Glucose response of dissolved-core alginate microspheres: towards a continuous glucose biosensor

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Microparticle optical sensors hold potential as implantable smart materials for *in vivo* analysis. In this work, the reversible response of dissolved-core alginate microspheres containing a homogeneous fluorescence resonance energy transfer (FRET)-based competitive binding assay for glucose was evaluated. The layer-by-layer self assembly technique was used to deposit multilayered nanofilm coatings on the alginate microspheres containing the assay, thereby stabilizing the sensor system when the alginate was de-crosslinked. The response to glucose was then determined in DI water and simulated interstitial fluid (SIF) using a flow cell to establish controlled, dynamic flow conditions for demonstrating reversibility. The glucose sensitivity under dynamic conditions was estimated to be 0.52%/mM glucose in DI water and 0.6%/mM glucose in simulated interstitial fluid; in both cases, the analytical response range was 0–30mM glucose, covering both physiological (normoglycemia) and pathophysiological range (hyperglycemia and hypoglycemia). The sensor demonstrated a repeatable and reproducible response when tested over a period of one month, under dynamic flow conditions. Finally, *in vitro* cytotoxicity assays performed with L929 mouse fibroblast cell lines suggested that the dissolved-core alginate microsphere sensor system with nanofilm coating has sufficient biocompatibility for use as implantable glucose biosensors.

Introduction

The ability to measure glucose continuously in real-time at the point of care facilitates both early diagnosis and management of diabetes. Unlike handheld glucometers, continuous glucose monitoring systems (CGMS) collect data on the magnitude, direction and frequency of fluctuations in blood glucose levels,[1] providing patients with an estimate of real-time glucose level, trend tracking, and alarms at times of rapid glucose fluctuations. These continuous glucose sensors may one day be combined with an insulin delivery system to implement the closed-loop control, which is considered to be the "Holy Grail" of diabetes management. Therefore, continuous glucose sensing represents the next step in the evolution of self-monitoring of glucose.

In the quest to demonstrate the benefits of continuous glucose monitoring, several sensors have recently been developed which allow continuous glucose monitoring for several days.[2] The most advanced sensors include the Medtronic Minimed CGMS® Gold System™ from Medtronic Diabetes (Northridge, CA), the STS® sensor from DexCom (San Diego, CA) and the FreeStyle Navigator® Continuous Glucose Monitor by Therasense/Abbott Diabetes Care (Alameda, CA). These commercially-available implantable sensors are based on electroenzymatic sensing platforms, which exhibit excellent analytical performance *in vitro*. However, they also require a permanent connection from the implanted sensor to an instrument outside the body, providing a potential infection pathway,[3] and have drawbacks of instability of the enzyme[4] electrochemical system, inaccuracy, low precision, extended warm-up period and frequent calibration requirements that make them more cumbersome for *in vivo* use.[5–8] Therefore, most diabetes patients still prefer to measure their blood glucose using a glucometer,[9] which involves pain. In fact, the poor patient compliance with recommended testing regimens due to the invasive nature of "finger-pricking," has further fueled the research for noninvasive and minimally-invasive technologies.

Fully-implantable sensors may be an ideal solution, particularly if optical interrogation can be used in lieu of RF communications. Microparticle "smart material" biosensors (also known as "smart tattoos") are one approach being investigated for this purpose. These materials, when implanted intradermally within the body, are exposed to glucose in the interstitial fluid (ISF): ISF glucose has been shown to correlate strongly with blood glucose levels.[10–12] Such implants may be interrogated noninvasively using simple optical instrumentation in the visible/near-infrared spectral range,[13,14] making their use all the more attractive. Several embodiments of potentially-implantable probes have been demonstrated in the past, including enzymatic assays.[15–20] The design and implementation of enzymatic sensors for long-term *in vivo* application is complicated due to the consumption of glucose and oxygen, generation of potentially toxic byproducts (gluconic acid, hydrogen peroxide), enzyme degradation, and the strong dependence of the glucose response on local tissue oxygen. Therefore, an alternative mechanism based on affinity binding is being investigated by many groups.[21–27]

The first affinity-based sensor proposed for monitoring glucose levels within the interstitial fluid was reported by Schultz *et al.*[22] Since that pioneering effort, a number of advancements

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toward *in vivo* use have been reported, including poly(ethylene glycol) (PEG) hydrogel microspheres[28] and fuzzy microshells.[27] Follow-up work has extended the optical interactions into the near infrared by labeling Con A and dextran with NIR dyes.[26,29,30] However, the true potential for use *in vivo* remains a question because of lingering concerns about Con A toxicity, aggregation and irreversible binding.[31] For this reason, alternative receptors have been studied for "smart tattoo" formats, including boronic acid derivatives,[32–34] apo-enzymes[35–39] and genetically-engineered glucose-binding proteins.[40–42]

To achieve high sensitivities with FRET-based schemes, the free movement of analyte, ligand and receptor is desired within the core of the template into which the sensing assay is encapsulated, such as can be obtained with hollow fiber membranes.[24,25] However, using relatively large hollow membranes has drawbacks in production, fouling, response time, and mechanical stability. Use of microporated PEG polymeric matrices with internal cavities to allow movement of sensing assay has shown promise, but issues of response time, leaching and reversibility persist.[28,43] In addition, while the encapsulation of sensing reagents within hollow nanoengineered capsules is elegant[27,38,44] and overcomes the major limitations of hydrogel-only entrapment, the diffusion-limited post-fabrication loading of the sensing assay into the microcapsules is not efficient. Thus, while the concept of implantable fluorescent sensors is attractive, realizing this goal remains a challenge.

To address these issues, we previously reported the initial design, development, and characterization of dissolved-core alginate microsphere glucose sensor system.[35,36] Here, we report the response of a continuously-responsive "smart tattoo" glucose sensor system under controlled and dynamic conditions. The system comprises multilayer-nanofilm-coated dissolved-core alginate microspheres incorporating the fluorescent dye labeled sensing assay; FITC-dextran and TRITC-apo-glucose oxidase. To further assess the potential for *in vivo* use, the *in vitro* toxicity of the particles with different nanofilm coatings was also determined.

