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Layer-by-Layer Adsorption of Biocompatible Polyelectrolytes onto Dexamethasone Aggregates

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Abstract—**Using Layer-by-Layer technology in drug delivery systems is advantageous because of its high precision, mild assembly conditions, and ease of use. In this study, we investigate the use of such a system using a micronized dexamethasone core as the building template. Dexamethasone was chosen because of its hydrophobic structure and role as a cellular differentiation factor. Structural characterization of the assembled structures shows particle size distribution between 3-10 μm with 20% more dissolution than free drug crystals. Additionally, as a measure of drug activity post-encapsulation, *in vitro* cell culture studies were performed. We suggest that the polyelectrolyte coatings enhance release and augment production of extracellular matrix proteins aggrecan and collagen II.**

I. Introduction

Conventiional drug delivery mechanisms require that several times more than the therapeutic dosage be administered to account for plasma protein coating and subsequent phagocytotic uptake [1]. Many efforts have been geared towards the development of delivery systems which avoid foreign recognition by the immune system and regulate the amount of drug released over time. Such systems offer decreased cost, since only therapeutic amounts of the drug are incorporated and increased patient compliance, as less frequent dosing is necessary [2].

A number of drug delivery systems have been developed based on linking the drug to a hydrophilic polymer in an effort to alter drug solubility and avoid foreign particle recognition [1]. Chemical bonding [3], physical entrapment [4], electrostatic interactions [2,5,6] and combinations thereof have been demonstrated as effective methods for incorporation of biomolecules into controlled release systems. [2, 5, 6, 7].

Of the aforementioned methods, the use of electrostatic interactions for complexing drugs within polymer shells is quite promising. This process, layer-by-layer (LbL) assembly, is an attractive method for fabrication of drug delivery systems for several reasons: it can be performed on a variety of substrate types and shapes, it is based on electrostatic interactions thus making the need for specialized chemical reactions obsolete, and it can be used to precisely control system dimensions on the order of nanometers [8].

Recently, Pargaonkar *et al.* and Zahr *et al.* described methods by which Dexamethasone (DEX) can be encapsulated using LbL assembly. In these studies, polyelectrolyte (PE) combinations of poly(dimethydiallyl ammonium chloride) (PDDA), poly(styrene sulfonate) (PSS), and poly(allylamine hydrochloride) (PAH) were investigated. It was shown that sonication of DEX crystals within polyelectrolyte solutions reduced the amount of aggregation, allowing for more uniformly distributed particle sizes [6]. Most importantly, the system release could be tuned by varying the shell thickness and the polyelectrolyte composition.

In this study, we describe the fabrication of solubilized dexamethasone microstructures via LbL assembly. Dexamethasone, a synthetic glucocorticoid, was chosen as the model drug because of its anti-inflammatory properties and use as a mesenchymal stem cell differentiation factor [6]. Direct surface modification of DEX crystals through deposition of biocompatible, linear polyelectrolytes, PSS and PS, (pH 4) demonstrated stable assembly (Fig. 1).

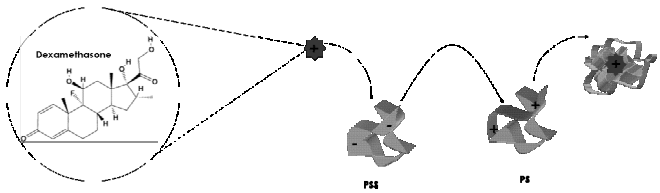

Figure 1. Schematic representation of LbL assembly onto DEX crystals

The shell buildup onto the drug crystals was monitored through quartz crystal microbalance (QCM) and zeta-potential measurements; drug release was determined using UV spectroscopy; and the geometry of the structures was characterized through confocal microscopy. To determine whether the drug maintained its activity after encapsulation, the structures were delivered to fibrochondrocytes in culture.

