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In vitro and *in vivo* evaluation of anti-inflammatory agents using nanoengineered alginate carriers: Towards localized implant inflammation suppression

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ABSTRACT

The aim of this research was to develop nanoengineered alginate microspheres for localized delivery of anti-inflammatory drugs (dexamethasone and diclofenac sodium) for implantable "Smart tattoo" glucose biosensor used for continuous glucose monitoring. The formulation was prepared and characterized for *in vitro* drug release from uncoated and polyelectrolyte-coated microparticles. Biocompatibility was then tested using L929 cell-line; pilot *in vivo* studies with Sprague–Dawley (SD) rat subjects were performed to test the suppression of inflammation and fibrosis associated with implantation and was analyzed using standard hematoxylin and eosin staining method. The drug-loaded microspheres were able to deliver the drug for 30 days at a controlled rate with zero-order kinetics. The layer-by-layer self-assembly technique was used to effectively limit the burst release of drug from the matrix. Cell culture studies prove that the material are not cytotoxic and showed acceptable >80% cell viability in all the tested samples. *In vivo* studies show that both drugs were successful in controlling the implant/tissue interface by suppressing inflammation at the implant site. It was clearly evident that the combined approach of drug loaded carriers along with implanted biosensor shows promise in improving sensor biocompatibility and functionality. Thus, suggesting potential application of alginate microspheres as "smart-tattoo" glucose sensors.

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1. Introduction

Currently, most diabetics measure their blood glucose concentrations by intermittent "finger-prick" capillary blood sampling, a method that is painful and uncomfortable. The development of technology for minimally or non-invasive and continuous glucose sensing is, therefore, considered a priority in diabetes care (Abel and Woedtke, 2002). Interest in implantable biosensors has gained popularity owing primarily to the continuous monitoring capability that aids in identifying trends in glucose excursions, and enables patients to take pre-emptive action to avoid dangerous hypoglycemia. Continuous blood glucose monitoring is also becoming a more integral part of diabetes management for understanding specific patient glucose patterns and aids in determining dosage frequency and time of administration of insulin dosage. However, there are significant problems with the performance of commercially available subcutaneously implanted *in vivo* glucose sensors, including inaccurate results, low precision, and requirements for frequent calibration (Pickup, 2004; Gilligan et al., 2004).

Another key issue with the use of these devices is the series of inflammatory events generated in response to tissue injury during implantation. This generally results in compromised device functionality and subsequent device failure (Sharkawy et al., 1997, 1998a,b). During the initial acute response, fluid carrying plasma proteins and inflammatory cells migrate to the implanted site. In many cases, proteins adsorb to the implant surface and then phagocytic cells (neutrophils, monocytes and macrophages) surround the biosensor, affecting its functionality (Anderson, 2001). Phagocytosis is the foremost body defense occurring upon implantation, since for large implants phagocytosis is not possible, the cells attack these implants by the release of reactive oxygen species and enzymes that are intended to degrade the implant. The exact timing, action, and intensity of the process are dependent on the nature of the foreign body, size, shape, and physical and chemical properties (Gerritsen, 2000). The acute response lasts about three days, after which a chronic inflammatory response may set in or a modified version of the healing process begins (Anderson, 2001; Gerritsen, 2000). Eventually, a fibrotic capsule is formed, which is a characteristic feature of the steady-state foreign body response. Fibrous encapsulation can impede transport of glucose and fluid to sensor causing compromised device functionality. Calcification and protein fouling of implanted biosensors may occur (Wisniewski and Reichert, 2000; Wisniewski et al., 2000) and these processes induce the outer mem-

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brane degradation and lead to increased permeability of sensing elements.

Currently, the longest *in vivo* functionally active FDA-approved implantable glucose-monitoring biosensor works for only seven days (Buckingham et al., 2007). 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* such as physical modifications to influence the tissue response or surface modification (Wisniewski and Reichert, 2000) of the biosensor e.g. hydrogels with polyethylene oxide (PEO)/polyethylene glycol (PEG) have been suggested to improve sensor stability by forming a layer of water that interacts with the hydrophilic coating, preventing protein penetration to the surface (Quinn et al., 1997). Biomimicry agents such as phospholipids and similar biomaterials have been used to increase the biocompatibility of sensors (Lewis, 2000). Flow-based systems are also used to prevent the biofouling by flowing fluid over the material-tissue interfaces (Rigby et al., 1996). Many other strategies have been tried e.g. Nafion (perfluorosulfonic acid polymer) membrane as biosensor coating (Moussy et al., 1994), surfactant-based modification (Neff et al., 1998), membranes derived from natural origin e.g. cellulose, chitosan, alginate and heparin.