Experimental

Materials

Alginate (Low viscosity, 2%), fluorescein isothiocyanate-dextran (FD, 70kDa, 150kDa, 500kDa), tetramethyl rhodamine isothiocyanate (TRITC, mixture of isomers, MW 443.53), glucose oxidase (160kDa, type VII from *Aspergillus niger*), β-D-Glucose(MW 180 Da), sodium poly (styrene sulfonate) (PSS, 70kDa), poly(allylamine hydrochloride) (PAH, 70kDa), diethylaminoethyl dextran hydrochloride (DEAE-dextran), chondroitin sulfate (CS), poly(L-lysine hydrochloride) (PLL), hyaluronic acid potassium salt (HA), phosphate buffer saline (PBS) tablets, dimethyl sulfoxide (FW 78.13), dimethylformamide (molar mass 73.09g/mole), SPAN 85(Sorbitane trioleate), TWEEN 85(Polyoxyethylene sorbitan Trioleate), 2,2,4-tri-methylpentane (iso-octane) and PD10 columns were purchased from Sigma-Aldrich. Bovine albumin fraction V powder (BSA) was purchased from Sisco Research Laboratories. Sodium azide was purchased from Loba Chemi (Mumbai). Calcium chloride, ammonium sulfate and sodium hydroxide were purchased from Merck (Mumbai). Sodium acetate was purchased from Ranbaxy Fine Chemicals Limited (Mumbai). Sodium bicarbonate, sodium chloride, potassium bicarbonate, magnesium sulfate and di-potassium hydrogen orthophosphate were purchased from SD Fine Chemicals Limited, Mumbai. Calcium gluconate was purchased from Loba Chemie, Mumbai. All chemicals were reagent grade and used as received.

Instrumentation

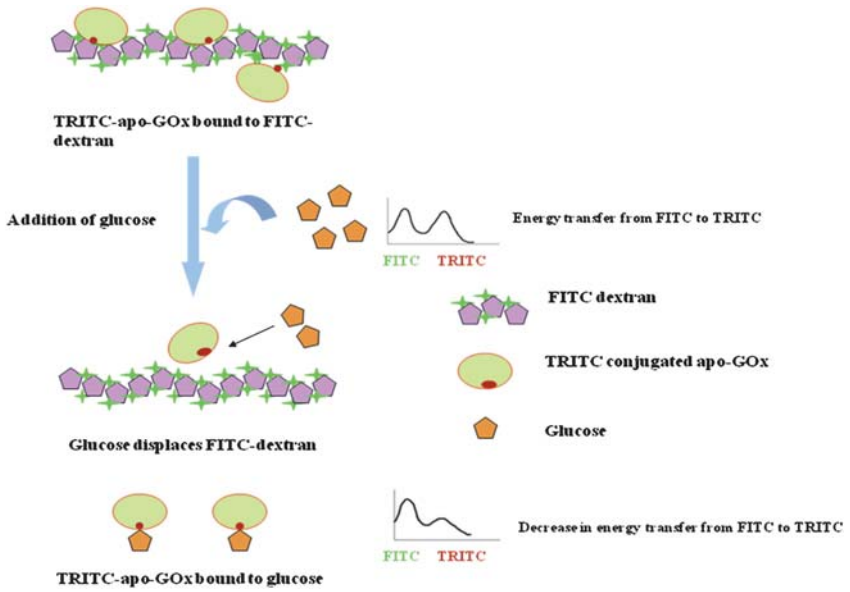
A Hitachi quartz LC micro-flow cell unit (90 μL) for fluorescence spectrophotometer (model no. 650-0163) was used for dynamic testing. A syringe pump (Multi-Phaser™, model NE-1000, New Era Pump Systems, NY) was used to pump the fluid into and out of the flow cell. A Nikon YS 100, Zeiss optical microscope with a digital camera was used for microscopic studies. A Helios Alpha UV-Vis spectrophotometer (double beam, Thermo Scientific) was used to collect absorbance spectra. The alginate microspheres were characterized using a Beckman Coulter laser diffraction particle size analyzer model LS 13 320 with universal liquid module (ULM2). A fluorescence spectrophotometer (Hitachi, F-2500) was used to collect emission scans. A fluorescence microscope (Nikon TE2000U, Japan) was used for obtaining images of alginate microspheres loaded with the sensing assay. FTIR spectra were obtained using a Nicolet spectrometer (Magna-IR 550, USA), wherein microspheres were completely dried and the powdered microsphere sample was then mixed with potassium bromide salt before taking measurements. A scanning electron microscope (S-3400 Hitachi, Japan) was used to obtain images of alginate microspheres.

Sensor design and description

As described in our previous work,[35] the apo-GOx retains its glucose binding capacity following deflavination, and will therefore bind dextran in solution. Apo-GOx retains its binding specificity for β-D-glucose, and the presence of low levels of other sugars like mannose, fructose, sucrose, α-D-glucose does not significantly interfere with accurate measurement of glucose concentration.[38] Therefore, when FITC-dextran and TRITC-apo-GOx are complexed, fluorescence resonance energy transfer (FRET) takes place from the excited donor dye (FITC) to the nearby acceptor (TRITC). When glucose is introduced into the system, it displaces FITC-dextran and binds to TRITC-apo-GOx resulting in a decrease in energy transfer. This manifests as a change in the relative emission intensity of FITC and TRITC, which can be correlated to the glucose concentration in interstitial fluid. Thus, the FRET monitoring approach is inherently ratiometric. This assay is illustrated in Scheme 1.