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II. Experimental Section

A. Materials

Micronized dexamethasone (MW 392.47) was purchased from Spectrum Chemicals (Gardena, CA). Anionic poly(sodium-4-styrene-sulfonate) (MW 70,000) and cationic protamine sulfate (Grade II) were obtained from Sigma Aldrich (St. Louis, MO). The polyelectrolytes were dissolved in 10 mM phosphate buffer pH 4 (PBS, Sigma Aldrich) and washed using purified deionized (DI) water. Fluorescein-5-isothiocyanate (FITC) was used to label the microstructures.

B. Layer by Layer Assembly onto Dexamethasone

A 2 mg/mL solution of PSS (pI=2) was prepared in pH 4 PBS. DEX (pI≈5) was added to the PSS suspension to generate a concentration of 5 mg/mL. The suspension was sonicated (VWR Model 50HT) for 30 minutes, centrifuged (Eppendorf 5804R) for 10 minutes, and washed in DI water. This series of steps completed adsorption of the first PE onto the DEX. Subsequently, 2 mg/mL of PS (pI=10) was added to the existing suspension, sonicated, centrifuged and washed, competing a full thin-film bilayer. The cycle was repeated again, generating an architecture of DEX/$(PSS/PS)_2$.

C. Characterization of Dexamethasone Microstructures

Verification of PE adsorption was determined by measuring the changes in surface charge of dexamethasone aggregates as PE layers were deposited. (Brookhaven Instruments Zeta Plus, Holtsville, NY). The average of five measurements was taken at 20 C.

A confocal microscope system (Leica TCS SP2, Wetzlar, Germany) was used to visualize fluorescently tagged samples; PSS was labeled with FITC prior to adsorbing onto the DEX aggregate. Confocal microscopy allowed for size determination as well as visualization of the structural geometry and fluorescence intensity as a function of geometry.

To determine the thickness of the PE layers, QCM data was obtained for PS and PSS layers. QCM measurements are based on frequency changes (ΔF) of a particular resonator as mass is deposited; the mass calculations (ΔM) can then be used to determine the thickness (Δt) of a particular PE layer [9].

$$-0.87\Delta F(Hz) = \Delta M(ng)$$

and

$$\Delta t(nm) \approx 0.017\Delta F(Hz)$$

D. Release Studies

Release studies were performed in Side-Bi-Side dissolution cells (PermeGear). Samples were suspended in 3 mL of HBSS with magnetic stir bars and the solution temperature was held constant at 25 C. Samples were taken at approximately 5 minute intervals for 24 hours. Drug concentrations over time were determined by correlation to UV/Vis absorbance measurements at 240 nm (Jasco V-530 UV/Vis Spectrophotometer, Easton, MD).

E. Cell Isolation and Culture

Bovine temporomandibular joint (TMJ) discs were routinely digested and filtered to isolate fibrochondrocytes from the posterior band. Cells were plated out at a density of 5 x 10^6 cells/mL and cultured at 37 C in 95% humidified air and 5% CO_2. Cultures were maintained in Dulbecco's modified eagle's medium (Medaitech, Inc. Hendon, VA), supplemented with 2mM L-glutamine, 10% fetal calf serum (Hyclone, Logan, UT), and 1% 1X penicillin/streptomycin (Mediatech, Inc.). The medium was changed every other day and cells were passaged at 80% confluence using Trypsin/EDTA. Cells in the fourth passage were used for experimentation.

Three groups were established: the first (No DEX) was grown in complete culture media only, while the others were further supplemented with dexamethasone (Free DEX) or dexamethasone-core microstructures (DEX structures). Free DEX and DEX structures were added to the media at a 1 nM concentration.

F. Assessment of In-vitro Cellular Response

Live-Dead staining (Biovision, Mountain View, CA) was used as a measure of cytotoxic response to the fabricated structures. Fibrochondrocytes were seeded on glass coverslips, supplemented with complete media, and allowed to attach overnight. After verifying attachment, the appropriate media was applied (No DEX, Free DEX, or DEX structures). The staining solution was prepared as per the manufacturer's protocol and incubated for 30 minutes at 37 C. Cells were observed using a fluorescent microscope (Nikon Eclipse TS 100) at 0, 12, 24, 36 and 48 post media change.

