

## “Smart Tattoo” Glucose Biosensors and Effect of Coencapsulated Anti-Inflammatory Agents

Rohit Srivastava, Ph.D.,<sup>1</sup> Rahul Dev Jayant, Ph.D.,<sup>1</sup> Ayesha Chaudhary, Ph.D.,<sup>1</sup>  
and Michael J. McShane, Ph.D.<sup>2</sup>

### Abstract

#### Background:

Minimally invasive glucose biosensors with increased functional longevity form one of the most promising techniques for continuous glucose monitoring. In the present study, we developed a novel nanoengineered microsphere formulation comprising alginate microsphere glucose sensors and anti-inflammatory-drug-loaded alginate microspheres.

#### Methods:

The formulation was prepared and characterized for size, shape, *in vitro* drug release, biocompatibility, and *in vivo* acceptability. Glucose oxidase (GOx)- and Apo-GOx-based glucose sensors were prepared and characterized. Sensing was performed both in distilled water and simulated interstitial body fluid. Layer-by-layer self-assembly techniques were used for preventing drug and sensing chemistry release. Finally, *in vivo* studies, involving histopathologic examination of subcutaneous tissue surrounding the implanted sensors using Sprague–Dawley rats, were performed to test the suppression of inflammation and fibrosis associated with glucose sensor implantation.

#### Results:

The drug formulation showed 100% drug release with in 30 days with zero-order release kinetics. The GOx-based sensors showed good enzyme retention and enzyme activity over a period of 1 month. Apo-GOx-based visible and near-infrared sensors showed good sensitivity and analytical response range of 0–50 mM glucose, with linear range up to 12 mM glucose concentration. *In vitro* cell line studies proved biocompatibility of the material used. Finally, both anti-inflammatory drugs were successful in controlling the implant–tissue interface by suppressing inflammation at the implant site.

#### Conclusion:

The incorporation of anti-inflammatory drug with glucose biosensors shows promise in improving sensor biocompatibility, thereby suggesting potential application of alginate microspheres as “smart tattoo” glucose sensors with increased functional longevity.

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**Author Affiliations:** <sup>1</sup>Department of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Powai, Mumbai, India; and <sup>2</sup>Biomedical Engineering Department, Texas A&M University, College Station, Texas

**Abbreviations:** (CB) competitive binding, (DI) distilled, (FITC) fluorescein isothiocyanate, (FITCD) fluorescein isothiocyanate dextran, (FRET) fluorescence resonance energy transfer, (GOx) glucose oxidase, (LbL) layer-by-layer, (MW) molecular weight, (NIR) near-infrared, (PAH) poly(allylamine hydrochloride), (PBS) phosphate buffered saline, (PSS) sodium poly(styrene sulfonate), (Ru(dpp)) ruthenium-tris(4,7-diphenyl-1,10-phenanthroline) dichloride, (SIF) simulated interstitial fluid, (TRITC) tetramethyl rhodamine isothiocyanate, (TRM) tissue response modifier, (w/v) weight in volume

**Keywords:** alginate, anti-inflammatory drugs, controlled release, glucose sensing, layer-by-layer self-assembly

**Corresponding Author:** Rohit Srivastava, Ph.D., Department of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Powai, Mumbai 400076, India; email address [rsrivasta@iitb.ac.in](mailto:rsrivasta@iitb.ac.in)

## Background

One effective way to manage diabetes is intensive insulin therapy, requiring frequent glucose monitoring. Over the years, continuous glucose sensors have been combined with an insulin delivery system in an attempt to implement closed-loop control, which is considered to be the "Holy Grail" of diabetes management.<sup>1</sup>

The most advanced glucose sensors to date are based on an electroenzymatic sensing platform and exhibit excellent sensor properties; however, they are short-duration transdermal glucose sensors, and their functionality is only guaranteed for few days post-implantation. They have associated drawbacks of instability of the enzyme electrochemical system,<sup>2</sup> inaccuracy, dependence on unstable oxygen levels, low precision, requirement of extended warm-up period, and frequent calibration.<sup>3–7</sup> At present, self-monitoring of blood glucose remains the most common approach to glucose monitoring for the majority of people with diabetes. However, there are important limitations to this approach, which is further fueling the research for minimally invasive technologies.<sup>8</sup>

One of the most promising optical, minimally invasive techniques is the implantation of glucose biosensors in the subcutaneous tissue, also known as "smart tattoos."<sup>9–13</sup> These implantable fluorescent microparticle sensors are intended for injection directly into the dermis and are exposed to the interstitial fluid and measure local changes in glucose that are correlated with blood glucose levels.<sup>14–16</sup> Such implants may be interrogated noninvasively using simple optical instrumentation.<sup>17,18</sup> The various factors affecting the sensor performance include the photon migration through skin, different implantation depths, size distribution and concentration of microparticles, optical properties of tissue, refractive index, absorption, and scattering.<sup>17</sup> Such optical sensors work on the principle of competitive binding (CB) and fluorescence resonance energy transfer (FRET).<sup>12</sup> When glucose analog conjugated with a donor fluorophore is bound to Apo glucose oxidase (GOx) conjugated to acceptor fluorophore, there is energy transfer from donor to acceptor fluorescent dye. When glucose is introduced into the system, it displaces glucose analog from Apo GOx, resulting in a decrease in the FRET, which is, in turn, estimated to measure the glucose concentration.<sup>12</sup>

But a critical problem that remains with such implantable glucose sensors is the inflammatory response of the body to tissue injury on implantation as suggested from the

preliminary *in vivo* studies. 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.<sup>19</sup> Currently, the longest *in vivo* functionally active Food and Drug Administration-approved implantable glucose-monitoring biosensor works for only 5–7 days.<sup>20</sup> Therefore, it is very important to control the device-tissue interface to minimize localized inflammation and ensure sensor functionality over a longer period of time. There are many strategies that have been applied for improving the sensor biocompatibility and functionality *in vivo* to influence the tissue response.<sup>21</sup> Among various strategies, localized controlled delivery of tissue response modifiers (TRMs), alone or in combination with other surface modifications, are attractive approaches to controlling the host response. Various TRMs (dexamethasone,<sup>22–24</sup> transforming growth factor alpha, antifibroblast antibody, and vascular endothelial growth factor) have been used for these purposes.<sup>25</sup> Localized delivery of TRMs has the advantages of reduced systemic side effects and improved therapeutic response of the drugs.