Preparation and characterization of calcium alginate microspheres

Alginate microspheres were prepared by the emulsification technique as reported earlier.[35,45–47] The oil phase was a mixture of 0.0424 gm of SPAN 85 and 1.85 gm isooctane. 1.25 gm of 3 wt % sodium alginate aqueous solution was added slowly drop wise to the oil phase while being continuously stirred. The mixture solution was allowed to stir for an additional 15mins at 800rpm. A solution containing 0.0226 gm of TWEEN 85 in 0.125 gm of isooctane was then added to the emulsion and stirred for another



Scheme 1 Schematic for principle of glucose sensing.

15mins at 800rpm to achieve stable water/oil emulsion droplets. After this, 500 μl of aqueous solution containing 5 wt % of calcium chloride was added to induce formation of ionic cross-links, and was allowed to stir for about 3 h at 300rpm. The microspheres were then diluted to prevent aggregation and further rinsed with DI water by successive centrifugation cycles and stored in DI water at room temperature. The microspheres were then diluted to prevent aggregation and further rinsed with DI water by successive centrifugation cycles and stored in DI water at room temperature.

The particle size distribution analysis of the microsphere sample was performed using particle size analyzer utilizing the laser diffraction technology. The samples were dispersed in water. The universal liquid module[48] was used for the analysis and particle size distributions were calculated using the Fraunhofer optical model. In addition, the alginate microspheres were also observed under SEM.

Preparation of fluorescent sensing assay

Apo-glucose oxidase is an inactive form of the glucose oxidase enzyme. It does not catalyze glucose oxidation, but retains its glucose binding affinity.[37] Apo-glucose oxidase is used as the glucose binding protein, so as to avoid analyte consumption and prevent by product formation. Apo-glucose oxidase was prepared by removal of flavin adenine dinucleotide (FAD) from GOx, using the Swoboda protocol[49] with the following modifications as reported earlier[35,36]: Glucose oxidase (20mg) was dissolved in 1mL of 0.4M sodium acetate buffer (pH = 5), and this solution was slowly added drop wise to 20mL of 25% saturated ammonium sulfate (pH = 1.4). The sample was then incubated on an ice bath with constant stirring for 2 h. After this, 100% saturated ammonium sulfate solution was added to the above solution to separate out the FAD from the glucose oxidase protein. The solution was then centrifuged twice at 16,000rpm for 15min at 0 °C. The yellow supernatant was then removed, and the precipitate was dissolved and neutralized by adding 2.5M sodium acetate buffer. The precipitated protein was finally redissolved in 0.1M sodium acetate buffer (pH = 5.6). The protein was eluted through PD10 columns to concentrate the protein and separate out the dissolved ammonium sulfate salt from the protein. Sodium bicarbonate buffer was used for elution so as to allow dye labeling of apoenzyme. A UV-Vis spectrophotometer was used to confirm the removal of FAD[36,37,50] and to determine the final concentration of the apoenzyme.

The obtained apo-GOx was conjugated with TRITC dye using a standard amine labeling procedure,[51] as reported previously.[35,36] Briefly, a solution containing 20mg ml^{-1} TRITC dissolved in DMSO was added to the apoenzyme solution under constant stirring. The reaction mixture was then incubated in the dark for 4 h at room temperature under continuous stirring. Finally, the mixture was eluted through PD-10 desalting columns to separate out the TRITC- labeled apo-GOx from the unconjugated dye. The concentration of the labeled apo-GOx was calculated to be 10 μM with degree of labeling of 1.

Fabrication and characterization of layer-by-layer coated alginate microsphere glucose sensors

Alginate microspheres loaded with the sensing assay were prepared using the technique described in the earlier section with the following modifications. A 1μM solution of FD was mixed with 10μM TRITC-apo-GOx solution and used for co-encapsulation. The sensing reagents were then mixed in 2%w/v sodium alginate solution in a ratio of 1 : 10 by volume. The solution was gently agitated for about 30min in the dark to prevent photobleaching of the dye while allowing complete mixing in the precursor solution. Calcium alginate microspheres were then prepared using the emulsification technique. Thereafter, multilayered thin film polyelectrolyte coatings (2 bilayers) were deposited over the alginate microspheres containing the fluorescent sensing assay using the layer by layer self assembly technique.

For the layer-by-layer (LbL) self-assembled coatings ([PAH/PSS]$_2$ multilayers), solutions of PAH (cationic) and PSS (anionic) were prepared in DI water at 2mg ml^{-1} with 250mM calcium chloride salt.[45–47] As the core alginate particles are negatively charged, they were initially dispersed in 2ml of 2mg ml^{-1} PAH solution for 20min, followed by two consecutive washing steps to remove excess polyelectrolyte. Particles were then suspended in the PSS solution for 20min to complete one bilayer.[35] The LbL-coated alginate microspheres were analyzed using FTIR spectroscopy. The microsphere pellet was dried completely to form powder, which was then mixed with potassium bromide powder for FTIR studies.

Stability of encapsulation

For potential use as glucose biosensors, it is important for the sensing chemistry to be stably retained in the partially-dissolved alginate microspheres over time. In this regard, different combinations of cationic and anionic polyelectrolyte coatings including [PAH/PSS]$_2$, [PLL/HA]$_2$ and [DEAE/CS]$_2$ were compared to estimate the loss of encapsulated material from these coated microspheres. Initially, leaching studies were performed with [PAH/PSS]$_2$ on three sets of microspheres containing FD 70kDa/TRITC-apo-GOx, 150kDa/TRITC-apo-GOx and 500kDa/TRITC-apo-GOx complexes. As expected, the FD 500kDa/TRITC-apo-GOx complex showed the minimum leaching, thereafter [PLL/HA]$_2$ and [DEAE/CS]$_2$ were analyzed to estimate the stability of encapsulation of the FD 500kDa/TRITC-apo-GOx complex. All samples were covered and stored under dark conditions at room temperature. Standard solutions of 70nM FD-150kDa, 30nM FD-500kD and 300nM TRITC-apo-GOx were also measured at each point to correct for instrumental drifts over time.