A second set of cells were seeded at a density of 200,000 cells/cm^2 and allowed to adhere overnight. Fresh differentiation media was applied containing either free DEX or DEX structures. The cells were washed with HBSS and fixed with 95% ethanol at 12, 24, 36, and 48 hours post media change. Immunocytochemistry for detection of aggrecan and collagen II (antibodies obtained from Calbiochem, LaJolla, CA) was performed using an anti-mouse kit purchased from Spring Biosciences (Fremont, CA) Staining was performed using the HistoMark ORANGE peroxidase substrate kit (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD).

III. RESULTS

The adsorption of polymer layers is confirmed by examining the overall surface charge of the microstructure. The surface charge is determined by ξ-potential measurements. Fig. 2 shows the resulting inversions in surface charge as PEs adsorb to the dexamethasone core. The mass deposition for each PE layer is given in Fig. 3. When using PSS, five times as much mass is deposited on the structure as compared to PS; correspondingly, the thickness of PSS layers are approximately 5 times greater as well. Fig. 4 shows the release profiles for Free DEX crystals vs. DEX microstructures (2 bilayers). The two samples follow the same general release pattern though the microstructures release approximately 20% more dexamethasone in the same time period.

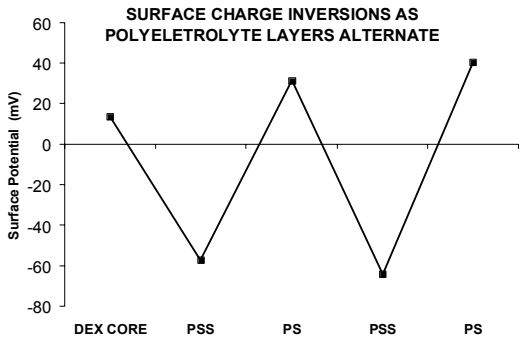

Figure 2. Surface charge inversions as PE layers alternate

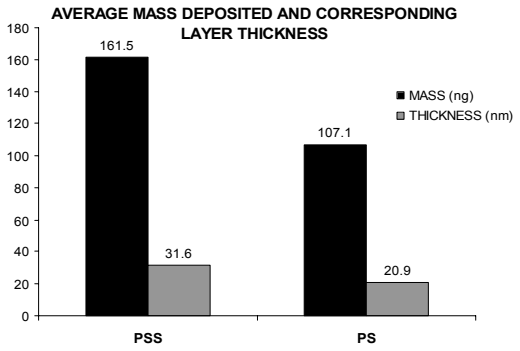

Figure 3. Mass deposition and layer thickness compared for PSS and PS

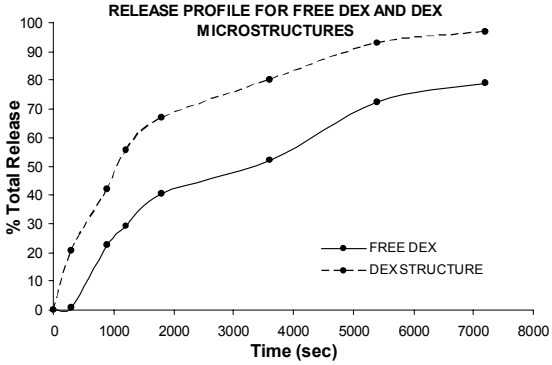

Figure 4. Release profiles for Dex and Dex microstructures

Finally, to visualize the PE layers distribution, the outermost layer of PSS was tagged with FITC before conjugation with the microstructure. Variations in fluorescence intensity as a function of structure length are shown in Fig. 4, with the greatest intensity on the outer edges. Fig. 5 shows a confocal image of a representative microstructure; note the color changes from the outer edges to the center.