The goal of this study was to develop the coimmobilized drug-sensor system that can concurrently deliver 100% anti-inflammatory drug encapsulated in alginate microspheres over a period of 3–4 weeks for improving biocompatibility and functionality *in vivo* the inflammatory response arising out of implantation of optical-based fluorescent "smart tattoo" biosensors and also sense the glucose continuously over a period of 1 month. Two different models of fluorescence glucose biosensors—GOx biosensors based on oxygen sensing and Apo GOx biosensors based on CB and FRET—were fabricated and used in this study as a model sensors.

## Materials and Methods

Low viscosity alginate [2% weight in volume (w/v), 350 cycles per second], diclofenac sodium [ $C_{14}H_{10}C_{12}NNaO_2$ ; molecular weight (MW) 318.14], dexamethasone-21-phosphate di-sodium salt (MW 517), GOx (G2133 from *Aspergillus niger*, type VII), fluorescein isothiocyanate dextran (FITCD; 70, 150, and 500 kDa), tetramethyl rhodamine isothiocyanate (TRITC; mixture of isomers, MW 443.53),  $\beta$ -D-Glucose (MW 180 Da), sodium poly(styrene sulfonate) (PSS; 70 kDa), poly(allylamine hydrochloride) (PAH, 70 kDa), phosphate buffered saline (PBS) tablets (0.1 M, pH 7.4), dimethyl sulfoxide (formula

weight 78.13), dimethylformamide (molar mass 73.09 g/mole), sorbitan trioleate 85, polyoxyethylene sorbitan trioleate 85, 2,2,4-tri-methylpentane (isooctane), and PD-10 columns were purchased from Sigma- Aldrich, India. Alexa Fluor-647, AF-750, dextran amino (500 kDa), and QSY-21 were purchased from Invitrogen, Molecular Probes, India. All chemicals were reagent grade and used as received.

### **Preparation of Glucose Biosensors**

#### Glucose-Oxidase-Based Alginate Microsphere Glucose Biosensors

The glucose sensors were prepared using a modified method acquired from Brown and colleagues.<sup>11</sup> The FITC-tagged GOx-loaded microspheres were prepared using a droplet generator as described, after which microspheres were loaded with oxygen-sensitive dye ruthenium-tris(4,7-diphenyl-1,10-phenanthroline) dichloride [Ru(dpp)] solution. Resuspension of the microspheres in neutral-pH aqueous solutions results in electrostatically mediated precipitation of the dye, leading to stable entrapment of Ru(dpp) inside the microspheres. The dye-doped microspheres were then rinsed with distilled (DI) water by consecutive centrifugation cycles. Poly(styrene sulfonate) and FITC-tagged PAH was alternately assembled on top of microspheres using the layer-by-layer (LbL) technique.<sup>26,27</sup> The sensors were imaged using a Olympus confocal microscope, and emission of Ru(dpp) and FITC were collected at 620 and 520 nm, respectively.

#### Apo-Glucose-Oxidase-Based Alginate Microsphere Glucose Biosensors

Apo GOx was prepared by removal of flavin adenine dinucleotide from GOx using the Swoboda protocol<sup>28</sup> as reported in our previous papers.<sup>29,30</sup> For visible dye sensors, the obtained Apo GOx was conjugated with TRITC dye using a standard amine labeling procedure,<sup>30</sup> as reported previously.<sup>29-31</sup> For near-infrared (NIR) dye sensors, dextran amino, which is a glucose analog having affinity for the glucose binding element Apo-GOx, was labeled with Alexa Fluor-647 donor dye, while the prepared Apo GOx was labeled with QSY-21 quencher acceptor dye and PAH polyelectrolyte was labeled with AF-750 reference dye using the standard amine labeling procedure.<sup>29</sup> Both visible dye sensors and NIR dye sensors were prepared by the emulsification technique as reported previously.<sup>30</sup> Subsequently, the core of alginate microspheres was partially dissolved using citrate treatment to provide free space inside alginate microspheres required for CB. The characterization of the dissolution process has already been reported in our previous work.<sup>29,30</sup> The polyelectrolyte coatings do not dissolve and thus stabilize the alginate

microspheres while simultaneously preventing the leakage of the sensing chemistry.

### **Sensor Response in Dissolved Core Alginate Microspheres**

The glucose response of alginate microspheres was tested in both DI water and simulated interstitial fluid (SIF).<sup>32</sup> A fluorescence spectrum of the dissolved-core alginate microspheres (600  $\mu$ l of diluted microsphere suspension) loaded with FITCD/TRITC Apo GOx complex dispersed in DI water/SIF was collected as the starting point. The effective dextran concentration inside the microspheres was calculated to be approximately 0.6  $\mu$ M, while that of Apo GOx was approximately 6  $\mu$ M. Fluorescence spectra were then collected after each addition of 3 to 60 mM  $\beta$ -D glucose solution to the microspheres. The percentage of change in FITC to TRITC peak intensity ratio (relative to the initial value without glucose) was calculated from each spectrum and plotted with respect to increasing glucose concentrations.