Alginate microsphere core dissolution

Alginate has a highly cross-linked network which causes physical restriction of the large dextran molecules resulting in an extremely slow spatial displacement of molecules and minimal changes in fluorescent intensities for the encapsulated assay. Therefore, non-dissolved alginate microspheres were disregarded for further development. Subsequently, we proposed an approach based on dissolved-core alginate microspheres formed by partial de-crosslinking.[35] To dissolve the core, 0.1M sodium citrate-TRIS HCl solution was added to a suspension of alginate microspheres and kept for 2–3 days. The removal of Ca^{2+} ions leads to solubilization of the high molecular weight alginate polymers.[19] A key aspect of our approach is that the polyelectrolyte coatings do not dissolve; thus, the nanofilm coatings stabilize the particles while preventing the leakage of the sensing chemistry.[38,52] It is noteworthy that some of the anionic alginate molecules remain electrostatically bound to the inner cationic polyelectrolyte coating.

Response of alginate microsphere glucose sensors under dynamic conditions in DI water and simulated physiological media

A Hitachi quartz LC micro- flow cell unit was used to accurately test the response of the alginate microsphere glucose biosensors to changes in glucose concentration. The flow cell unit provides real-time measurements of the glucose concentration under dynamic flow conditions. The flow cell is equipped with an inlet and an outlet that allows continuous flow of the sensor suspension within the chamber. A syringe pump equipped with a 10ml syringe attached to silicone tubing was used to pump the sensor suspension into and out of the flow cell chamber. The experimental setup is illustrated in Fig. 6(a) and (b). The flow rate of the pump was maintained at 4ml min^{-1}. Glucose was added into the microsphere suspension to a desired concentration, and the equilibrated suspension was then passed through the flow cell chamber after 2 min of reaction. Fluorescence spectra were continuously recorded for 5 mins during flow. The concentration of glucose was then returned to 0mM and fluorescence emission spectra were again recorded for 5 mins. Measurements were performed on the same batch of microspheres so as to demonstrate the reversible nature of the response. Overall, the same set of alginate microsphere sensors were subjected to incremental glucose concentrations from 0–30mM glucose and tested similarly under dynamic conditions.

Likewise, the dynamic response of glucose sensitive dissolved core alginate microsphere biosensors was evaluated in simulated interstitial fluid (SIF) with similar intracellular composition as those of the human interstitial fluid (Na^+ = 143.0 meq/L, K^+ = 4.0 meq/L, Mg^{2+} = 3 meq/L, Ca^{2+} = 5 meq/L, $C1^-$ = 117 meq/L, HCO_3^- = 27 meq/L, HPO_4^{-2} = 2 meq/L, SO_4^{-2} = 1 meq/L, organic acid = 6 meq/L, protein = 2 meq/L).[53] The SIF was prepared by dissolving the reagents NaCl, $NaHCO_3$, $KHCO_3$, K_2HPO_4, $MgSO_4$, Ca gluconate and BSA in distilled water and the fluid was buffered to pH 7.4. In addition, the sensors were stored in simulated interstitial fluid and tested for their stability. SEM imaging was used to study the effect of SIF on the morphology of the alginate microsphere sensors during storage.

In vitro cytotoxicity studies

Biocompatibility studies on an implantable device require complex experiments both *in vitro* and *in vivo* in order to test the local and systemic effects of the material on culture cells, tissue sections and the whole body. *In vitro* cell culture tests are considered as one of the most fundamental tests for biocompatibility.[54] Such tests are sensitive, reliable, convenient and reproducible and are therefore used to screen the biocompatibility of implantable devices.

The cytotoxicity of the plain alginate microspheres and [PAH/PSS]$_2$-coated alginate microspheres loaded with the fluorescent glucose sensing assay were evaluated by using the sulforhodamine-B (SR-B) semi-automated assay. An *in vitro* biocompatibility study of these samples was performed using L929 (Mouse fibroblasts) cell lines obtained from National Centre for Cell Science (NCCS), Pune, India. The cells were grown in modified DMEM (Dulbecco's modified essential medium, Sigma, USA) supplemented with 10% FBS (fetal bovine serum, Sigma, USA) and 1% antibiotic/antimycotic solutions (Himedia, India) and incubated at 37 °C temperature under 5% CO_2 and saturated humid environment. Nearly confluent cells in 25cm^2 tissue culture flasks were trypsinized by trypsin-EDTA (ethylenediaminetetraacetic acid) solution and centrifuged at 1000g for 10min. The cell pellet was then resuspended in fresh media. Cells were counted and cell count was adjusted accordingly to the

titration readings so as to give an optical density in the linear range (from 0.5 to 1.8). Samples were tested in 96 well plates in 3 triplicates, each well receiving 90 µl of cell suspension with a concentration of 1×10^4 cells per well. The plate was then incubated at 37 °C in CO_2 incubator for 24 h. Afterwards, 10µL of diluted (10^5 microspheres/100µl) plain, polyelectrolyte coated and alginate microsphere glucose biosensors were added to the 96 well-plate and further incubated for 48 h. Finally, the experiment was terminated by gently layering the cells in the wells with 50µl of chilled 50% TCA (trichloroacetic acid) for cell fixation. Plates were kept in a refrigerator (4 °C) for 1h. The plates were washed thoroughly with tap water for at least 5 times and air dried. For the assay, plates were stained with 50µl of 0.4% SR-B for 20 min then washed with 1% acetic acid at least 5 times and air dried. Finally, the bound SR-B was eluted with 100µl of Tris (10 mM, pH 10.5) for 10min. Thereafter, the plates were shaken for 1 min using an automated shaker and the absorbance (O.D.) of each well was read in a micro plate reader (Thermo Electron Corporation, USA) at 540nm with reference to 690nm against blank culture media without any cells.

