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Dissolved Core Alginate Microspheres as "Smart-Tattoo" Glucose Sensors

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Abstract— **The feasibility of multilayer thin film coated dissolved-core alginate-templated microsphere sensors based on fluorescence resonance energy transfer and competitive binding, was explored in simulated interstitial fluid, using glucose as a model analyte. The glucose sensitivity was observed to be 0.89%/mM glucose with a linear response in the range of 0-50mM glucose. The sensor response was observed to be completely reversible in nature with a response time of 120 seconds. The system was further demonstrated to respond similarly using near-infrared dyes (Alexa Fluor-647-labeled dextran as donor and QSY-21-conjugated apo-GOx as acceptor) which exhibited a sensitivity of 0.94%/mM glucose with a linear response in range of 0-50mM glucose, making the sensor more amenable to *in vivo* use, when implanted in scattering tissue.**

I. INTRODUCTION

The current management of diabetes involves certain frequent glucose self monitoring technologies like finger-prick analysis of capillary blood samples, reagent strips tests, and glucometers. Diabetes, with its high prevalence and mortality rate, ranks among the world's deadliest diseases. Therefore, non-invasive or minimally invasive optical sensors also known as "smart tattoos" [1]-[14] intended for injection directly into the dermis [15]-[17] may prove to be an attractive alternative to established methods. Such implants may be interrogated non-invasively using simple optical instrumentation [4], [18] making their use all the more attractive.

The first affinity-based sensor proposed for monitoring glucose levels within the interstitial fluid was reported by Schultz *et al* [1], [2]. Since that pioneering effort, a number of advancements toward *in vivo* use have been reported, including the first examples of "smart tattoo"-type devices, which involve encapsulation of tetramethyl rhodamine isothiocyanate-Concavalin A (TRITC-Con A) and fluorescein isothiocyanate-dextran (FITC-dextran; FD) into poly(ethylene glycol) (PEG) hydrogel microspheres [3] and fuzzy microshells wherein the sensing assay was entrapped within self assembled multilayer films [19]. Follow-up work has extended the optical interactions into the near infrared by labeling Con A and dextran with Near infra-red (NIR) dyes [20]-[22].

These glucose biosensors based on Con A demonstrated feasibility, but their true potential for use *in vivo* remains a question because of lingering concerns about Con A toxicity, aggregation and irreversible binding [23]. Therefore, recently alternative receptors like apo-glucose oxidase (apo-GOx) [24]-[26] have been used in "smart tattoo" formats; for example, several generations of microcapsule-based glucose biosensors employing fluorescence resonance energy transfer have been reported [3], [8], [10], [12], [13], [24], [27]-[29].

In this study, we assessed and compared the response of two different systems (FITC-dextran–TRITC-apo-GOx and AF-647-dextran–amino-QSY-21-apo-GOx) encapsulated within dissolved core alginate microspheres. The glucose sensitivity of these dissolved core microspheres was studied in simulated interstitial fluid using fluorescence spectroscopy and characterized using confocal laser scanning microscopy (CLSM), scanning electron microscopy (SEM), fourier transform infra red spectroscopy (FTIR) and energy dispersive X- ray analysis (EDX). These alginate microsphere glucose sensors can be implanted intra-dermally within the body, where they measure the interstitial glucose levels that can be correlated with blood glucose levels [15]-[17]. The sensors can be monitored from outside the body using simple optical instrumentation [4], [18].

II. METHODOLOGY

A. Materials

Alginate (Low viscosity, 2%), FD (150kDa/500kDa), TRITC , glucose oxidase (160 kDa), β-D-Glucose(MW 180 Da), sodium poly (styrene sulfonate) (PSS, 70 kDa), poly (allylamine hydrochloride) (PAH, 70 kDa), phosphate buffer saline (PBS) tablets, dimethyl sulfoxide (FW 78.13), SPAN 85, TWEEN 85, 2,2,4-tri-methylpentane (iso-octane) and PD10 columns were purchased from Sigma-Aldrich (India). Alexa Fluor-647, dextran amino (500kDa) and QSY-21

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were purchased from Invitrogen. NAP5 columns were purchased from GE Healthcare. All chemicals were reagent grade and used as received.