Among all strategies, localized controlled deliveries of tissue response modifiers (TRMs) – alone or in combination with surface modifications – are attractive approaches to controlling the host response. Various TRMs dexamethasone (Norton et al., 2005; Hickey et al., 2002; Galeska et al., 2005), transforming growth factor alpha (TGF-α), anti-fibroblast antibody, vascular endothelial growth factor (VEGF) have been used for these purposes (Buckingham et al., 2007; Zolnik and Burgess, 2008). Localized delivery of TRM's has the advantages of reduced systemic side effects and improved therapeutic response of the drugs. Kroll and Tchou (2000) have used a small reservoir of dexamethasone at the tip of pacemaker to prevent the deposition of proteins on the surface. Similarly, sensors could also incorporate a layer that would slowly degrade *in vivo* and release the drug as it happened in drug eluting stents. Burgess et al. have used degradable microspheres for site specific, controlled delivery of both small molecular weight drugs (e.g. dexamethasone) (Hickey et al., 2002; Galeska et al., 2005) and proteins, including VEGF (Buckingham et al., 2007). Recently, poly(vinyl alcohol) hydrogels have been used for enhancement of glucose sensing (Vaddiraju et al., 2009). But, the main disadvantages with the above mentioned strategy is their inability to provide 100% drug release combined with a low burst release. Therefore, research is needed in preparing a system that has better control over drug release profile for longer duration typically more than 3–4 weeks so that maximum amount is released in the induction period.

Thus, the goal of this study was to a develop the system that can concurrently deliver 100% drug (anti-inflammatory) encapsulated in alginate microspheres over a period of 3–4 weeks to overcome the inflammatory response arising out of implantation of optical based luminescent "smart tattoo" biosensors used for continuous glucose monitoring (Russell et al., 1999; McShane et al., 2000; McShane, 2002; Brown et al., 2006; Russell et al., 1999; Chinnayelka and McShane, 2006). Layer-by-layer (LBL) self-assembly technique (Decher et al., 1994a,b) was used for minimizing the burst release and to achieve controlled release of encapsulated drug while retaining the sensing chemistry. Different formulations with controls (i.e. Plain microspheres (positive control), drug loaded coated and uncoated microspheres, glucose oxidase based (GOx) sensors and dexamethasone loaded (dexa) and diclofenac loaded (diclo) formulation with the glucose sensors) were used for the evaluation for anti-inflammatory efficacy in Sprague–Dawley (SD) rats using standard hematoxylin and eosin (H&E) staining methods.

2. Materials and methods

Low viscosity alginate (2%, 250 cps), diclofenac sodium salt (MW-318.13), dexamethasone-21-phosphate di-sodium salt (MW-516.4), and glucose oxidase (G2133 from *Aspergillus niger*, Type VII) were purchased from Sigma, India. Phosphate buffer saline tablets (PBS tablets, pH-7.4) and polyelectrolytes, including sodium poly(styrene sulfonate) (PSS, 70 kDa), poly(allylamine hydrochloride) (PAH, 70 kDa), poly(acrylic acid) (PAA, 45 kDa) and poly(diallyldimethylammonium chloride) (PDDA, 20–35 kDa) were also purchased from Sigma, India. Sodium azide was purchased from Loba Chemie, Mumbai (India). Calcium chloride and dialysis membrane (10–14 kDa) were purchased from Merck, Mumbai (India) and Hi-Media Laboratories, Mumbai (India), respectively. All chemicals were reagent grade and used as received.

2.1. Instrumentation

Encapsulation unit Variation J30 (Nisco Engineering AG, Zurich, Switzerland) and syringe pump (Multi-PhaserTM, model NE-1000, New Era Pump Systems, New York, USA) has been used for preparing alginate microspheres. "Cuvette" Helos (Sympatec, CUV-50ML/US, Germany) was used for particle sizing based on dynamic light scattering principles. Nikon YS 100 (Melville, New York, USA) optical microscope with a digital camera and scanning electron microscope (Hitachi S3400, Tokyo, Japan) were used for microscopic imaging studies. Zeta potential of uncoated and coated microspheres was measured using Zetaplus (Brookhaven Instruments, New York, USA). Helios Alpha double beam UV-VIS spectrophotometer (Thermo Scientific, Surrey, UK) was used for quantifying drug release.

2.2. Preparation of 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 (Jayant and Srivastava, 2007). The mixture was then extruded at a flow rate of 20 ml/h under 75 mbar pressures into a vessel containing 250 mM calcium chloride solution while continuously stirring. After allowing 10 min for the completion of external gelation, the hardened drug loaded alginate microspheres were separated and washed by centrifugation (1000 rpm for 1 min).

2.3. Preparation of glucose sensors

The glucose sensors were prepared using a modified method acquired from Srivastava et al. (2005a,b). Glucose oxidase (GOx) loaded microspheres as glucose sensors were prepared using the droplet generator as described, after which microspheres were placed in ruthenium-tris (4,7-diphenyl-1,10-phenanthroline) dichloride (Ru(dpp)) solution (3.5×10^{-5} M) in a 10 vol.% methanol in water solution at pH 12 for 3 h. 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 de-ionized (DI) water by consecutive centrifugation cycles. Poly(styrene sulfonate) and fluorescein isothiocyanate (FITC) tagged-poly(allylamine hydrochloride) was alternately assembled on top of microspheres as described by Srivastava et al. (2005a,b).

