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Development of PLGA Microsphere/PVA Hydrogel Composite Coatings for Long-Term Biosensor Functioning

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This work focuses on the development of poly (lactic-co-glycolic acid) (PLGA) microsphere/polyvinyl alcohol (PVA) hydrogel composite coatings to permit long-term glucose sensor functionality. Two aspects were addressed: *1)* prevention of the foreign body reaction (FBR) for 6 months; and *2)* investigation of the long-term effects of such coatings on glucose transport.

PLGA microspheres were prepared *via* blending low (RG503H, 25 kDa) and high (DLG7E, 113 kDa) molecular weight (MW) polymers. "Real-time" *in vitro* studies demonstrated that the dexamethasone release profiles were dependent on the polymer ratios. The duration of drug release lasted for approximately 2-6 months with varied burst release and lag phase. A discriminatory accelerated *in vitro* release method was developed to shorten drug release from 6 months to less than 2 weeks. One formulation exhibited continuous dexamethasone release *in vitro* as well as *in vivo* efficacy for 4.5 months.

A central composite design was applied to generate predictive mathematical models of drug loading and burst release to facilitate optimization of microsphere composition. The optimized composition for long-term drug release with suitable burst release and drug loading was DLG7E/RG503H/dexamethasone = 21/4/5 (w/w/w). The optimized microspheres showed continuous dexamethasone release *in vitro* and anti-FBR for 6 months. It was also determined that the released dexamethasone was stable for the entire 6-month period. Through analysis of an

in vitro drug release heat map together with the *in vivo* histological data, it was determined that the coatings should release approximately 0.1 µg dexamethasone per mg daily in order to counter chronic inflammation in rats.

To evaluate the effect of microsphere degradation in the coatings on glucose transport, two types of coatings were prepared incorporating different types of microspheres (*i.e.* microspheres with/without a lag phase). The patterns of pore formation and microsphere swelling were evaluated for both coatings. The coating thickness increased as a result of microsphere swelling and this was considered to contribute to a decrease in glucose transport.

In conclusion, the long-term dexamethasone releasing coatings developed here as well as the understanding of microsphere degradation within the coatings will facilitate the application of such coatings for implantable glucose sensors.

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Development of PLGA Microsphere/PVA Hydrogel Composite Coatings for Long-Term Biosensor Functioning

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Dedication

To my wonderful family

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Chapter 1

Introduction

1.1. Background

Diabetes mellitus is a chronic metabolic disease affecting about 10% of the adult population in the U.S. Fluctuation of blood glucose levels is a typical symptom of diabetic patients due to underproduction or underutilization of insulin.[1, 2] It is critical for diabetic patients to closely monitor their blood glucose levels in order to control disease progression and prevent severe complications.[3-6] At present, most diabetic patients rely on glucose strips along with hand held glucose meters to measure blood glucose levels *via* finger pricking.[7, 8] This painful process can only measure the glucose concentration for a few time points during a day and is highly user dependent. It is easy to miss important fluctuations in glucose using this technique. Continuous glucose monitoring provides the advantage of accurately monitoring the blood glucose trend for precise calculation of insulin dose therefore eliminating the possibility of hypo/hyperglycemic conditions.[9] Currently, commercially available continuous glucose monitoring devices can only function for up to 7 days with a glucose oxidase based transcutaneous amperometric sensor.[10] This sensor loses functionality after one week due to the foreign body reaction (FBR) which is a series of sequential events that ultimately rejects the implanted biomaterials.[11, 12] Initial biofouling and sequential inflammatory cell attack can affect enzyme stability and reduce sensor sensitivity. Fibrous encapsulation, the final event of FBR, will deprive the sensor from adequate analyte supply leading to loss of sensor signal. Inhibition of local FBR is one of the most promising strategies to extend sensor lifetime.[13]