Identical experiments were conducted with NIR dye pair involving AF-647-dextran amino/QSY-21 Apo GOx complexes in DI water. Initially, a fluorescent emission scan was collected for the dissolved core alginate microspheres suspension loaded with AF-647-dextran amino and QSY-21 Apo GOx. Fluorescence spectra were then collected after titration of increasing concentrations of 3 to 60 mM  $\beta$ -D glucose solution into the alginate microspheres sample to observe the change in energy transfer.

### **Preparation of Anti-Inflammatory-Drug-Loaded Alginate Microspheres**

Drug-loaded calcium alginate microspheres were prepared using a droplet generator (Var J30, Nisco Engineering AG, Zurich). Briefly, 10 ml of 2% w/v sodium alginate solution was mixed with 250 mg/ml dexamethasone and 750 mg/ml diclofenac sodium salt, respectively, according to our previous paper.<sup>33</sup> Briefly, the mixture was extruded at a flow rate of 20 ml/h under 75 mbar pressures into a vessel containing 250 mM calcium chloride solution with continuous stirring. After allowing 10 min for the completion of external gelation, the hardened drug-loaded alginate microspheres were separated by centrifugation (1000 rpm for 1 min) and used for the experiments.

### **In Vitro Drug Release Study**

*In vitro* drug release studies were performed on uncoated microspheres and polyelectrolyte-coated microspheres using dialysis membrane with MW cutoff of 10–14 kDa.

Dexamethasone- and diclofenac-loaded uncoated and coated microspheres were transferred to a vessel containing 150 ml of 0.01 M PBS and SIF (pH 7.4) and 0.01% w/v sodium azide. The samples were incubated in a 37 °C incubator under sink condition for the release studies. At preset time intervals, the release medium was collected and replaced with a fresh buffer solution. The cumulative percentage release profile was obtained by taking the ratio of the amount of drug released to the total drug content and was determined spectrophotometrically at  $\lambda_{\text{max}}$  of 242 nm for dexamethasone and 236 nm for diclofenac. Statistical analysis of the data was performed using analysis of variance (single factor). Difference was considered significant when  $p < .05$ .

### *In Vitro Biocompatibility Studies*

The cytotoxicity of the uncoated, drug-loaded, (PAH/PSS)<sub>1</sub>-coated microspheres and Apo GOx and GOx alginate sensor were evaluated by sulforhodamine-B semiautomated assay using L929 mouse fibroblast cells (National Centre for Cell Science, Pune, India). Plain uncoated and unloaded microspheres were used as positive control as described in Jayant and associates.<sup>34</sup>

### *In Vivo Experiments to Assess Pharmacodynamic Changes*

All animal studies were conducted at Omega Laboratories (Lonand, Maharashtra, India) in accordance with the animal ethics committee and the committee for the purpose of control and supervision of experiments on animals committee guidelines using an approved protocol (Resolution no/03/09 of 2009). Food and water were provided to animals *ad libitum*. Sprague–Dawley Rats (weighing ~250 g) were caged in random pairs in polycarbonate cages and maintained in accordance with the standards set forth by the Animal Welfare Act. Animal were divided in five groups, and each group consisted of six rats. Three injections were made per animal, i.e., positive control, vehicle, and formulation. Rats were anesthetized with a 4.5% (volume per volume) mixture of isoflurane in oxygen. The microspheres were injected dorsally in shaved locations lateral to midline. Then 100  $\mu\text{l}$  of microspheres dispersed in vehicle were injected subcutaneously using 20 gauge needles. Microspheres were administered at 1 mg/ml dexamethasone and 1 mg/ml diclofenac dose per animal. Plain microspheres were used as a positive control, and untreated subcutaneous tissue samples were used as a negative control. Rats were sacrificed at each of the following time intervals: 7, 14, 21, and 30 days for controls, drug-loaded microspheres, and plain microspheres.

Standard hematoxylin and eosin staining protocols were used to characterize and quantify the inflammation-mediating cells in the vicinity of the microspheres in response to the inflammation induced by sensor implantation for longer duration of time period.

## Results

### *Alginate Microspheres Biosensor Fabrication*

#### Glucose-Oxidase-Based Alginate Microsphere Glucose Biosensors

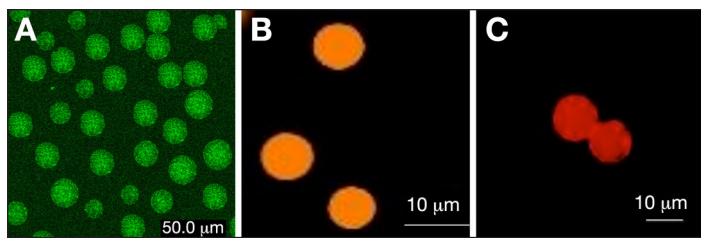
The FITC-tagged GOx-loaded microspheres as glucose sensors were fabricated with a size range of  $60 \pm 5\mu\text{m}$  and characterized using confocal microscopy. Results prove that GOx was successfully encapsulated as shown in **Figure 1A**; enzyme loading was calculated  $\sim 90 \pm 4\%$  (estimated by using Bradford assay). The GOx activity was monitored using a colorimetric assay based on the oxidation of o-dianisidine through a peroxidase-coupled system and the (PAH/PSS)<sub>1</sub>-coated microspheres lose 20% activity over a period of 4 weeks. Thus this proves that the application of LbL on top of the microspheres not only helps in drug release, but also enhances the enzyme retention in the matrix.