Results and discussion

Alginate microsphere fabrication

The response time (time to reach steady-state) of the glucose biosensor will depend on particle size and consistency (particle-to-particle) will depend on size distribution. Therefore, it is significant to have a uniform size distribution. The alginate microspheres prepared by the emulsification technique were characterized using the laser diffraction technology. The mean diameter was determined to be 17µm (as illustrated in Fig. 1). Using SEM imaging, it was confirmed that the microspheres were in the range of 10–20µm diameter (as depicted in Fig. 2). The particles retained a spherical shape with a relatively smooth surface while imaging, indicating the presence of the polymeric core prior to de-crosslinking. Alginate microspheres loaded with the fluorescent sensing assay were prepared using the modified technique described earlier. Representative fluorescence microscopy images of the alginate microspheres loaded with FD500kDa-TRITC-apo-GOx sensing assay are shown in Fig. 3a and Fig. 3b, illustrating the encapsulation of the sensing assay. In all of the particles observed, both fluorescent reagents were approximately homogeneously distributed throughout the particle interior.

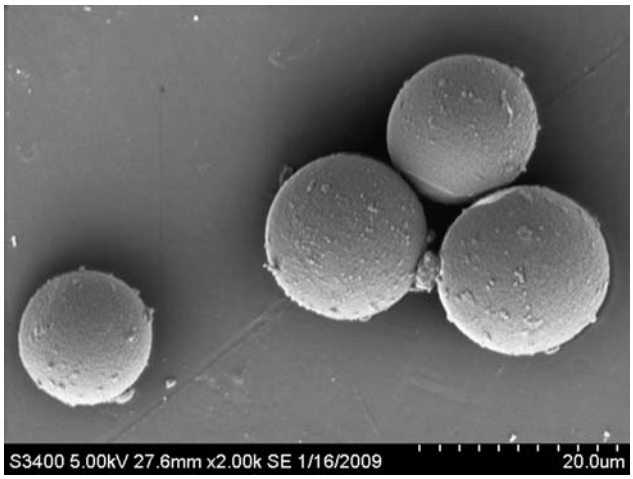


Fig. 2 SEM micrograph of alginate microspheres.

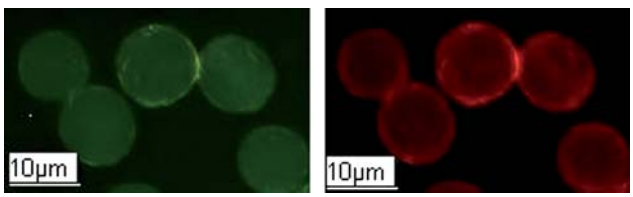


Fig. 3 Fluorescence microscopy images of alginate microspheres loaded with FD (500kDa)-TRITC-apo-GOx.

FTIR spectra analysis of LbL coated alginate microspheres

FTIR analysis performed on the LbL-coated alginate microspheres yielded the vibrational absorbance spectra, which reveal the bonding characteristics of the materials. As illustrated in Fig. 4, the most significant peak in the infrared spectrum for alginate (spectrum a) was at 1600 cm^{-1}, attributed to carbonyl groups on alginate molecules as reported in literature.[55] Spectrum b is the absorbance of PAH, while spectrum c is the absorbance of PSS. The peaks at 1590 cm^{-1} and 1470 cm^{-1} are attributed to N–H and C–H bending respectively. The peaks at 3400 cm^{-1} and 3150 cm^{-1} also indicate the presence of $–NH_2$ groups, while the peaks at 1115 cm^{-1} and 1170 cm^{-1} indicate presence of $–SO^{2-}$ groups. For [PAH/PSS] coated alginate microspheres, same features from $–NH_2$ indicating complexation of carboxylic acid groups on alginate with amine groups on positively charged polyelectrolyte (PAH), are observed at

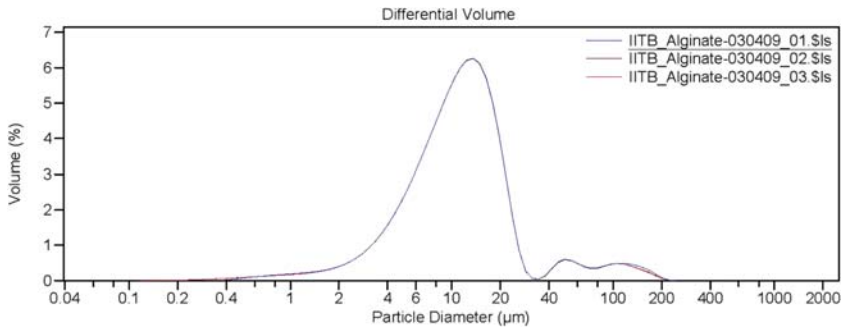


Fig. 1 Laser diffraction technology based particle size analysis.

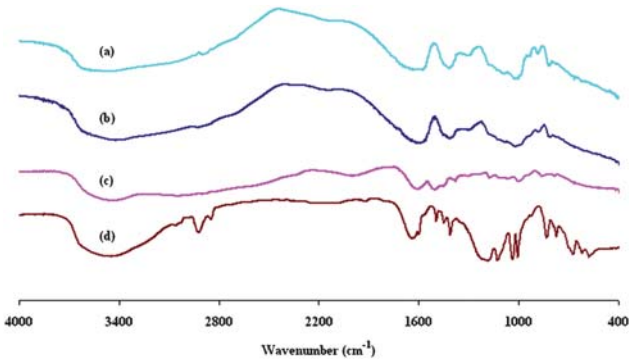


Fig. 4 FTIR spectra of (a) alginate micropsheres (b) PAH (c) PSS (d) PAH/PSS coated alginate microspheres.