2.4. Characterization of microspheres

The mean particle size and size distributions of the microspheres were measured using optical microscopy, scanning electron microscopy (SEM), and particle sizing systems (Sympatec, Cuvette Helos, CUV-50ML/US). A small volume of suspended microspheres, usually 0.5 or 1 ml was added to 50 ml of continuously stirred distilled water. Each measurement reported is the mean and standard deviation of three samples per batch of microspheres for at least three different batches. The sensors were imaged using a Olympus confocal microscope, emission of Ru(dpp) and FITC were collected at 620 and 520 nm, respectively.

2.5. Drug loading efficiency

Drug loading efficiency was calculated using the standard calibration curve of both drugs (dexamethasone and diclofenac), which were plotted to define the quantitative relationship between observed absorbance and concentration of the drug. For this, the amount of drug present in the supernatant after centrifugation was determined by UV-spectrophotometry at λ_{max} of 242 nm (dexamethasone) and 236 nm (diclofenac). All the measurements were completed in triplicate and the mean values and standard deviations were calculated.

2.6. Polyelectrolyte coating of alginate microspheres

The LbL coatings were analyzed using an FTIR spectrometer wherein the microspheres were completely dried and mixed with potassium bromide before measurements. Further confirmation of polyelectrolyte nanofilms assembly was confirmed using Zeta potential analysis. The ζ-potential was calculated from the electrophoretic mobility using the Smoluchowski relation. For this, 50 μl sample solution containing the polyelectrolyte coated microspheres was diluted in 2 ml of distilled water and analyzed using Zetaplus.

2.7. In vitro drug release study

Dexamethasone and diclofenac (sodium salt) loaded alginate microspheres were prepared as described previously (Jayant and Srivastava, 2007; Jayant et al., 2009). The microspheres were introduced in dialysis membrane (molecular cutoff: 10–14 kDa) and transferred to a glass beaker containing 100 ml of 0.01 M PBS (pH 7.4) and 0.01% (w/v) sodium azide. The samples (in triplicate) were incubated at $37 \pm 0.5\,^{\circ}$C with constant agitation of 250 rpm for the release studies. The 250 rpm agitation speed was chosen in order to suspend majority of the microspheres in the dialysis membrane to avoid their agglomeration. 1 ml of buffer was periodically withdrawn from the beaker and replaced to maintain the drug concentration below 10% solubility (sink condition). The amount of released drugs in the collected medium was determined spectrophotometrically at λ_{max} of 242 nm for dexamethasone and 236 nm for diclofenac. All *in vitro* release studies were conducted in triplicate, mean values and standard deviations were calculated for all measurements.

2.8. In vitro biocompatibility studies

The cytotoxicity of the uncoated, drug loaded, $(PAH/PSS)_1$ coated alginate microspheres and GOx based glucose sensor was evaluated by using sulforhodamine-B (SR-B) semi automated assay using L929 mouse fibroblast cells (National Centre for Cell Science (NCCS), Pune, India). Plain uncoated and unloaded microspheres were used as positive control. 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) at 37 °C temperature under 5% CO_2 and saturated humid environment. Nearly confluent cells in 25 cm^2 tissue culture flasks were trypsinized by trypsin-EDTA (ethylene diamine tetra acetic acid) solution and centrifuged at $1000 \times g$ for 10 min. 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 triplicate using 96 well plates, 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. 10 μl of diluted polyelectrolyte-coated and uncoated alginate microspheres were added after 24 h incubation 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 50 μl of chilled 50% TCA (trichloroacetic acid) for cell fixation. Plates were kept in the refrigerator (4 °C) for 1 h, followed by thorough washing with water and air drying. For the assay, plates were then stained with 50 μl of 0.4% SR-B for 20 min then washed 5× with 1% acetic acid and air dried. Finally the bound SR-B was eluted with 100 μl of tris (10 mM, pH 10.5) for 10 min. 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 540 nm with reference to 690 nm against blanks culture media without any cells.

2.9. In vivo experiments to assess pharmacodynamic changes

All animal studies were conducted at Omega Laboratories (Lonand, Maharashtra, India) using an approved protocol in accordance with Animal Ethics Committee (AEC) and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) committee guidelines (Resolution No/03/09 of 2009). Food and water were provided to animals *ad libitum*. Microspheres were prepared and suspended in a viscosity enhanced diluent (30 mg/ml carboxymethylcellulose sodium salt, 9 mg/ml NaCl and sterile water) to prevent microspheres from sticking in syringes/needles during injection. Animals were divided in 5 groups and each group was consisted of 6 rats. Three injections were made per animal i.e. positive control, vehicle and formulation. Rats were anesthetized with a 4.5% (v/v) mixture of isofluorane in oxygen. The microspheres were injected dorsally in shaved locations lateral to midline. 100 μl 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 and plain microspheres.