#### Apo-Glucose-Oxidase-Based Alginate Microsphere Glucose Biosensors

The alginate microspheres were in the range of 10–20  $\mu\text{m}$ . Representative fluorescence microscopy images of the alginate microspheres loaded with visible dye and NIR-dye-sensing assay are shown in **Figures 1B** and **1C**. For Apo GOx, visible dye sensors were imaged using an Olympus confocal microscope; the images were captured using a dual wavelength filter (488/550 nm).

### *Sensor Response in Dissolved Core Alginate Microspheres*

The dissolved core alginate microspheres loaded with FITCD (150/500 kDa)/TRITC Apo GOx complex were tested for glucose sensing both in DI water and SIF. A fluorescence spectrum was collected after each addition of 3 to 60 mM  $\beta$ -D glucose, and corresponding percentage change in FITC/TRITC peak ratio was calculated for each case. It can be observed from **Figure 2** that the glucose sensitivity of the encapsulated FITCD 150 kDa/TRITC Apo GOx assay in DI water was estimated to be 0.84%/mM glucose, while that in SIF was observed to be 0.9%/mM glucose, with an analytical response range of 0–40 mM glucose. The glucose sensitivity of the encapsulated FITCD 500 kDa/TRITC Apo GOx assay in DI water was estimated to be 0.33%/mM glucose, while that in SIF



**Figure 1.** Fluorescence microscopy images of alginate microspheres containing (A) FITC-tagged GOx sensor and (B and C) visible dye and NIR dye Apo GOx sensor.

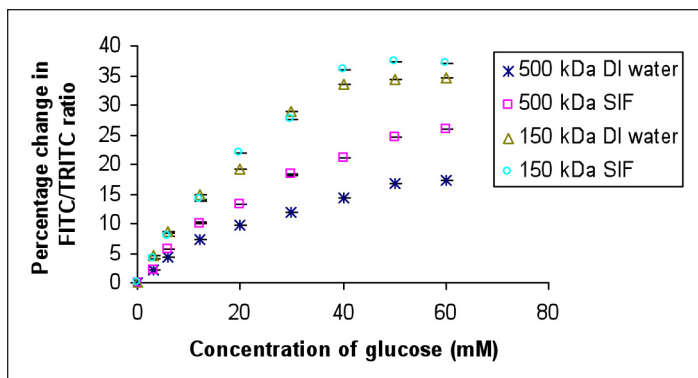
was observed to be 0.5%/mM glucose, with an analytical response range of 0–50 mM glucose. The response is observed to be linear up to 12 mM glucose concentration, which covers the range required to accurately predict glucose concentrations in hypoglycemia. The results have been summarized in **Table 1**.

Likewise, glucose sensitivity experiments were performed on the microspheres loaded with AF-647-dextran amino and QSY-21 Apo GOx complexes. These microspheres were coated with AF-750-labeled PAH polyelectrolyte to facilitate reference dye measurement. These microspheres were tested for glucose sensing both in DI water and SIF. The fluorescence spectrum was collected after each addition of 3 to 60 mM β-D glucose to microspheres. The AF-647 fluorescence was normalized with respect to the AF-750 peak fluorescence. It was observed that, with the addition of glucose, there is an increase in the AF-647 fluorescence, attributed to the increase in distance between AF-647 and QSY-21 due to which QSY-21 is no more able to quench the fluorescence of AF-647. It can be observed from **Figure 3** that the glucose sensitivity of the NIR sensor system suspended in DI water was estimated to be 0.73%/mM glucose with an analytical response range of 0–50 mM glucose, while that in SIF was observed to be 0.8%/mM glucose with an analytical response range of 0–50 mM glucose.

In both the cases, the response is observed to be linear up to 12 mM glucose concentration, which covers the range required to accurately predict glucose concentrations in hypoglycemia. In addition, the glucose sensor response was also investigated under controlled and dynamic conditions using a microflow cell unit (results are not shown here).

**Drug Loading and In Vitro Drug-Release Experiments**

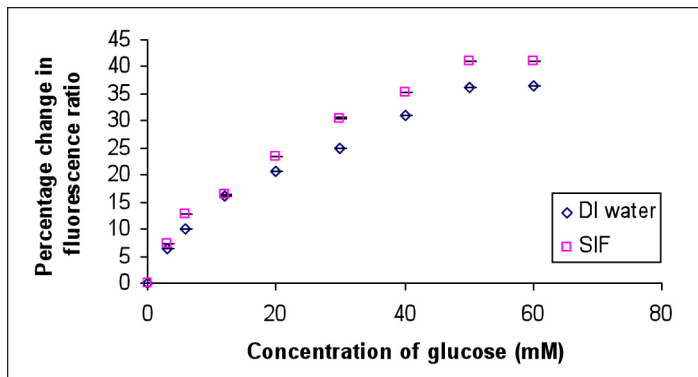
The average encapsulation efficiency for both drugs was approximately 77 ± 8%. The burst release observed for encapsulated dexamethasone for 0.25, 0.5, 0.75, and 1 mg/ml



**Figure 2.** Glucose sensitivity curve for visible dye sensor in DI water and SIF. Mean ± standard deviation (n = 5).

**Table 1.** Summary of Glucose-Sensing Results of Visible Dye Apo Glucose Oxidase Sensor (Alginate Microspheres Sensor Response)

Sensor system (alginate microsphere biosensor)	Response range (mM)		Glucose sensitivity (% change/mM glucose)	
	DI water	SIF	DI water	SIF
FITCD 150 kDa / TRITC Apo GOx	0–40	0–40	0.84	0.9
FITCD 500 kDa / TRITC Apo GOx	0–50	0–50	0.33	0.5