1400–1600 cm^{-1}. Likewise features from $–SO^{2-}$, indicating the presence of negatively charged polyelectrolyte (PSS), are observed at 1100–1200 cm^{-1}. Therefore, spectrum d suggests that alginate/[PAH/PSS] produces a combining spectra of alginate microspheres and PAH and PSS polyelectrolytes, thereby illustrating the adsorption of oppositely-charged polyelectrolytes over the alginate microsphere templates. The band positions reported here are in agreement with those previously reported.[19]

Stability of encapsulation

The loss of FD and TRITC-apo-GOx molecules from dissolved core alginate microspheres was quantified using fluorescence spectroscopy studies, where release of the encapsulated molecules from the microspheres was observed as a percentage increase in the fluorescence intensity of the supernatant (as demonstrated in Fig. 5). The leaching for 70kDa complex was very rapid and no significant fluorescence was observed after the LbL coating process. Leaching was higher for the microspheres loaded with FD-150kDa/TRITC-apo-GOx relative to FD-500kDa/TRITC-apo-GOx; this matched expectations, as diffusion is inversely proportional to the square root of molecular mass. Most importantly, there was only a small amount of leaching; around 4% during the first 15 h of the leaching studies for the encapsulated assay. Thereafter, no change in the fluorescence intensities from the supernatant solutions was observed. Thus, the encapsulation of the assay with the largest molecular weight dextran was considered to be most stable and preferred for long-term, repeated use where the same reagents must be maintained.

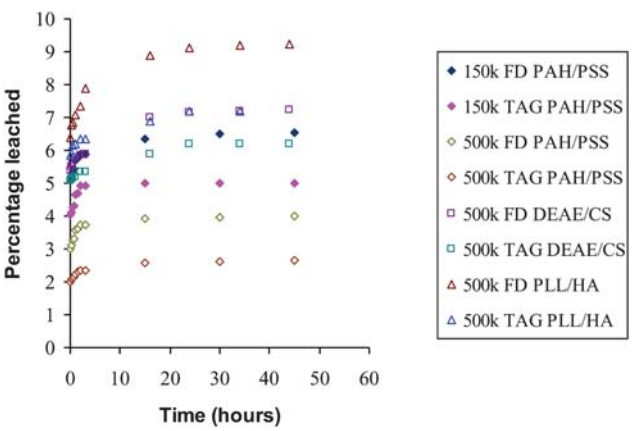


Fig. 5 Leaching curves for $[PAH/PSS]_2$, $[PLL/HA]_2$ and $[DEAE/CS]_2$ coated alginate microsphere biosensors.

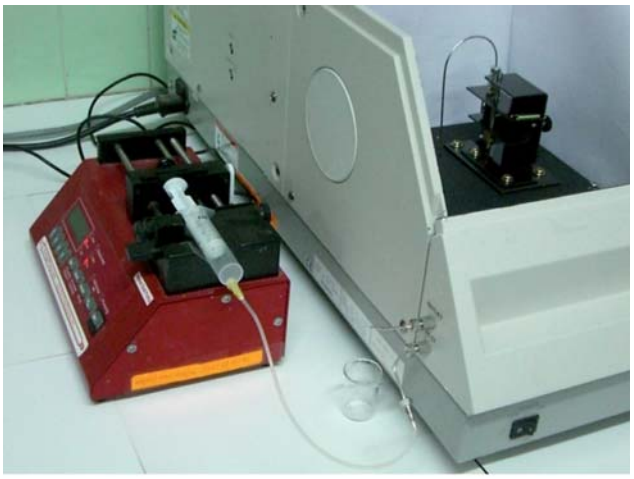
Of the three different coatings compared ($[PAH/PSS]_2$, $[PLL/HA]_2$ and $[DEAE/CS]_2$), $[PAH/PSS]_2$ yielded the most stable encapsulation. $[PLL/HA]_2$- and $[DEAE/CS]_2$-coated alginate microspheres leached 9% and 7% of encapsulated material, respectively, over 30–40 h compared to the 4% for the synthetic polymer coatings. The higher percentage of leaching may be attributed to the fact that these polypeptide/polysaccharide films exhibit an exponential growth regime where the thickness increases exponentially with the number of deposited layers. It has been suggested that such a growth regime results from an "in" and "out" diffusion of polyelectrolyte chains through the film during their buildup,[56,57] which in turn is a result of greater porosity and permeability within such polyelectrolyte films. These polypeptide multilayer films form secondary structures such as α-helix and β-sheets at neutral pH.[48,58] As a result of the more stable behavior, only particles with $[PAH/PSS]_2$ coatings were used for further testing.

Response of alginate microsphere glucose sensors under dynamic conditions

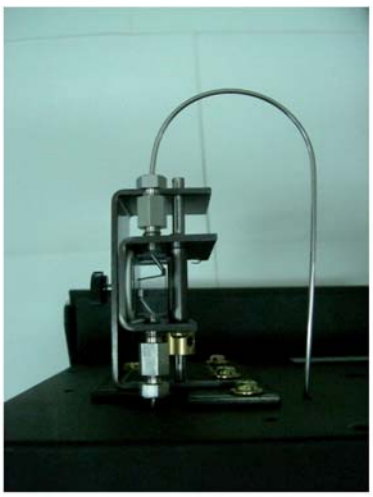
For dissolution of the alginate core, the alginate microsphere glucose sensors were subjected to citrate treatment as previously reported in our earlier work,[35] wherein the microspheres were suspended in 0.1M sodium citrate-TRIS HCl solution for 2–3 days. These microspheres were then tested for glucose sensing under dynamic conditions.

The continuous glucose sensing profile to assess the response of the alginate microsphere glucose sensors under dynamic conditions was obtained using a fluorescence spectrophotometer and a flow cell unit as illustrated in Fig. 6(a) and (b). The dynamic sensor response to step changes in the bulk glucose concentrations was tested both in DI water and simulated interstitial fluid. Fig. 7(a), (c) demonstrate real-time continuous acquisition of emission spectra with constant irradiation under dynamic glucose concentration changes in DI water and SIF, respectively. The response data were collected during sensor exposure to random bulk glucose levels ranging from 0 to 30mM glucose.