In order to achieve long-term continuous glucose monitoring, a fully implantable glucose biosensor is under development by a multi-disciplinary team of UConn scientists. As part of the team, our group has developed biocompatible coatings composed of poly (lactic-co-glycolic acid) (PLGA) microspheres embedded in a polyvinyl alcohol (PVA) hydrogel for our glucose

biosensors.[14-17] The PVA hydrogel acts as a hydrophilic base to support the microspheres and allows glucose diffusion to the biosensor. PLGA microspheres serve as drug reservoirs to continuously release dexamethasone to inhibit local inflammation, therefore, preventing the foreign body reaction. However sustained release of dexamethasone is required over the entire sensor lifetime since a delayed tissue reaction can develop after exhaustion of the drug.[15] Applying the concept of blending different polymers, it is possible to achieve microspheres which can sustain dexamethasone release for a longer time period with effective dose. Polymer blends of 25 KD PLGA and 70 KD PLGA were used to prepare microspheres to sustain the release of dexamethasone for approximately 3 months.[18]

From our previous study, microspheres with approximately 7.6% (w/w) loading, 10 days lag phase and 40% (w/w) burst release were able to prevent the FBR for 1 month.[17] Based on the *in vivo* performance of the 1-month formulation, approximately 3 to 6 times the total dose (excluding the amount released in the initial burst phase) is required for a 6-month formulation. However, due to the limited space in the PVA hydrogel which restricts the maximum amount of microspheres embedded, the total dose can be increased by increasing the loading and reducing the burst release of dexamethasone in the microspheres. The microsphere formulation can be optimized to achieve approximately 15% loading and 10% burst release. The initial burst release is required to inhibit the acute inflammatory reaction resulting from sensor implantation. In the case that 10% burst release is insufficient, then the dose will be adjusted by loading additional dexamethasone directly into the PVA hydrogel. In order to achieve the desired release properties, a quality by design approach was used and risk analysis was performed to the microsphere preparation process shown in Figure 1.1. As determined from the risk analysis, the most important factor affecting drug release is the formulation composition, which is the ratios of drug and the different polymers in the

microspheres. The preparation process can also affect drug loading, burst release and the lag phase by generating particles with different morphology. However, obtaining a 6-month release formulation from a 1-month release formulation, composition adjustment in the polymer blends is necessary and should be investigated as a priority. Therefore**, the first objective** of the current research was to develop a dexamethasone loaded PLGA microsphere formulation with 6-month continuous drug release using polymer blends by adjusting the formulation composition.

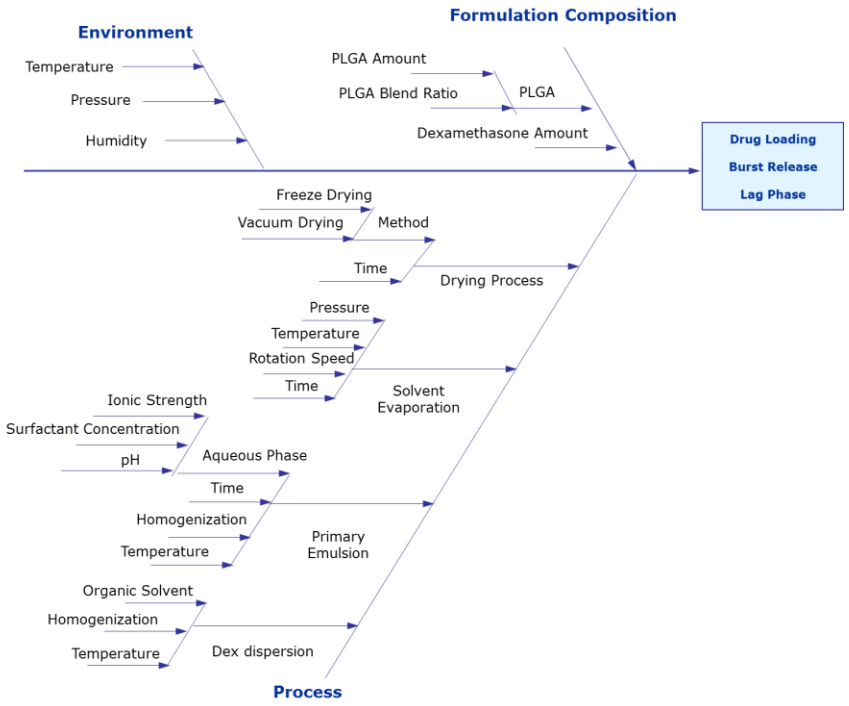

Figure 1.1. Ishikawa diagram for loading, burst release and lag phase of microsphere preparation