**Figure 3.** Glucose sensitivity curve for NIR sensor in DI water and SIF. Mean ± standard deviation (n = 5).

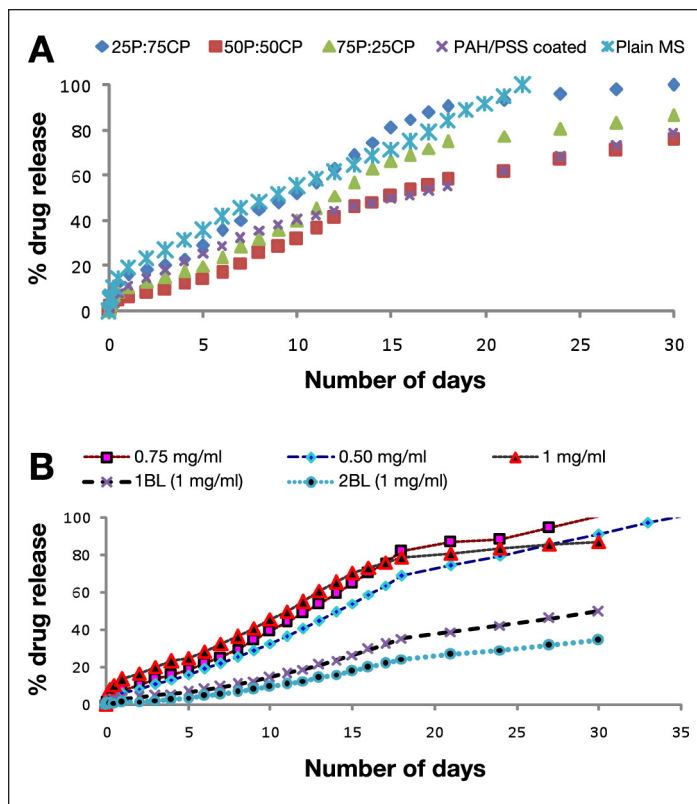
loaded microspheres was 19%, 24%, 28%, and 33%, respectively; 100 % cumulative release was observed in 22 days for microspheres loaded with 0.25 mg/ml dexamethasone, while other concentrations showed <80% drug release in 30 days. To achieve the desired release profile of 100% drug release in 30 days, drug-loaded microspheres were added in different ratio combinations of uncoated and polyelectrolyte-coated microspheres, i.e., 25C:75P, 50C:50P, and 75C:25P, where P stands for plain and C stands for polyelectrolyte coated particles; results for the same are summarized in **Table 2**.

Table 2. Summary of Drug Loading and <i>In Vitro</i> Drug Release <sup>a</sup>			
Formulation	Drug loading (%)	Burst release % (duration in days)	Controlled release % (duration in days)
Uncoated DEXA MS (0.25 mg/ml)	77 ± 8%	19% (1)	100% (22)
(PAH/PSS) <sub>1</sub> coated DEXA (0.25 mg/ml) MS		11% (1)	79% (30)
(25P+75CP) DEXA (0.25 mg/ml) MS		6.5% (1)	100% (30)
(50P+50CP) (0.25 mg/ml) DEXA MS		10.5% (1)	86.5% (30)
(75P+25CP) (0.25 mg/ml) DEXA MS		16.5% (1)	75.7% (30)
Uncoated Diclo MS (0.50 mg/ml)	79 ± 5%	8.5% (2)	100% (30)
Uncoated Diclo MS (0.75 mg/ml)		11.5% (2)	100% (35)
Uncoated Diclo MS (1 mg/ml)		20 % (2)	86.7% (30)
(PAH/PSS) <sub>1</sub> coated (0.75 mg/ml) Diclo MS		9.5% (2)	50.9% (30)
(PAH/PSS) <sub>2</sub> coated (0.75 mg/ml) Diclo MS		9.0% (2)	34.1% (30)

<sup>a</sup> MS, microspheres; DEXA, dexamethasone; Diclo, diclofenac; P, uncoated MS; CP, polyelectrolyte coated

In the case of diclofenac-loaded microspheres, different concentration of drugs, i.e., 0.5, 0.75, and 1 mg/ml, were used as initial drug loading concentrations, which showed 12%, 9%, and 8%, burst release rates, respectively. A 30-day period shows a cumulative release of 87% for 1 mg/ml and 100% for 0.75 mg/ml. In the case of 0.5 mg/ml drug loading, 100% cumulative drug release was achieved in only 24 days; results for both the drugs is shown in **Figure 4B**. Results for polyelectrolyte, i.e., (PAH/PSS)<sub>1</sub>- and (PAH/PSS)<sub>2</sub>-coated microspheres, is also shown in **Figure 4B**, which shows a cumulative drug release of 86.70%, 50.92%, and 34.11%, respectively, in 30 days. There was a significant ( $p < .05$ ) difference in the rate and extent of drug release when comparing uncoated and coated microspheres as shown in **Table 2**.

The release profiles of both drugs showed zero-order release kinetics after a burst release period, which lasted for 1 day. The data were fitted to kinetic equations such as zero-order kinetic release equation. The value of regression coefficient ( $R^2$ ) for uncoated and various polyelectrolyte-coated microspheres indicated that drug release followed the diffusion control mechanism.



**Figure 4.** (A) Comparative release profile of dexamethasone-loaded uncoated and (PAH/PSS)<sub>1</sub>-coated alginate microspheres. (B) Comparative release profile of uncoated and (PAH/PSS)<sub>1,2</sub>-coated diclofenac-loaded alginate microspheres in PBS (pH 7.4) containing sodium azide (0.01%) at 37 °C. Mean ± standard deviation ( $n = 3$ ).