The percentage change in peak ratio, calculated relative to the baseline (no glucose) fluorescence ratio, is plotted as a function of time at different incremental glucose concentrations from 0 to 30mM glucose. It can be clearly observed from the graph that the step changes in glucose concentration elicit a significant increase in FITC/TRITC peak ratio. Furthermore, exposing the sensors to glucose-free buffer caused the peak ratio to return to the baseline value, demonstrating reversible sensor response. The reversibility of a sensor was assessed by the baseline change before and after exposure to glucose and was quantified by calculating the average change in the baseline value before and after a step change in glucose concentration. The average baseline shift was determined to be 1.03% with SD of 0.61 in DI water and 1.3% with SD of 0.67 in SIF, thus demonstrating a highly reversible sensor response. The small initial drift may be



(a)



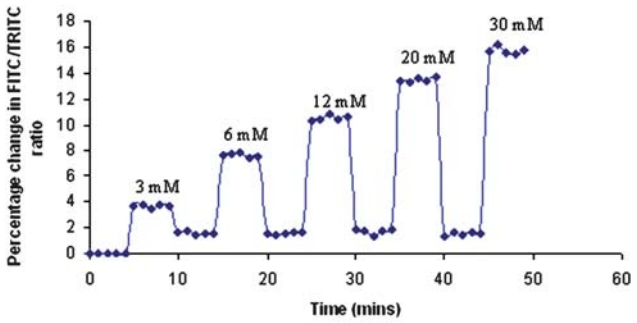
(b)

Fig. 6 (a) Experimental set up for glucose sensing under dynamic conditions. (b) Enlarged view of the micro-flow cell chamber.

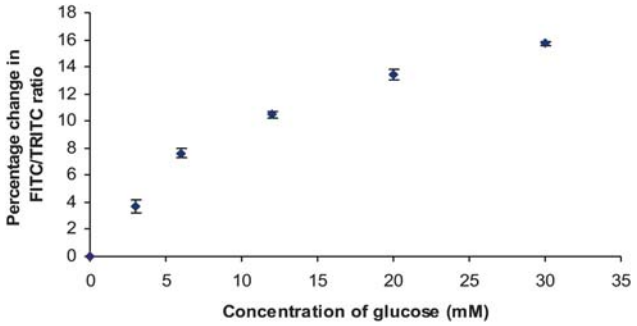
attributed to a small amount of leaching of the free dye from the microspheres. Any instrumental variations in the form of source fluctuations and minor changes in sensor concentration were accounted by the fluorescent ratiometric analysis. As seen in the repeated measurements, the response appears very stable.

The response time of a glucose sensor is critical as sensors must respond within the physiological time scale of glucose fluctuations to ensure accuracy. The sensor response time under dynamic conditions was observed to be 2 mins (maximum response time), which is identical to that observed in steady-state, making these sensors adequate to monitor fluctuations in blood glucose, which usually occur over a period of 30 mins time.[59]

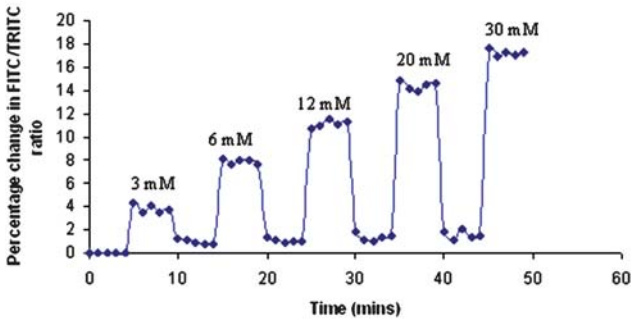
Sensitivity curves were obtained by plotting the percentage change in FITC/TRITC peak intensity *versus* glucose concentration and response sensitivity values calculated for each case as illustrated in Fig. 7 (b), (d). The glucose sensitivity under dynamic conditions was estimated to be 0.52%/mM glucose in DI water and 0.6%/mM glucose in simulated interstitial fluid, with an analytical response range of 0–30mM glucose in both cases. This range of response covers the complete expected physiological range (normoglycemia) and pathophysiological range



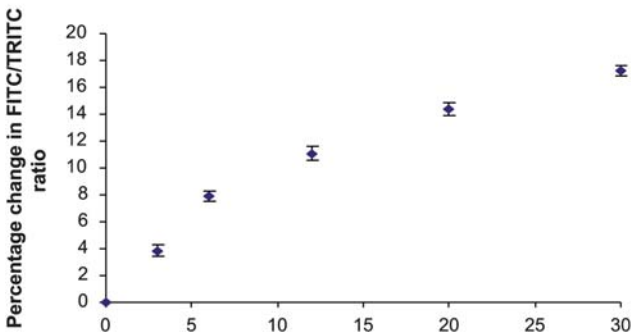
(a)



(b)



(c)



(d)

Fig. 7 Continuous sensing profile and sensitivity curve of alginate microsphere glucose sensors under dynamic conditions in (a), (b) DI water and (c), (d) SIF. Mean ± SD ($n = 3$).

(hyperglycemia and hypoglycemia). The response is observed to be linear up to 6mM glucose concentration, which is the range required to accurately predict glucose concentrations in hypoglycemia. The glucose response sensitivity under dynamic conditions was found to be comparable to the steady state

glucose response reported earlier in our previous paper,[60] when compared statistically using a student's paired t-test ($\alpha = 0.05$). These findings suggest that these dissolved-core alginate microsphere glucose sensors are stable under static and dynamic flow conditions, further supporting their potential for monitoring glucose under physiological conditions.

Repeatability, reproducibility and stability of biosensors

The repeatability of the alginate microsphere glucose biosensors was obtained by testing the response of the sensors to same glucose concentration for five successive readings. The standard deviation was calculated to be 1%. Additionally, to evaluate reproducibility, three different sets of the biosensors were independently prepared and tested using the same conditions; these experiments yielded a standard deviation value of 1.5%, suggesting the fabrication and measurements are highly consistent.