Our implantable glucose sensors operate by measuring the electrochemical signal from glucose oxidation by glucose oxidase deposited on the surface of the sensor.[19] Sufficient glucose diffusion to the glucose oxidase layer is necessary to ensure sensor performance.[20] The presence of composite coatings increased sensor response time and reduced sensor sensitivity due to glucose diffusion change caused by the coating.[21] Two major factors affecting glucose diffusion through the composite coating are the water content in the coating and the inner structure of the

composite.[22, 23] Higher water content in the swollen hydrogel can lead to increased diffusion coefficient of hydrophilic molecules.[24] Microsphere degradation in the composite coating was shown to create macroscopic porosity which can counter biofouling-induced sensor degradation for 1 month *in vitro*.[25] The microsphere degradation was shown to follow a hydration and auto-catalysis process with a pH gradient from the interior (low pH) to the surface (high pH).[26, 27] However, the microscopic structure change (swelling and pore formation) during the degradation has not been fully understood. Since porosity and hydrophilicity are the two most important factors determining glucose diffusion through the coating, these changes are important for the understanding of analyte diffusion through the coating as well as biosensor performance when coated. Therefore**, the second objective** was to investigate the microscopic changes within the composite coating to understand its effect on glucose diffusion.

1.2. Objectives:

The present research focuses on further optimization of the PLGA microsphere/PVA hydrogel composite coating to allow long-term dexamethasone release and sufficient glucose transportation. The objectives of the present work include: *1)* optimizing PLGA microspheres using polymer blends to achieve a formulation suitable for long-term dexamethasone release with 6-month *in vivo* efficacy; and *2)* exploring the dynamic changes of composite coatings during microsphere degradation which can affect glucose transport.

The following specific aims were developed to achieve the objectives mentioned above:

Specific Aim 1 (Chapter 2)

To determine *in vitro* and *in vivo* performance of dexamethasone loaded PLGA microspheres prepared using polymer blends

Specific Aim 2 (Chapter 3)

To optimize dexamethasone release from PLGA microspheres prepared with polymer blends using a design of experiment approach.

Specific Aim 3 (Chapter 4)

To develop a PLGA microsphere/PVA hydrogel composite coating for glucose sensors to suppress the foreign body reaction for 6 months

Specific Aim 4 (Chapter 5)

To evaluate the effect of PLGA microsphere degradation on glucose transport through composite coatings

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Chapter 2

In vitro and *in vivo* performance of dexamethasone loaded PLGA microspheres prepared using polymer blends

Abstract

The foreign body reaction is the major cause of the dysfunction and relatively short lifetime associated with implanted glucose biosensors. An effective strategy to maintain sensor functionality is to apply biocompatible coatings that elute drug to counter the negative tissue reactions. This has been achieved using dexamethasone releasing poly (lactic-co-glycolic acid) (PLGA) microspheres embedded in a poly vinyl alcohol (PVA) hydrogel coating. Accordingly, the biosensor lifetime relies on the duration and dose of drug release from the coating. To achieve long-term drug release mixed populations of microspheres have been used. In the current study, microspheres were prepared by blending low (25 KDa) and high (113 KDa) molecular weight PLGA at different mass ratios to overcome problems associated with mixing multiple populations of microspheres. "Real-time" *in vitro* studies demonstrated dexamethasone release for approximately 5 months. An accelerated method with discriminatory ability was developed to shorten drug release to less than 2 weeks. An *in vivo* pharmacodynamics study demonstrated efficacy against the foreign body reaction for 4.5 months. Such composite coatings composed of PLGA microspheres prepared using polymer blends could potentially be used to ensure long-term performance of glucose sensors.