A. Methods

Preparation of labeled sensing elements

Apo-glucose oxidase was prepared by the Swoboda protocol [30] as reported earlier [12]. A UV-Vis spectrophotometer was used to confirm the removal of FAD [12], [27], [28] and to calculate to concentration of the apoenzyme. Thereafter apo-GOx was conjugated with TRITC dye using a standard amine labeling procedure [31]. Subsequently, for the preparation of NIR sensing assay, dextran amino was labeled with Alexa Fluor-647 donor dye while apo-glucose oxidase was labeled with QSY-21 quencher acceptor dye using a standard amine labeling procedure.

Fabrication of alginate microsphere biosensors

Alginate microspheres loaded with the labeled sensing assay (visible and NIR) were prepared using the emulsification technique reported earlier [32]-[34] with some modifications. The prepared calcium alginate microspheres were imaged using fluorescence microscopy to confirm encapsulation. Layer by layer (LbL) self assembly technique was then used to fabricate multilayer thin film coatings over alginate micropsheres [35], [36]. The adsorption of LbL coatings was analyzed using FTIR studies.

Partial Dissolution of Alginate Microsphere Core

To allow free movement of the sensing chemistry during competitive binding partial dissolution of the alginate microsphere core was performed wherein 0.1M sodium citrate-TRIS HCl solution was added to a suspension of alginate microspheres and kept for 2-3 days. As the Ca^{2+} ions are removed, the crosslinking in the gel decreases and this leads to solubilization of the high molecular weight alginate polymers [37]. The polyelectrolyte coatings do not dissolve and thus stabilize the alginate microspheres simultaneously preventing the leakage of the sensing chemistry [8]. Visualization of core dissolution was confirmed using SEM, CLSM and finally EDX was used to analyze the resulting structures following core dissolution.

Stability of Encapsulation

To estimate the quantity of encapsulated material lost from the coated microspheres, leaching studies were performed on microspheres containing FD 150kDa-500kDa/TRITC-apo-GOx complexes. All samples were covered and stored under dark conditions at room temperature.

Sensor response in dissolved core alginate microspheres

The glucose response of alginate microspheres was tested in simulated interstitial fluid (SIF). The SIF had almost equal ion concentrations as those of the human interstitial fluid (Na^{+}=143.0, K^{+}=4.0, Mg^{2+}=3, Ca^{2+}=5, $C1^{-}$=117, HCO^{3-} =27, HPO_4^{2-}=2, $SO4^{2-}$=1, organic acid = 6, protein = 2) [38]. The simulated interstitial fluid was prepared by dissolving the reagents NaCl, $NaHCO_3$, $KHCO_3$, K_2HP0_4, $MgSO_4$, Ca-gluconate in distilled water and pH buffered to 7.4.

Reversibility of alginate microsphere glucose biosensor

To test the reversibility of the sensor system, alginate microsphere suspension was exposed to random glucose concentrations (3mM to 70mM glucose). The change in FITC/TRITC peak ratio was obtained at each step and percentage change in peak ratio was plotted to demonstrate reversibility of sensor.

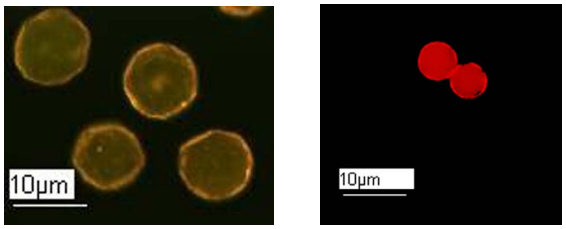
Time response of alginate microsphere glucose biosensor

To calculate the time response of the sensor, a desired concentration of glucose was added to the alginate microsphere suspension. After addition of glucose, fluorescence spectra were collected after every 60 seconds for about 2-3 minutes. The change in FITC/TRITC peak ratio was calculated and a time response curve was plotted for the experiment.