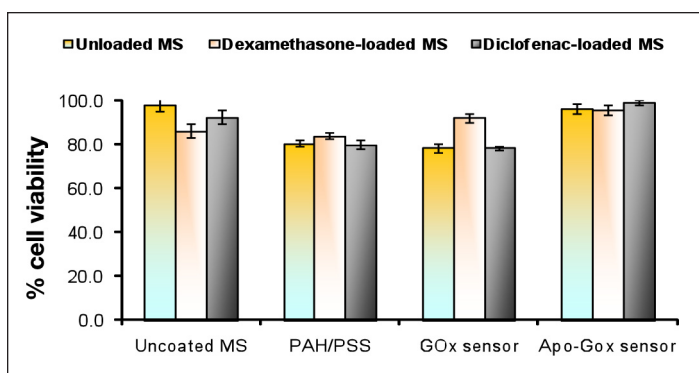
Studies were also conducted in SIF that mimicked the release behavior inside the body. Cumulative release of 96.5% and 97.2% was observed as compared to 100% in 30 days with 25P:75C combination of dexamethasone loaded (0.25 mg/ml) and diclofenac loaded (0.75 mg/ml), respectively.

### *In Vitro* Biocompatibility Studies

*In vitro* biocompatibility of drug-loaded polyelectrolyte-coated and uncoated alginate microspheres and Apo GOx and GOx sensor was evaluated using L929 mouse fibroblasts cell line. The relative percentage of viability of the cells was approximately 100% with uncoated alginate microspheres and ~80% for our formulation compared to controls, indicating no cytotoxicity. Both types of sensor also showed good percentage of viability as shown in **Figure 5**.

### Results of *In Vivo* Experiments to Assess Pharmacodynamic Changes

A localized delivery of anti-inflammatory agent reduces the immunostimulatory cascade of events and eases the wound-healing process. To investigate the effect of

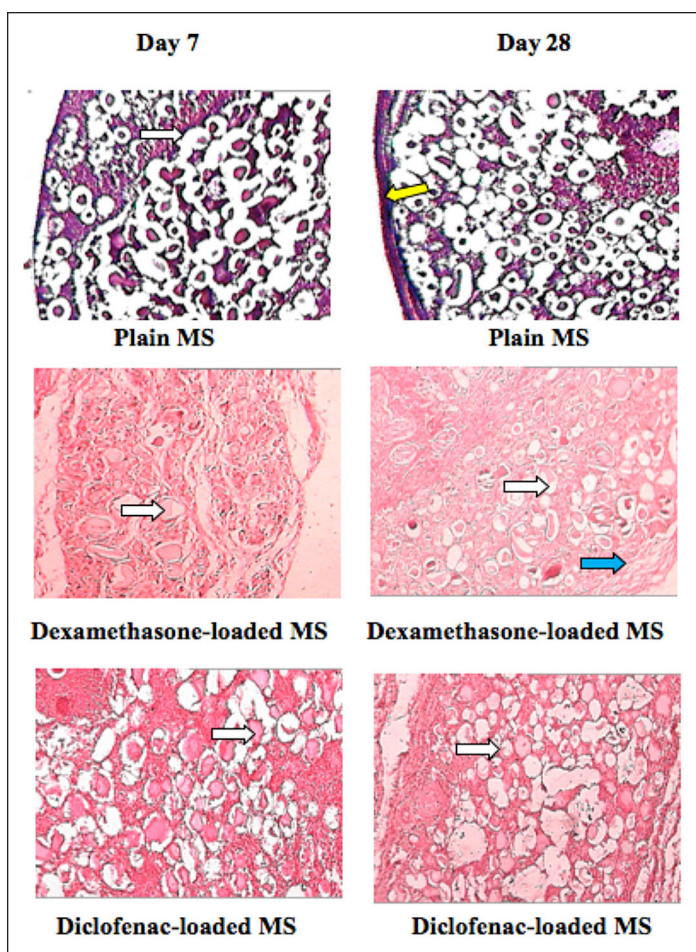


**Figure 5.** Percentage of cell viability for alginate microspheres using L929 mouse fibroblast cell line. Cells were cultured for 48 h.

the dexamethasone and diclofenac sodium salts on the local tissue environment, the following controls were studied: (1) drug-free microspheres, (2) combination of coated (C) and uncoated (P) microspheres in the ratio of 25P:75CP with dexamethasone, (3) diclofenac (0.75 mg/ml)-loaded microspheres, (4) GOx-loaded microspheres, (5) combination of GOx-loaded microspheres with dexamethasone and diclofenac-loaded microspheres in 50:50 ratios, and (6) combination of Apo-GOx-sensor-loaded microspheres with dexamethasone- and diclofenac-loaded microspheres in 50:50 ratios.

Tissue samples surrounding the microspheres containing no drug displayed negative response associated with the implantation procedure as shown in **Figure 6**. Plain (unloaded) microspheres were used as positive control, and by the end of 4 weeks, a very thick fibrotic capsule formed that entirely surrounded the implant, as observed in **Figure 6A**. On the other hand, drug-releasing microspheres prevented the inflammatory response and stopped the progression into chronic inflammatory phase, which was evident by a decrease in inflammatory cells and absence of fibrotic tissue around the implant on week 4 (**Figures 6B and 6C**).