2.10. Pharmacodynamic study of drug loaded microspheres

Tissue samples from the experiment described above were fixed in 10% formalin, embedded in paraffin, and cut using a microtome (5 μm). Standard hematoxylin and eosin (H&E) staining protocols were used. This method was 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. Inflammation mediating cells and blood vessels were counted in chosen section of equal part in each photomicrograph taken. For inflammation cell counts, the final values reported are an average from 5 to 6 regions counted in photomicrographs from five different rats per time point ± standard

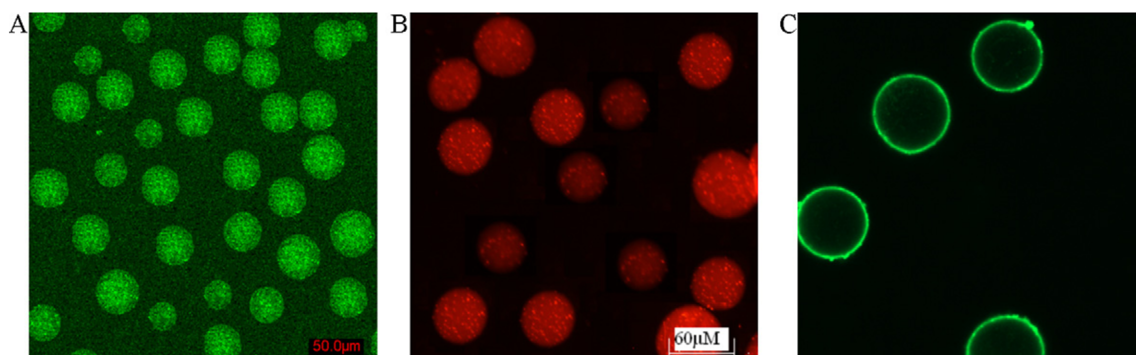

Fig. 1. Confocal images of (A) FITC tagged GOx loaded microspheres and (B) Ru(dpp) loaded uncoated alginate microspheres and (C) PAH-tagged FITC/PSS coated alginate microspheres.

deviation. Photomicrographs of the histology slides were taken and digitally stored using a microscope at 10–100× magnification. Visual counting of the purple stained nuclei of the inflammatory cells was performed to reduce error.

2.11. Statistical analysis

Pearson correlation coefficients were used to evaluate the extent of a relationship between two data sets. Coefficients of determination were calculated. Statistical differences among groups were analyzed using ANOVA. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Preparation and characterization of microspheres

Drug loaded (dexamethasone and diclofenac) loaded alginate microspheres were prepared using the droplet generator technique as described earlier by Jayant and Srivastava (2007). The particle sizing confirms that particles were in the range of 60 ± 10 μm (Saurter mean diameter, SMD at 90% cumulative distribution), further microsphere size were confirmed using optical microscopy and scanning electron microscopy (SEM) and it was observed that particles were in the size range of 60 ± 10 μm ($n > 400$). The ζ-potential value for uncoated alginate microspheres was −28.0 ± 1 mV, which reversed upon addition of positive polyelectrolyte, PAH to +34 ± 2 mV and which again reversed on addition of PSS to −25.0 ± 2 mV, confirming that nanofilm assembly is taking place. The FITC tagged GOx loaded microspheres as glucose sensor (model sensor) were fabricated using above mentioned protocol. They were then characterized using confocal microscopy, from which it was observed that GOx was successfully encapsulated inside alginate matrix as shown in Fig. 1(A). Also oxygen sensitive dye Ru(dpp) was encapsulated in the alginate microspheres uniformly as shown in Fig. 1(B) and (C) shows the deposition of reference dye (FITC) within LbL self-assembly.

3.2. Drug loading and in vitro release experiments

The average encapsulation efficiency for both drugs was approximately 77 ± 8%. The burst release data for different dexamethasone loading concentrations in alginate microspheres i.e. 0.25, 0.5, 0.75, and 1 mg/ml were 19%, 24%, 28% and 33%, respectively. 100% cumulative release of the encapsulated drug was observed in just 22 days for a loading concentration of 0.25 mg/ml dexamethasone, while for other concentrations, <80% dexamethasone release was observed as shown in Fig. 2. In the case of diclofenac loaded microspheres, different drug concentration also tried i.e. 0.5, 0.75 and 1 mg/ml showed 12%, 9%, and 8%, burst release rates, respectively. Whereas, cumulative release of 87% and 100% over 30 days was observed for loading concentrations of 1 mg/ml and 0.75 mg/ml, respectively. For 0.5 mg/ml drug loading, 100% cumulative drug release was achieved in more than 30 days (Fig. 2).