III. RESULTS

Fabrication of alginate microsphere biosensors

Representative fluorescent images of the LbL coated alginate microspheres loaded with both FD500kDa-TRITC-apo-GOx sensing assay and AF-647-dextran amino(500kDa)-QSY-21-apo-GOx sensing assay (green excitation filter of 560nm used) are shown in Fig. 1(a), (b). It is evident that the assay molecules have been encapsulated within the microspheres. SEM images indicated that the microsphere were in the range of 10-20µm diameter as shown in Fig. 2(a).



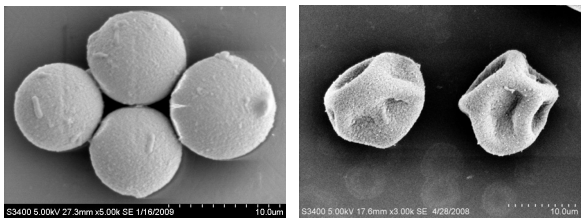
(a) (b)

Fig1. Fluorescent images of alginate microspheres loaded with (a) visible (b) NIR sensing assay.

Partial core dissolution of alginate microsphere

The alginate microspheres appear to have collapsed partially after 3 days of citrate treatment particularly when compared to the undissolved particles shown in Fig. 2(a) and 2(b). Approximately 95% of the microspheres were observed under CLSM to have a dissolved core after a 3-day citrate treatment (results not shown here). It is noteworthy that the microspheres maintain integrity and the LbL coatings prevent rupture. We hypothesized that at least some of the alginate remains complexed with the cationic inner layer of PAH, providing additional bulk and

mechanical strength. EDX analysis was used to calculate the percentage composition of alginate microspheres before and after the citrate treatment. The percentage of calcium ions was found to decrease considerably from 4% to 0.5% after the citrate treatment. Also, a very small decrease was observed in the carbon and oxygen percentages from 62% to 58% for carbon and 34% to 31% for oxygen. This may be due to fractional loss of alginate from the microspheres; however, clearly significant amount of the alginate remains inside the microsphere after dissolution of core.



(a) (b)

Fig2. SEM images of (a) citrate untreated and (b) citrate treated microsphere

Stability of Encapsulation

The release of the encapsulated molecules from the microspheres was observed as an increase in the fluorescence intensity of the supernatant. Most importantly, there was only a small amount of leaching of ~4% during the first 15 hours of the leaching studies for the sensor incorporating 500kDa dextran as illustrated in Fig. 3. Thereafter no change in the fluorescence intensities from the supernatant solutions was observed which proved the stability of the assay.

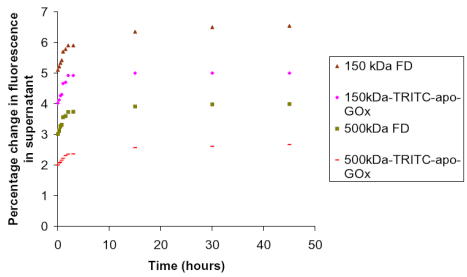


Fig3. Leaching curve of alginate microsphere biosensors

Sensor response to glucose addition in microspheres

Microspheres loaded with FD (150kDa/500kDa)/TRITC-apo-GOx complex were tested for glucose sensing in SIF. A fluorescence emission spectrum was collected after each addition of 3mM to 60mM β-D glucose and corresponding % change in FITC/TRITC peak ratio was calculated for each case. It can be observed from Fig. 4 that the glucose sensitivity of the encapsulated TRITC-apo-GOx /FITC-dextran (500kDa) assay in simulated interstitial fluid was

observed to be 0.89%/mM glucose with a linear response in the range of 0-50mM glucose. The glucose sensitivity of the encapsulated TRITC-apo-GOx /FITC-dextran (150kDa) assay in simulated interstitial fluid was observed to be 0.91%/mM glucose with a linear response in the range of 0-40mM glucose. It can therefore be concluded that the calcium ions present in the SIF do not result in substantial re-crosslinking of residual alginate so as to interfere with the response to glucose. In addition, the glucose response sensitivity of the biosensor depends largely on the concentration of receptor and ligand encapsulated inside the alginate microspheres, which is approximately uniform.