The stability of the biosensors was examined by intermittently measuring the dynamic response to successive glucose concentrations at every 7th day for over a 30-day period. The alginate microsphere glucose sensors (FD 500kDa/TRITC-apo-GOx) were suspended in SIF. Fluorescence spectra were collected after each addition of 3mM to 30mM β-D glucose and corresponding % change in FITC/TRITC peak ratio was calculated for each case. The biosensors were stored at room temperature in a dark place when not in use. The glucose sensitivity was observed to remain constant (0.6%/mM) over this entire time, with a standard deviation of 1.1%. Therefore, it can be concluded that the alginate microspheres were stable in the simulated interstitial fluid when tested over a period of one month, confirming that the sensing assay was stabilized in a favorable microenvironment. It is also noteworthy to state that the data were not corrected for photobleaching, indicating the system exhibits a high degree of photostability. In addition, the SIF does not apparently affect the morphology of alginate microspheres as has been demonstrated in SEM micrograph in Fig. 8. The particles were washed with water 5–6 times to remove salt deposition before taking a SEM micrograph.

In vitro cytotoxicity studies

Cytotoxicity studies were performed using the L929 mouse fibroblast cell line as a means of assessing plain microspheres and polyelectrolyte-coated microspheres containing the fluorescent glucose sensing. The results, depicted in Fig. 9, indicate that the viability of cells was approximately 100% with plain alginate microspheres as compared to the control (cells without

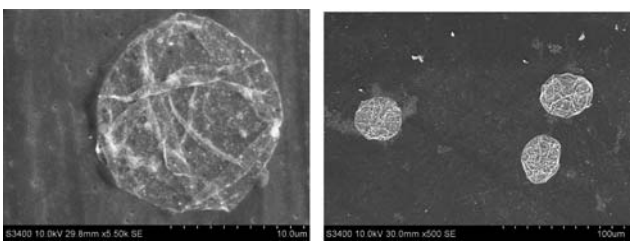


Fig. 8 SEM micrograph of dissolved-core alginate microsphere glucose biosensors suspended in SIF.

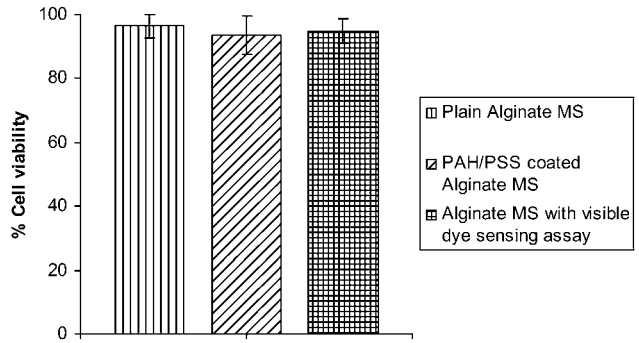


Fig. 9 Cytotoxicity results of plain and polyelectrolyte coated sensing assay loaded alginate microspheres. Mean ± SD ($n = 3$).

microsphere sample), indicating that there was no cytotoxicity to cells. In the case of $[PAH/PSS]_2$-coated alginate microspheres, the viability was again observed to be around 100%. Finally, the dissolved-core alginate templated microspheres loaded with the fluorescent dye sensing assay also demonstrated 100% viability of cells. Therefore, it can be concluded from the cytotoxicity studies that the dissolved-core alginate templated microsphere glucose biosensors do not cause significant cell death to the L929 mouse fibroblast cell line, suggesting that the system has acceptable basic biocompatibility to allow their *in vivo* evaluation for use as "smart tattoos".

Conclusion and outlook

Preliminary glucose sensing studies under dynamic flow conditions have demonstrated the feasibility of dissolved-core alginate microspheres for continuous monitoring of glucose. The glucose sensitivity under dynamic conditions was estimated to be 0.52%/mM glucose in DI water and 0.6%/mM glucose in simulated interstitial fluid and both demonstrated an analytical response range of 0–30mM glucose, covering both physiological (normoglycemia) and pathophysiological range (hyperglycemia and hypoglycemia). The sensor demonstrated a reversible, repeatable and reproducible response when evaluated under dynamic flow conditions. The glucose response of alginate microsphere glucose sensors was comparable under static and dynamic conditions suggesting that alginate-templated microspheres are a facile and effective means of encapsulating fluorescent sensing reagents. These sensor systems have potential to be used as implantable glucose biosensors for *in vivo* glucose sensing in diabetics.

However, the application of visible dyes with relatively short wavelength is limited because skin exhibits substantial interfering fluorescence at shorter wavelengths.[5] Therefore, the incorporation of long wavelength near infrared dyes is underway. The long wavelength NIR dyes enable more efficient excitation through scattering tissue,[5,61] which effectively improves the odds of successful use of alginate microsphere "smart tattoo" glucose sensors *in vivo*.

Another critical problem which remains with such implantable glucose sensors is the inflammatory response of the body to tissue injury on implantation. When the sensors are exposed to the biological system, they cause tissue injury, which triggers a cascade of inflammatory responses that compromise implant functionality and ultimately lead to implant failure.[62,63] This

localized inflammation must be minimized to ensure sensor functionality and a long lifetime. We therefore propose a plausible solution towards minimizing the triggered inflammatory response of the host body that would lead us to increase the functional longevity of the glucose biosensors. The incorporation of dexamethasone loaded alginate microspheres[64,65] along with the sensor system would provide localized drug delivery to suppress the inflammatory response arising out of the implantation procedure, thereby improving the biocompatibility and functional longevity of the sensor. An ideal glucose sensor with a real-time continuous response along with a long operational lifetime under physiological conditions remains elusive, but there is significant progress.

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