2.1. Introduction

Continuous glucose monitoring (CGM) devices can provide tremendous assistance in the management of diabetes through the availability of real-time blood glucose levels [1]. The current CGM technology is based on semi-implantable glucose sensors with limited life-time (maximum 7 days). Those sensors suffer from instability as a result of the tissue trauma caused by implantation and a series of negative tissue responses triggered by the persistent presence of the implant [2]. The series of immunological events that include acute inflammation (*e.g.* infiltration of inflammatory cells), chronic inflammation (*e.g.* activation of fibroblasts), and fibrous encapsulation (*e.g.* collagen capsule formation surrounding the implants) are called the foreign body reaction (FBR) [3]. Various approaches have been attempted to overcome the foreign body reaction to extend the life-time of glucose sensors. These include application of biocompatible coatings [4, 5], release of anti-inflammatory drugs [6-9] as well as delivery of angiogenic agents [10, 11].

Local delivery of dexamethasone, a potent corticosteroid, has been shown to effectively control the FBR in normal rats, diabetic/fatty rats as well as mini pigs [4, 5]. However, delayed tissue reaction can develop after exhaustion of the drug [12]. A dexamethasone loaded PLGA microsphere/PVA hydrogel coating has been developed to control the FBR for 3 months [9]. This was achieved using a mixture of two populations of microspheres, one prepared with low Mw PLGA and the other with high Mw PLGA. Dexamethasone release from PLGA microspheres typically exhibits a tri-phasic profile with a burst release phase followed by a lag phase and a secondary release phase. A long lag phase is usually associated with high Mw PLGA based microspheres because the time required for sufficient bulk erosion to allow drug release. For example, the lag phase for dexamethasone from the microspheres was extended from 10

days to 41 days when the Mw of PLGA was changed from 25 KDa to 70 KDa [13]. The limited drug release during the long lag phase of high Mw PLGA microspheres was compensated by drug release from the low Mw PLGA microspheres during that period and thus a 3-month effective formulation was achieved. Further extending drug release beyond 3 months using this strategy would be difficult since this would probably require at least three batches of microspheres and the drug release profiles of these batches would need to match one another to achieve continuous release with no lag phase. In addition, this would provide challenges in terms of processing and product quality control. An alternative strategy is therefore needed to produce a single formulation with a short lag phase and long duration of drug release.

Drug release from PLGA microspheres is controlled by polymer degradation, which is a result of the polymer backbone hydrolyzing into oligomers and monomers. Bulk erosion of the PLGA polymer matrix usually occurs from water penetration followed by autocatalysis of the ester bonds. The long lag phase associated with the degradation of high Mw PLGAs is a result of the relatively slow water penetration into these polymers due to their increased hydrophobicity compared to low Mw PLGAs. This can be shortened by enhancing the hydrophilicity of the polymer using approaches such as decreasing polymer Mw and introducing hydrophilic groups. Blending low Mw PLGA with high Mw PLGA is an intriguing prospect since the low Mw PLGA may facilitate degradation of the high Mw PLGA *via* increased water absorption and generation of acidic oligomers that will result in autocatalysis of the polymer matrix. A design of experiment (DoE) approach was used to generate a design space to optimize drug release profiles of PLGA microspheres prepared using polymer blends.[14] Sustained dexamethasone release for approximately 3 months *in vitro* was achieved by blending 25 KDa PLGA with 70 KDa PLGA [13].

Drug release profiles are important product performance indicators. Since real-time release testing of the target product would last for months, it is essential to develop accelerated release testing methods for quality control purposes. Accelerated release testing for microsphere products can be achieved by changing various release conditions (such as pH, temperature, organic solvent, and surfactant). For example adjusting the media to acidic pH conditions has been shown to expedite drug release as PLGA degradation is catalyzed under acidic conditions [15].However the drug release mechanism was shown to change from bulk erosion to surface erosion when lower pH condition were used. Elevating temperature can facilitate drug diffusion from the microspheres as well as expedite polymer matrix degradation. It has been shown that accelerated drug release from PLGA microspheres can be achieved under elevated temperature conditions [16-18].