To prove the efficacy of the drug formulation in suppressing the inflammation upon sensor/implant implantation, sensor samples were mixed with drug-loaded microspheres in 50:50 ratios and were implanted subcutaneously. Samples were taken periodically every week for 4 weeks. The GOx and Apo GOx microspheres with no drug were taken as positive control. As expected, upon plain sensor implantation, very high immunostimulatory response in the surrounding area near the sensor–tissue interface was observed as shown in **Figures 7A and 7B**. The inflammatory response to the sensors was significantly more severe than that for plain microspheres alone as shown in **Figure 6A**. But, upon



**Figure 6.** Pharmacodynamic changes in representative Sprague–Dawley rat subcutaneous tissue sections on 7 and 28 days. Inflammation-mediating cells and normal cells are stained purple and pink, respectively (hematoxylin and eosin staining). The white arrows show alginate microspheres, the yellow arrow shows connective tissue capsule, and the blue arrow shows mononuclear cell infiltration (leucocytes).

mixing sensors with drug loaded formulations, was no sign of inflammation was found after 4 weeks as shown in **Figures 7C–F**.

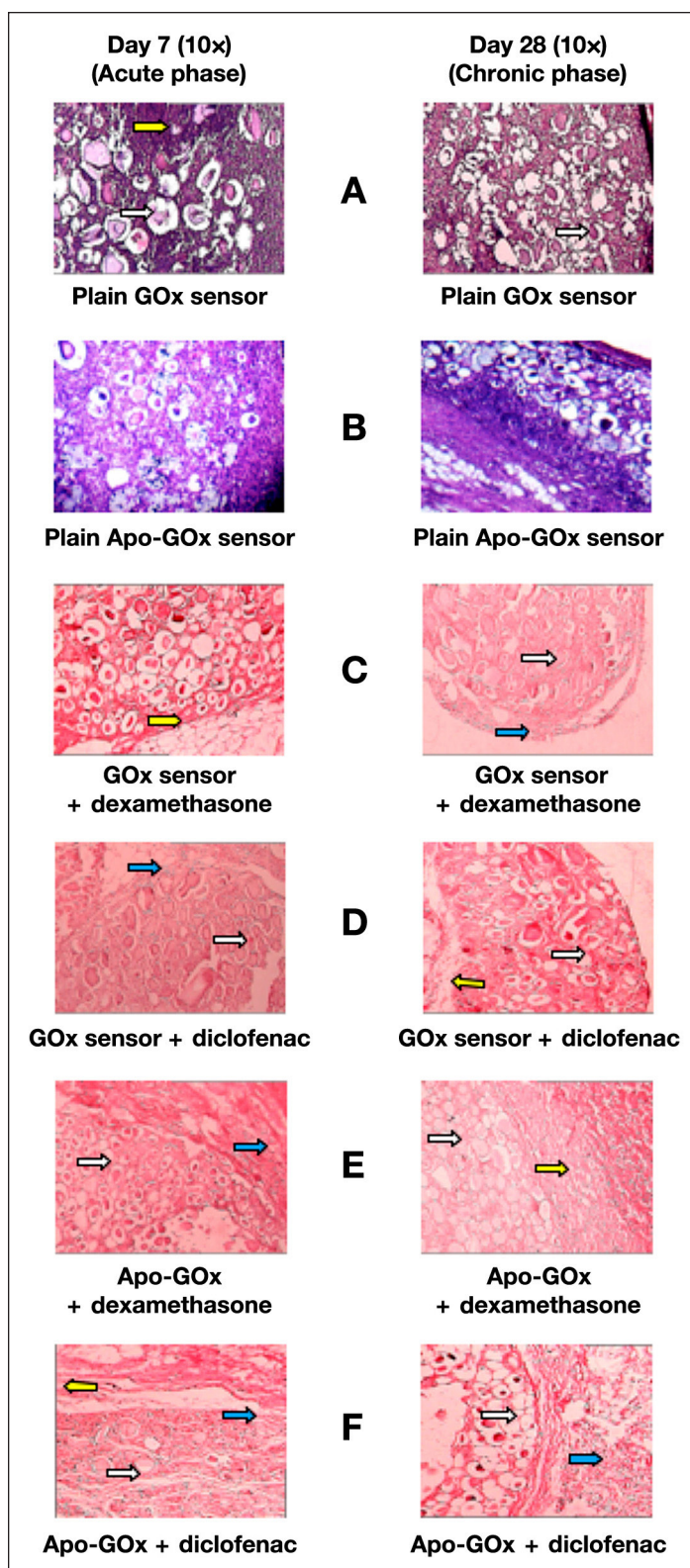
## Discussion

The GOx enzyme was successfully encapsulated into alginate microspheres, to be used as implantable glucose biosensors, and FTIR and confocal microscopy were used to confirm the encapsulation of GOx and Ru(dpp) and FITC dye in alginate microspheres. The (PAH/PSS)<sub>1</sub>-coated enzyme-loaded microspheres show less leaching of enzyme and good enzyme stability as compared to uncoated and other polyelectrolyte coatings pairs (results not shown). *In vitro* biocompatibility studies also showed good percentage of viability for GOx-loaded sensor. In the case of the Apo GOx sensor, citrate treatment was done to create the free space between the cross-linked alginate

microspheres for the free movement of sensing assay. The citrate (sodium citrate-Tris-HCl solution)-treated alginate microspheres showed uneven distribution of fluorescence, which clearly indicates a change in the structure and fluorescence-sensing assay distribution, thus confirming the dissolution of alginate microsphere core. The dissolved-core alginate microspheres were designed to decrease the time response as well as to increase the glucose response sensitivity by partially dissolving  $Ca^{2+}$  cross linkages to provide free space inside alginate microspheres required during CB.