The cumulative *in vitro* release profile of dexamethasone loaded uncoated and $(PAH/PSS)_1$ coated microspheres is shown in Fig. 2. It was observed in case of dexamethasone release that $(PAH/PSS)_1$ polyelectrolyte coating enabled control over the release as compared to other polyelectrolyte pairs like PAA/PAH, PDDA/PSS, etc as shown in our previous work (Jayant et al., 2009). Different ratios of uncoated and PAH/PSS-coated dexamethasone loaded microspheres (i.e. 25C:75P, 50C:50P and 75C:25P, where 'P' stands for plain and 'C' stands for polyelectrolyte coated particles) were used to achieve 100% drug release in desired period of 30 days (Fig. 2). Similarly, based on previous results with different polyelectrolyte for the diclofenac release study, $(PAH/PSS)_1$ was used as final coating. The *in vitro* diclofenac release profile using uncoated and $(PAH/PSS)_1$ coated microspheres showing cumulative release of 86.70% and 50.92%, respectively, in 30 days. There was a significant ($P < 0.05$) difference in the rate and extent of drug release when comparing uncoated and coated microspheres. The release profiles of both drugs showed zero-order release kinetics after a burst release period, which lasted for 1 day. The data was fitted to kinetic equations like 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.

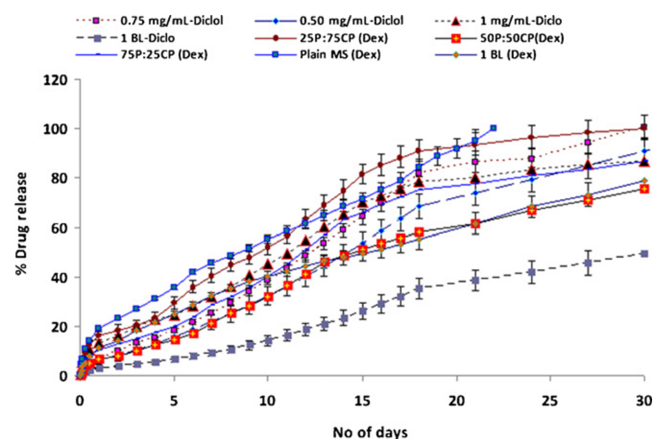

Fig. 2. Comparative release profile of dexamethasone and diclofenac loaded uncoated and $(PAH/PSS)_1$ coated alginate microspheres in PBS (pH-7.4) containing sodium azide (0.01%, w/v) at 37 °C, Mean ± SD ($n = 3$).

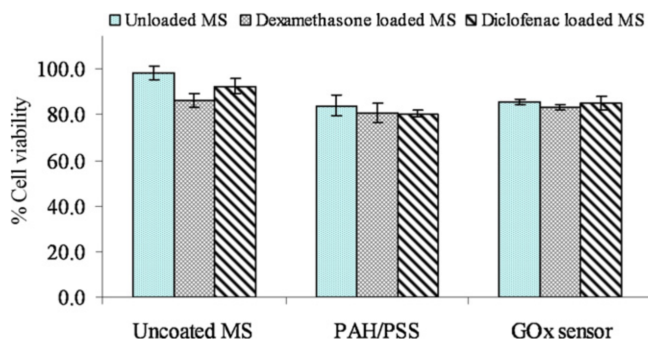

Fig. 3. Cell viability for alginate microspheres with different drugs and coatings.

Studies were also conducted in simulated interstitial body fluid (SIF) to assess whether the expected release behavior might be altered inside the body due to the presence of body fluid ions and proteins. Cumulative release of 96% and 97% was observed in 30 days with dexamethasone (0.25 mg/ml) and diclofenac (0.75 mg/ml), respectively, as compare to 100% dug release in PBS. Hence, it was concluded that microspheres would release the drug at the same rate and kinetics as determined *in vitro* and all further tests were performed with PBS only.

3.3. *In vitro biocompatibility studies*

The cell viability was approximately 100% with uncoated alginate microspheres compared to unexposed control cultures, indicating no cytotoxicity. On the other hand, dexamethasone-loaded and diclofenac-loaded particles yielded 86% and 90% cell viability, respectively. The percent viability for $(PAH/PSS)_1$ coated particles was ~80% for both the drugs (diclofenac and dexamethasone) loaded microspheres. GOx-loaded microspheres alone and in presence of dexamethasone and diclofenac were also tested, from which 82% viability was observed (Fig. 3). Material combinations yielding >80% cell viability were considered for further studies.