In the current study, the *in vitro* and *in vivo* performance of microspheres prepared by blending low (25 KDa) and high (113 KDa) Mw PLGA was investigated. The microspheres were prepared with different polymer mass ratios and characterized for physicochemical properties such as particle size, glass transition temperatures and morphology. “Real-time” and accelerated release testing was performed. The accelerated release method was able to shorten the duration of drug release while maintaining discriminatory ability. The “Real-time” release profiles provided guidance for formulation screening towards *in vivo* testing. The selected formulation was evaluated *in vivo* and histological results demonstrated that the foreign body reaction was inhibited for at least 4.5 months.

2.2. Material and Methods

2.2.1. Materials

Dexamethasone was purchased from Cayman Chemical (Ann Arbor, MI), poly (vinyl alcohol) (PVA, Mw 30–70 KD), sodium chloride (NaCl, ACS grade), sodium azide (NaN_3), sodium phosphate dibasic dihydrate ($Na_2HPO_4 \cdot 2H_2O$), sodium phosphate monobasic (NaH_2PO_4) and dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich (St. Louis, MO). PVA (99% hydrolyzed, Mw 133 KD) was purchased from Polysciences, Inc. (Warrington, PA). PLGA Resomer® RG503H 5050 (RG503H, inherent viscosity 0.32–0.44 dl/g) was a gift from Boehringer-Ingelheim. PLGA 9010 DLG7E (DLG7E, inherent viscosity 0.6-0.8 dL/g) was purchased from Lakeshore Biomaterials (Birmingham, AL). RG503H has carboxylic acid end groups and DLG7E is end-capped with a lauryl ester group. Methylene chloride (DCM), acetonitrile (ACN, HPLC grade), and tetrahydrofuran (THF, HPLC grade) were purchased from Fisher Scientific (Pittsburgh, PA). NanopureTM quality water (Barnstead, Dubuque, IA) was used for all studies.

2.2.2. Methods

2.2.2.1. Preparation of microspheres and composite coated implants

Dexamethasone loaded microsphere formulations were prepared using an oil-in-water (o/w) emulsion solvent extraction/evaporation technique according to the compositions listed in Table 2.1. The PLGA polymer was dissolved in 2 ml of methylene chloride and dexamethasone was dispersed in this solution. Following 20-minute sonication in a bath sonicator, the dispersion was further dispersed using a T 25 digital ULTRA-TURRAX homogenizer (IKA Works, Inc., Wilmington, NC) at 10,000 rpm for 1 min. In order to form an emulsion, the organic phase was homogenized into a 10-ml PVA solution (1% (w/v), average Mw 30–70 KDa) at 10,000 rpm for 2.5 min. The emulsion was then transferred into a 125 ml aqueous PVA solution (0.1% (w/v), Mw 30-70 KDa) and stirred at 600 rpm under vacuum. After 2.5 hours, hardened microspheres

were transferred to 50 mL centrifuge tubes and collected through centrifugation under 1500 rpm for 2 minutes. The microspheres were then washed three times with deionized water (10 mL each time), recollected using the same centrifugation procedure and dried *via* freeze drying. The prepared microspheres were stored at 4°C until further use. Blank microspheres were prepared following the same procedure except that dexamethasone was not added.

Cylindrical implants were prepared using a two-piece grooved mold based method after three freeze-thaw cycles [19]. Briefly, 150 mg of microspheres (F-3 in Table 2.1) was suspended using 1 ml of 5% w/w PVA solution (133 KDa) and filled in a 1-mL syringe. The PVA solution was pre-filtrated using 0.22-μm sterile filters to ensure sterility. The suspension was sonicated in a bath sonicator for 10 seconds followed by one freeze–thaw cycle (2 hour at - 20 °C and 1 hour at ambient temperature). The partially thickened suspension was fed into a 2-piece mold with 1.5-mm grooves. The dummy sensors (silicon chips with dimension of 5 × 0.5 × 0.5 mm) were sandwiched between the two mold pieces and were then subjected to additional two freeze thaw cycles. The coated dummy sensors were air dried and cut into 7-mm length implants. The grooves in the mold are 1.5-mm diameter. To ensure size consistency of each implant, dummy sensors are placed in the center of the groove between the two pieces of the mold. Sterile tools (*e.g.* vials, tubes, twizzles and containers, *etc.*) were used in the coating process. All the procedures were conducted in a laminar flow hood under sterile conditions. Blank formulations were also prepared using blank PLGA microspheres without dexamethasone.