A difference was observed in the glucose sensitivity for different molecular weight FD molecules for the encapsulated FD/TRITC-apo-GOx system. The 500kDa FD system exhibited a wider range of response but lower sensitivity than 150kDa FD system. It can be concluded that the differences in response are due to the number of glucose residues present. A higher molecular weight of FD molecule has a longer chain with more saccharides that can bind more TRITC-apo-GOx molecules, which effectively decreases the sensitivity to glucose and simultaneously increased the linear range for glucose sensing.

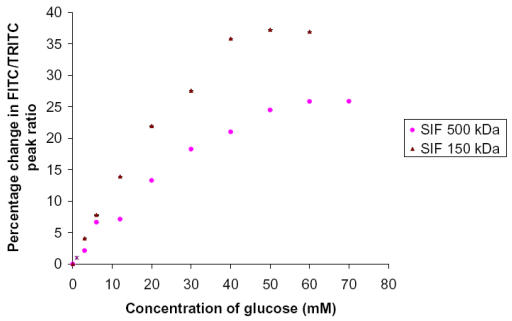


Fig4. Glucose sensitivity curve of alginate microsphere biosensors loaded with visible dye sensing assay in SIF.

Finally, glucose sensitivity experiments were performed on the microspheres loaded with AF-647-dextran amino and QSY-21-apo-GOx complexes. The fluorescence spectrum was collected after each addition of 3mM to 60mM β-D-glucose to microspheres and corresponding change in AF-647 peak was plotted. On plotting the glucose sensitivity curve, the glucose sensitivity was calculated to be 0.94%/mM glucose with a linear response in the range of 0-50mM glucose as illustrated in Fig. 5.

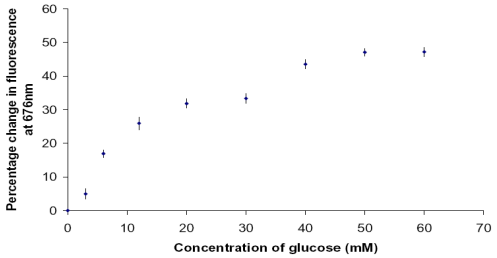


Fig5. Glucose sensitivity curve of alginate microsphere biosensors loaded with NIR sensing assay

These findings suggest that alginate templated microspheres are a facile and effective means of encapsulating fluorescent sensing reagents. Other ongoing studies include sensor response characterization in continuous flow through system, long-term stability analysis and in vitro biocompatibility studies on dissolved-core alginate microspheres. Incorporation of a reference dye inside the polyelectrolyte coating will allow ratiometric analysis with NIR dyes; such as attachment of the succinimidyl ester form of AF-750 to amine residues of PAH, involves a simple procedure and has recently been demonstrated in a similar hybrid microcapsule system [13]. The overall advantage of using a reference dye is that the sensing mechanism would be inherently ratiometric and therefore less sensitive to noise and fluctuations in measurement apparatus properties.

Reversibility of alginate microsphere glucose biosensor

Alginate microsphere biosensors were observed to be completely reversible with a response sensitivity of 0.65%/mM glucose and a linear response over glucose range of 0-50mM (results not shown here).

Time response of alginate microsphere glucose biosensor

The sensor response was observed to reach steady state within 2 min. A detailed study of the dynamic response was not performed; this will be completed in future experiments. There was no variation in response time with the variation in assay elements concentration and ligand molecular weight.

IV. Conclusion

Preliminary glucose sensing studies with fluorescent dissolved-core alginate microspheres have demonstrated the feasibility of encapsulating working fluorescent reagents with a simple and efficient process. It can be concluded that these dissolved core alginate microspheres, used here for glucose sensing, are suitable as carriers for other assay chemistry. These novel systems have potential to be used as implantable glucose biosensors for in vivo glucose sensing. The incorporation of long wavelength NIR dyes was also demonstrated, enabling more efficient excitation through scattering tissue, which effectively improves the odds of successful use in vivo.

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