3.4. *Results of in vivo experiments to assess pharmacodynamic changes*

To evaluate the efficacy of drugs (i.e. dexamethasone phosphate and diclofenac sodium salt) in controlling the implant associated inflammation around the local tissue environment, the following formulations were tested: (1) drug-free (plain) MS (positive control); (2) dexamethasone loaded MS (0.25 mg/ml) in the ratio of 25P:75CP (i.e. 25% plain and 75% $(PAH/PSS)_1$ coated); (3) diclofenac loaded (0.75 mg/ml) MS; (4) glucose biosensor (GOx) and (5) glucose sensor (GOx) with dexamethasone and diclofenac formulation in 50:50 ratios. Tissue samples surrounding the microspheres containing no drug displayed strong neutrophilic acute inflammatory reaction associated with the implantation as shown in Figs. 4 and 5. The inflammation mediating cells stained purple and normal tissue stained pink as evaluated using standard histology staining (H&E) method. To improve the sensor functionality, as mentioned earlier, site specific localized and controlled delivery of tissue response modifier (TRM) can control the tissue response. *In vivo* results shows that the drug containing microspheres prevents the acute inflammatory phase progression to the chronic inflammatory phase, which was evident by the lack of fibrotic tissue surrounding the implant on 1 week as shown in Figs. 4 and 5 (1C and 1D) and looks similar to normal tissue (pink in colour). Finally, glucose sensors were tested for their biocompatibility *in vivo*. GOx loaded microspheres (model sensor) with drug loaded microspheres was taken as positive control. Sensor samples were mixed with drug loaded microspheres in 50:50 ratios and were implanted subcutaneously using 20 gauge syringe needle. Samples were taken periodically every week for 4 weeks. As expected, upon implantation, GOx loaded microspheres leads to an immunostimulatory response in the surrounding area of the implant as shown in Figs. 4 and 5 (2A and 2B) due to the presence of dyes and enzymes. The inflammatory response to these sensors was significantly greater than that for the unloaded and uncoated microspheres alone. But, when these GOx loaded alginate microspheres were mixed with drug formulations, they resulted in lesser immunogenic response as were shown in Figs. 4 and 5 (2C and 2D).

4. Discussion

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 ζ-potential values clearly demonstrated that the surface charge of the microspheres reverses upon coating of alternately charged on PAH/PSS coating proving that multilayer build-up is taking place. Results showed that LbL helps in reducing the initial burst and prolongs the period of release in induction phase without significantly affecting the release rate.

The desired system is expected to achieve complete release of the drug with in a time period of 3–4 weeks to overcome the inflammatory response of the body to the implantable glucose sensor. To achieve 100% drug release over a period of one month with zero-order release kinetics, different concentrations of dexamethasone and diclofenac were used in the precursor alginate solution. It was observed that % drug release was significantly affected by change in drug content. As the drug content increases, there is influence on both type of release (i.e. the cumulative amount of drug released at any time, such as burst release) and the total % drug release during the induction period. Results suggest that high drug loading is not required and an optimal amount of drug will serve the requirements of the desired system. It was also noted that high drug loading leads to an increase in the induction period, resulting in longer duration release. The desired system is expected to achieve complete release of the drug with in a time period of 3–4 weeks to overcome the inflammatory response of the body to the implantable glucose sensor. To achieve an approximate zero-order release profile and 100% drug release over a period of 3–4 weeks. 0.25 mg/ml (dexamethasone) and 0.75 mg/ml (diclofenac) concentration was chosen, as there was no significant difference was found in the initial burst release profile when compared to higher concentration. The release profiles can be altered by selection of polymer, particle size, and surface along with drug–matrix interactions within the system. In order to obtain the desired release profile several influencing parameters were altered and the main problem of decreasing the initial burst drug release was achieved using the deposition of polyelectrolyte coatings using LbL self-assembly technique. However, when results were compared for drug release in SIF and PBS, there was no significant difference was found in the release pattern. Hence, it may be concluded that microspheres would release the drug at the same rate and kinetics as determined *in vitro*.

Cytotoxicity testing of implantable devices is recommended by regulatory bodies and is considered as one of the most fundamental tests for biocompatibility. The aim of cytotoxicity studies was to evaluate the *in vitro* biocompatibility of drug loaded polyelectrolyte coated and uncoated alginate microspheres and GOx loaded microspheres using L929 mouse fibroblasts cell line. Different cellular aspects were analyzed in order to determine the cell viability during the culture of L929 fibroblasts with alginate microspheres, namely, adhesion, proliferation and morphology. The results pro-