A difference was observed in the glucose sensitivity for 150/500 kDa MW FITCD molecules for the encapsulated FITCD/TRITC Apo GOx system. The 500 kDa FITCD system exhibited a wider range of response but lower sensitivity than the 150 kDa FITCD system. These differences are due to the number of glucose residues present. A higher MW of FITCD molecule has a longer chain with more saccharides that can bind more TRITC Apo GOx molecules. Thus, for the same molar concentration of dextran, the longer chains have more glucose residues and more free glucose is required to displace the same amount of dextran. This effectively decreases the sensitivity to glucose and simultaneously increases the glucose-sensing range. In case of NIR dye sensors, it was observed that, with the addition of glucose, there is an increase in the AF-647 fluorescence, attributed to the increase in distance between AF-647 and QSY-21 due to which QSY-21 is no more able to quench the fluorescence of AF-647. Studies done in a dynamic continuous flow-through system also suggest that these dissolved core alginate microsphere glucose sensors are stable under both static and dynamic flow conditions. The glucose response sensitivity under dynamic conditions was found to be comparable to the steady state glucose response reported earlier, when compared statistically using Student’s paired *t*-test ( $\alpha = 0.05$ ). Sensing studies conducted in SIF shows very comparable results with DI water sensing results, thus suggesting that the calcium ions present in the SIF do not result in substantial recrosslinking of residual alginate so as to interfere with the response to glucose.

For *in vitro* release studies, drug-loaded uniform-size alginate microspheres were produced by a commercially available droplet generator and tested for their *in vitro* release behavior as well as their *in vivo* efficacy. The most important optimized parameters affecting release behavior were identified and optimized. The  $\zeta$ -potential values clearly demonstrated that the surface charge of the microspheres reverses upon coating of alternately charged



**Figure 7.** Pharmacodynamic changes in representative Sprague–Dawley rat subcutaneous tissue sections on 7 and 28 days. Inflammation-mediating cells and normal cells are stained purple and pink, respectively (hematoxylin and eosin staining). The white arrows show alginate microspheres, the yellow arrows show connective tissue capsules, and the blue arrows show mononuclear cell infiltration (leucocytes).



PAH/PSS coating, proving that multilayer buildup is taking place (results not shown here). The LbL self-assembly technique helps in reducing the initial burst of drug and also prevents enzyme leaching from the microspheres. For our system requirement, the desired system is expected to achieve complete release of the drug within a time period of 3–4 weeks to overcome the inflammatory response of the body to the implantable glucose sensor. Thus, in order to achieve an approximate zero-order release profile and 100% drug release over a period of 1 month to combat localized inflammation, the drug-loading demonstrating lowest burst release was chosen for further studies. As we know, release profiles can be altered by selection of polymer, particle size, drug loading, and surface charge, so to achieve 100% drug release over a period of 1 month with zero-order release kinetics, different concentrations of dexamethasone and diclofenac were used in the precursor alginate solution, and it was observed that the percentage of drug release was significantly affected by changes in drug content. As the drug content increases, there is influence on both types of release (i.e., the cumulative amount of drug released at any time, such as burst release) and the total percentage of drug released during the induction period. Results suggest that high drug loading is not required, and optimal amount of drug will serve the requirements of the desired system. The release profiles can be altered by selection of polymer, particle size, and surface along with drug-matrix interactions within the system. The main problem of any controlled drug delivery system is initial burst release, to overcome this problem and to maximize the amount of drug to be released in the induction period. Release studies in SIF showed no significant difference in the release patterns as compared to PBS. Hence it was concluded that microspheres would release the drug at the rate and kinetics as determined *in vitro*. Cell culture results of all formulations showed good adhesion, growth, morphology, and percentage of viability of cells on uncoated, polyelectrolyte-coated, GOx, and Apo GOx alginate sensor, hence proving the biocompatibility of the formulations and the material used for the same.

To improve the sensor functionality, as mentioned earlier, site-specific localized and controlled delivery of TRM can be used to control the tissue-implant response. To test this theory and to evaluate the efficacy of drug formulations, drug-loaded microspheres along with sensors were injected subcutaneously and histopathologic changes at the implant site were compared with positive control. As reported by Wisniewski and Reichert,<sup>21</sup> the main reason for implantable sensor malfunction is the issue

associated with healing of the tissue surrounding the implanted device, such as inflammation, encapsulation, and wound repair. *In vivo* results show that coimplantation of drug-loaded carriers along with the sensor helps in controlling the immunostimulatory response upon sensor implantation. Also, these results clearly confirm that site-specific local delivery of anti-inflammatory drugs not only prevents the negative immunogenic response to the sensor, but also increases the *in vivo* acceptability of the implanted biosensor.

## Conclusion

Drug-loaded alginate microspheres were tested for their *in vitro* release behavior and associated *in vivo* effectiveness in limiting inflammation to assist in development of a "smart tattoo" glucose biosensor. It was observed that nanofilm coatings help lower the burst release and adjust the long-term drug release to follow approximately zero-order release kinetics over a 4-week period. *In vitro* cell culture studies proved that the materials involved are not toxic and showed acceptable percentage of viability in all the samples tested. These properties, while sufficient to enable the *in vivo* studies here, can be further optimized with more investigation of coating materials and assembly procedures. *In vivo*, concurrent release of anti-inflammatory agents was observed to effectively reduce inflammation and also inhibit fibrosis at the implant site. Most interestingly, even with reactive implants that consume substrate and release potentially toxic byproducts (e.g., enzymatic sensors), this combination of anti-inflammatory agents managed the host response to remain at levels equivalent to those observed for nonreactive implants. Hence the strategy of combining drug release with sensor implantation showed an apparent decrease of the "break-in" period, which should generally enhance the sensor acceptability and functionality. As a result, this approach of localized delivery of TRMs is a promising approach to controlling the tissue-biosensor interface and is worthy of further consideration in expanded studies alongside *in vivo* sensor testing.

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