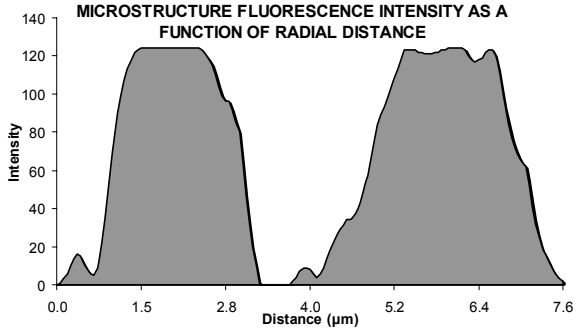

Figure 5. Fluorescence intensity as function of geometry

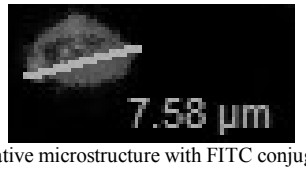

Figure 6. Representative microstructure with FITC conjugated PSS

Aggrecan and collagen II staining at 48 hours post media change is shown in Fig. 7. It may be observed that the Free DEX group shows the least amount of staining for either Aggrecan or collagen II. For both assays, it is also noted that cultures with DEX structures produce approximately equal amounts of aggrecan and collagen II as the control cultures.

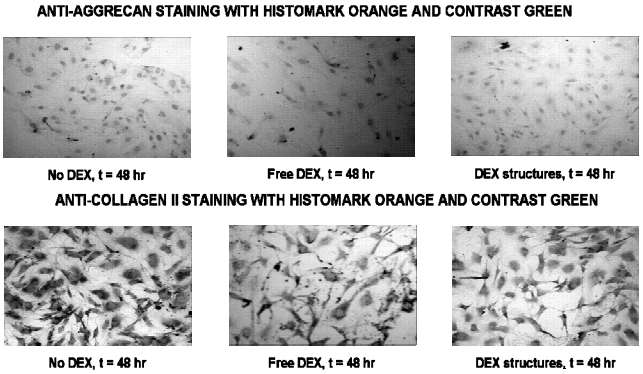

Figure 4. Aggrecan and collagen II staining for No DEX, Free DEX, and DEX structures at 48 hours

Live/Dead staining of the cells (results not shown) indicated there was no cytotoxic response to the microstructures over a 48 hour period.

IV. Discussion

The adsorption of PEs was confirmed by ζ-potential measurements at each step of the microstructure assembly. On average, PSS layers exhibit -60 mV of charge on the surface while PS layers exhibit 30 mV. Since both PEs are strongly charged at pH 4.0, the difference in charge magnitude may be explained by greater "charge area" for PSS than PS. This hypothesis is supported by the mass deposition data shown in Fig. 3. PSS is a larger, bulkier PE and produces (5X) thicker layers than PS; the added thickness and mass potentially leads to the greater surface charge observed.

Dissolution results indicate that DEX structures release more dexamethasone than free DEX within the same time period. This observation is most likely due to increased motility of the crystals. Dexamethasone is naturally insoluble in aqueous media; however, adsorption of water-soluble PEs onto the crystals increases the solubility of dexamethasone and the solvated DEX can more easily diffuse. Increased delivery from the DEX structures when compared to free DEX can also be documented through staining for extracellular matrix proteins specific to the fibrochondrocyte phenotype. Aggrecan staining is markedly stronger for DEX structures when compared to the free DEX (Fig 7). Similarly, collagen II is present in high quantities for the cells exposed to the DEX structures as opposed to the free DEX. Overall staining for collagen II was greater than that for aggrecan. This is reasonable since collagens comprise the bulk of the extracellular matrix proteins associated with chondrocytes. The ratio of live cells to dead cells as obtained through fluorescence imaging was similar for cells exposed to free DEX and DEX structures. Previous work in our lab has shown that cells grown on substrates with a terminating layer of PSS exhibited only a slightly cytotoxic response [10]. We postulate that the adsorption of PS onto PSS is able to mask its cytotoxic effects, making the surface more biocompatible.

V. Conclusions

The results from this study are promising though further testing is needed to fully characterize the microstructures. Additionally, long-term cultures could provide a more detailed view of the mechanisms occurring as DEX structures release. A promising application of this technology is its use in tissue engineering, particularly TMJ disc tissue, where controlled-release of proteins and growth factors is desirable. Using biocompatible polymers such as PSS and PS also makes it promising in stealth delivery applications.

Acknowledgment

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