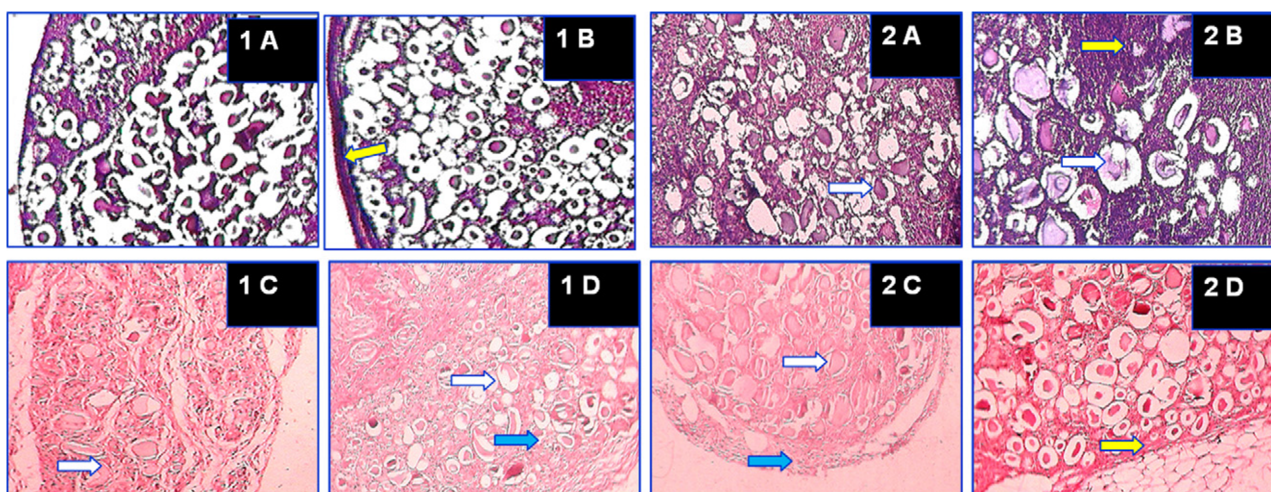

Fig. 4. Pharmacodynamic changes in representative Sprague–Dawley (SD) rat subcutaneous tissue sections on 7 day (1A – Plain MS, 1C – Plain MS + Dexa MS in 50:50 ratio, 2A – Plain GOx sensor and 2C – GOx sensor + Dexa (25P:75CP) in 50:50 ratio) and 28 days (1B – Plain MS, 1D – Plain MS + Dexa MS in 50:50 ratio, 2B – Plain GOx sensor and 2D – GOx sensor + Dexa (25P:75CP) in 50:50 ratio). Inflammation-mediating cells and normal cells are stained purple and pink, respectively (H&E staining). Scale 100 μm. (⇨) Alginate microspheres, (⇨) connective tissue capsule, and (⇨) MNC infiltration (leucocytes). (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

vided evidence of good adhesion, growth, morphology and viability of cells on uncoated and coated alginate microspheres.

The standards pertaining to evaluating the biocompatibility of biomaterials and acceptability of devices have been established by American Society for Testing and Materials (ASTM, 2001), United States Pharmacopoeia (USP, 2002) and International Organization for Standardization (ISO-10993-1 (E), 1992). To a large degree, implantable sensor malfunction can be associated to the actions linked with healing of the tissue surrounding the implanted device, such as inflammation, encapsulation, and wound repair (Koschwanez and Reichert, 2007). Various approaches have been used to avoid or control tissue remedial process with the goal of improving *in vivo* sensor performance. Localized drug delivery approaches are gaining attention as strategies to prevent implant inflammation. For electrochemical sensors, it has been noted that the duration of *in vivo* studies must extend past the "break-in" period (14–18 days) as testing before this time does not allow the sensor to fully stabilize within the body (Wisniewski and Reichert, 2000; Koschwanez and Reichert, 2007). One of the strategies to improve biocompatibility and longevity of implantable devices is concurrent drug delivery incorporated within the sensor or site-specific local delivery of anti-inflammatory agents, as demonstrated in this work.

A localized delivery of anti-inflammatory agents reduces the immunostimulatory cascade of events and eases wound healing process. Sensor implantation stimulates immunogenic response that is differentiated by instant penetration of inflammation-mediating cells, especially neutrophils at the site and followed by macrophages, polymorphonuclear leukocytes, monocytes, fibroblasts and eventually giant cells form. Extended *in vivo* implant reside causes the acute inflammatory responses to progress to the chronic phase, which is characterized by the fibrotic tissue deposition around the implant and a decrease in neutrophils count. In case of blank microspheres i.e. positive control, by the end of week 4, there was a thick fibrotic capsule deposited around the implant, which was characterized by high amount of purple color (due to

