one week, with no further reduction in activity after 4 weeks (Fig. 5a). However, it is also apparent from the figure that the effective activity of uncoated microspheres dropped dramatically after 24 weeks. Although the activity is normalized to unit mass, the observed differences are likely due to the higher initial activity of weakly bound or free GOx within the microspheres vs. the decreased effective activity of the remaining immobilized GOx in weeks one and four; the decreased effective activity after 24 weeks is due to the destruction of the spheres in the phosphate buffer storage solution, leaving only fragments of the spheres containing immobilized GOx. Thus, the effective activity of the microspheres can be viewed as a superposition of the effective activity of mobile encapsulated GOx and the effective activity of immobilized encapsulated GOx, and differences in overall effective catalytic activity can be attributed to varying contributions of both. For uncoated microspheres, the process can be described as such: Initially, the microspheres contain a large amount of mobile GOx as well as a smaller amount of GOx firmly immobilized to the alginate matrix, resulting in high effective activity. However, after one week, most of the mobile GOx has leached from the microspheres, leaving only the immobilized GOx, which is expected to have lower effective catalytic activity than free GOx. This immobilized GOx is remarkably stable, and there is no further loss of enzyme (and thus effective activity) after 4 weeks; however, after 24 weeks, the effective activity of the enzyme is reduced due to deactivation. Thus, the goal of multilayer nanofilm coatings is to retain as much as possible of the initially encapsulated mobile GOx within the microspheres, resulting in higher catalytic activity.

The utility of uncrosslinked and crosslinked DAR/PSS coatings to achieve this goal is shown in Figure 5b–d). The application of uncrosslinked {DAR/PSS}₁ nanofilms resulted in decreased loss of activity over one week, with only 53% loss in initial activity (Fig. 5d). Crosslinking of the {DAR/PSS}₁ nanofilms resulted in a significant increase in effective activity over uncrosslinked films ($P = 0.07$), with only 51% reduction in initial activity over one week for crosslinked {DAR/PSS}₁ nanofilms. Similar results were obtained for {DAR/PSS}_{2–3} nanofilms ($P = 0.01$ and 0.1 , respectively). After 4 weeks, there was no statistically significant difference between uncrosslinked and crosslinked films of 1, 2, or 3 bilayers. This suggests that, while the microspheres with uncrosslinked coatings experienced a faster loss of mobile GOx than those with crosslinked coatings, after 4 weeks the amount of mobile GOx in each is relatively the same.

Thus, for shorter-term applications, it seems that there is no practical improvement in effective catalytic activity gained by crosslinking the DAR/PSS films. However, these microspheres are intended for use as engines in implantable biosensors, and as such, will require much longer term application. Thus, the activity of the microspheres was assessed after 6 months of storage to determine whether there is any long-term effect of crosslinking the sphere coatings. These results are also shown in Figure 5b–d. While the {DAR/PSS}₁ films resulted in no significant difference between uncrosslinked and crosslinked films, both the {DAR/PSS}₂ and {DAR/PSS}₃ crosslinked films exhibited higher effective activity than their uncrosslinked counterparts ($P = 0.1$ and 0.01 , respectively). This suggests that after 6 months, the crosslinked films of 2–3 bilayer thickness are more effective at retaining effective catalytic activity than uncrosslinked films. Further observation of the figure reveals that regardless of crosslinking, the application of nanofilm coatings resulted in the prevention of the dramatic decrease in effective activity after 6 months seen for the uncoated microspheres, confirming that they act as structural supports to prevent the spheres from degradation. The better performance of the crosslinked films is due to their ability to retain more of the mobile GOx for longer, as evidenced by their slower leaching rates and higher effective activity.

In summary, these results suggest that {DAR/PSS}-crosslinked films are preferable for catalytic stability over uncrosslinked films over a one week period; after 4 weeks, there is no statistically significant difference between uncrosslinked and crosslinked films of any number of layers investigated. However, after 6 months, 2- and 3-bilayer crosslinked DAR/PSS films exhibit higher effective activity than uncrosslinked films. Furthermore, both uncrosslinked and crosslinked {DAR/PSS} nanofilms are superior to bare alginate alone, and thicker films result in more higher effective activity over 6 months regardless of whether or not the films are photo-crosslinked.

CONCLUSION

Alginate microspheres ($5\ \mu\text{m}$) were used to encapsulate glucose oxidase enzyme using emulsification, and the spheres were modified using {DAR/PSS} multilayers in an attempt to improve the stability of the microsphere reactors. The experimental results show that the application of {DAR/PSS} multilayer thin films was effective in reducing loss of the encapsulated enzyme compared to bare microspheres, as well as increasing retention of enzymatic activity. Crosslinking the {DAR/PSS} films did not generally affect the retention of catalytic activity after 4 weeks, most likely due to the fact that the crosslinked films are as permeable to enzyme co-substrates as uncrosslinked films, although less permeable to the enzyme itself as evidenced by the leaching studies. However, after 6 months, the utility of crosslinking the film is shown as evidenced by the higher effective activity. These results show that crosslinked {DAR/PSS} films may be used to decrease leaching of enzyme from alginate microspheres, and protect the spheres from degradation, without adversely influencing effective activity in longer term applications for enzyme based bioreactors and biosensors.

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Figure 1. (a) Emulsification technique for the production of alginate microspheres. (b) Confocal image of FITC-glucose oxidase loaded microspheres with a scale bar of 16 μm. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Figure 2. Zeta potential measurements on microspheres during DAR/PSS assembly depicting charge reversal at each step. (Error bars $\pm$ one standard deviation, $n = 3$). [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Figure 3.
 (a) UV-Vis absorption spectra of $\{DAR/PSS\}_3$ multilayer coated alginate microspheres after irradiation with UV light for 0 s, 1 s, 5 s, 10 s, and 20 s. (b) Schematic diagram of enzyme encapsulation in crosslinked DAR-PSS coated alginate microspheres. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Figure 4. (a) Comparison of coating on leaching of glucose oxidase from alginate microspheres. (b) Comparison of coating on leaching rate of glucose oxidase from alginate microspheres (Error bars $\pm$ one standard deviation, $n = 3$). [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Figure 5. Comparison of coating on activity per unit mass over time of glucose oxidase inside alginate microspheres. (a) Activity of bare microspheres over time, (b) activity of microspheres coated with 1 bilayer of uncrosslinked (DAR/PSS) and crosslinked (DAR/PSS CR) films, (c) activity of microspheres coated with 2 bilayers of uncrosslinked (DAR/PSS) and crosslinked (DAR/PSS CR) films, (d) activity of microspheres coated with 3 bilayers of uncrosslinked (DAR/PSS) and crosslinked (DAR/PSS CR) films. (Error bars $\pm$ one standard deviation, $n = 3$). [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]