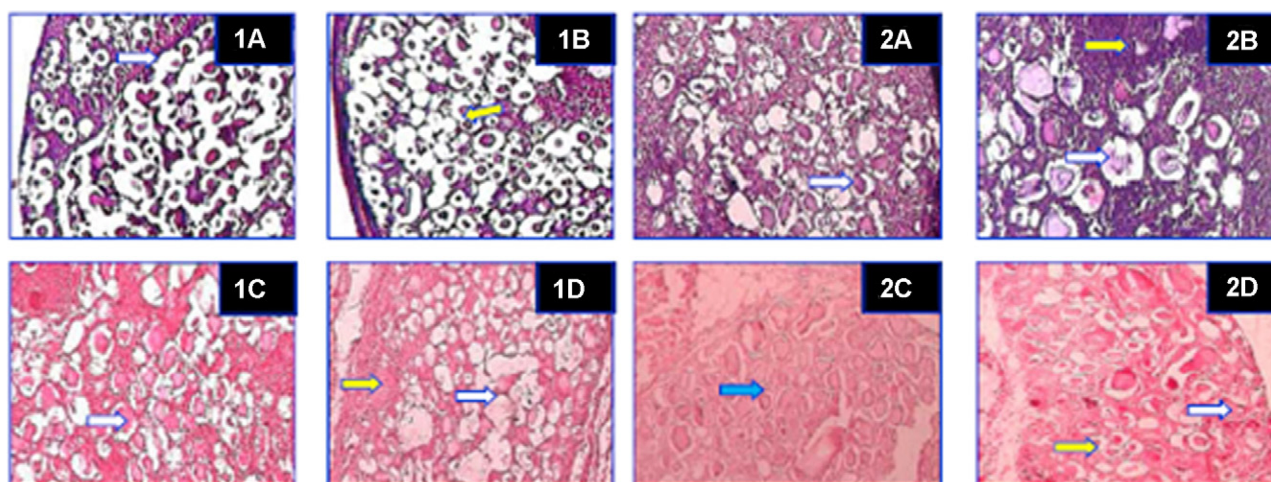

Fig. 5. Pharmacodynamic changes in representative Sprague–Dawley (SD) rat subcutaneous tissue sections on 7 day (1A – Plain MS, 1C – Plain MS + Diclo MS in 50:50 ratio, 2A – Plain GOx sensor and 2C – GOx sensor + Diclo (0.75 mg/ml) in 50:50 ratio) and 28 days (1B – Plain MS, 1D – Plain MS + Diclo MS in 50:50 ratio, 2B – Plain GOx sensor and 2D – GOx sensor + Diclo (0.75 mg/ml) in 50:50 ratio). Inflammation-mediating cells and normal cells are stained purple and pink, respectively (H&E staining). Scale 100 μm. (⇨) Alginate microspheres, (⇨) connective tissue capsule, and (⇨) MNC infiltration (leucocytes). (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

Table 1
Average numbers of inflammations mediating (cells/hpf with an area = 0.66 cm) of subcutaneous tissue surrounding implant microspheres over a period of 4 weeks.

Formulation type	Day 7 Acute inflammation phase Infiltration of neutrophils (cells /hpf)	Day 28 Chronic inflammation phase Fibrous capsule condition
Plain MS[a]	70 ± 5 + mononuclear cells	Thick fibrous capsule + mature fibroblast cells
25P:75C Dexa MS	40 ± 4 + mononuclear cells	No fibrous capsule present
Diclofenac MS	45 ± 7 + mononuclear cells	No fibrous capsule present
Glucose sensor	100 ± 5 + mononuclear cells	Thick fibrous capsule + mature fibroblast cells
Glucose sensor + Dexa MS (50:50)	41 ± 5 + mononuclear cells	No fibrous capsule present
Glucose sensor + Diclo MS (50:50)	52 ± 7 + mononuclear cells	No fibrous capsule present

[a] MS: Microspheres, P: uncoated microspheres, C: coated microspheres, hpf: high power field, DEX: dexamethasone, and Diclo: diclofenac.

high acidic structures i.e. neutrophils, etc.) as shown in Figs. 4 and 5 (1A and 1B). Similar immunological reactions have been previously shown to occur in the presence of drug delivery systems as well as polymeric materials such as polyester and polyethylene (Sanders et al., 2002). This cellular inflammatory cascade changed the preferred pharmacological effects. Fibrous capsule formation is very disadvantageous for biosensors as the capsule may render it impermeable for small molecular weight analytes for e.g. glucose in case of glucose biosensor. Therefore, it is very important to maintain fibrosis-free surroundings to make sure successful performance of implantable device. To test the efficacy of both the formulations, drug loaded microspheres were injected subcutaneously and histopathological changes at the implant site were compared with positive control (blank MS). As expected, initially, even for the drug releasing implants there was some negative immunogenic response observed but the controlled and continuous drug release from the formulation prevents the acute inflammatory phase progression to the chronic inflammatory phase, which was characterized by the lack of neutrophils and fibrotic tissue deposition around the implants. This looks similar to normal tissue (pink and red in color) by the end of 4 weeks as shown in Figs. 4 and 5 (1C and 1D). Localized elution of drugs, led to a statistically significant decrease in the number of inflammation cells surrounding the implant during the acute as well as chronic phases of inflammation, results for the entire study are compiled in Table 1. Thus, concurrent drug release with sensor implantation is capable of showing efficacious control of 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 of sensor, but also helps in increase the shelf life of the implanted biosensor.

5. 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. Nanofilm coatings help to lowering the burst release and helps in the long-term drug release to follow approximately zero-order release kinetics over a four-week period. Cell culture studies showed that the materials involved are not toxic and showed good % viability for 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 by-products (e.g. enzymatic sensors), this combination of anti-inflammatory agents managed the host response to remain at levels equivalent to those observed for non-reactive 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 TRM (tissue response modifiers) 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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