2.2.3. *In vitro* characterization of microspheres and composite coatings

2.2.3.1. Drug loading

Approximately 5 mg of dexamethasone-loaded PLGA microspheres was dissolved in 10 ml THF for drug loading determination. A previously reported HPLC method was used for analysis of

dexamethasone concentration for the loading calculation [14]. Briefly, the solution was filtered (Millex® HV, PVDF 0.45 μm syringe filter) and subjected to HPLC analysis with 5 μl of injection volume. A Perkin Elmer series 200 HPLC system (Shelton, CT) was equipped with a UV absorbance detector (240 nm wave length for dexamethasone analysis). Acetonitrile/water/phosphoric acid (35/70/0.5, v/v/v) was used as the mobile phase. A Zobax C18 (4.6 mm × 15 cm, Agilent, Santa Clara, CA) analytical column was used with a flow rate of 1 ml/min. The chromatographs were analyzed by PeakSimple™ Chromatography System (SRI instruments, Torrance, CA).

Drug loading was determined as: % drug loading = (weight of drug loaded/weight of microspheres) × 100%.

2.2.3.2. Microsphere characterization

The particle size and size distribution was determined using an AccuSizer 780A autodiluter particle sizing system (Nicomp, Santa Barbara, CA). Approximately 5 mg of microspheres were dispersed in 1 ml of 0.1% (w/v) PVA solution (30-70 KDa) and 100 μl of the dispersion was injected into the system for particle size analysis. All measurements were conducted in triplicate.

A TA Q1000 differential scanning calorimeter (DSC) (TA, New Castle, DE) was used to determine the glass transition temperature (Tg) of the prepared microspheres. Modulated DSC was performed with the cycle below: the samples were heated at a rate of 3 °C/min from 4°C to 80 °C at a modulating oscillatory frequency of 1°C/min. The thermograms were analyzed using Universal Analysis software (TA Instruments) to determine the glass transition temperature.

The morphology of the microspheres were determined using a scanning electron microscopy (a JEOL JSM-6335F unit). Samples were mounted on carbon taped aluminum stubs and sputter coated with gold for 1.5 min at 6 mA before imaging.

2.2.3.3. *In vitro* release testing

In vitro release testing was performed on the PLGA microsphere/PVA hydrogel (99% hydrolyzed, Mw 133 KD) composites since the purpose was to test the performance of the coatings rather than the microspheres alone. The composites were prepared using a freeze-thaw method described previously [13]. Briefly, approximately 75 mg of PLGA microspheres were dispersed into the PVA hydrogel (5% w/v) solution, then this suspension was filled into a pre-made mold (15×38×2 mm) and subjected to three freeze-thaw cycles consisting of 2 h freezing at −20°C followed by 1 h thawing at room temperature to form a physically crosslinked hydrogel resulting from PVA crystallization. The composites were then air dried. Approximately 8 mg composite samples were immersed in 5 ml of 10 mM PBS (pH 7.4) with 0.1% NaN_3 and incubated at both elevated temperature (45 and 53 °C) and body temperature (37 °C) under constant agitation. At pre-determined time points, all the release media was removed and replenished with the same volume of fresh media. Sink conditions were maintained throughout. The samples were filtrated through 0.45 μm syringe filters and the concentration of dexamethasone in each sample was determined using the HPLC method as described above. The release profiles were plotted as cumulative % release *vs.* time. Cumulative percent release at a given time point was calculated as: cumulative percent release = (total amount released at sampling time/total amount loaded) × 100. The values are reported as mean ± standard deviation (n = 3).

2.2.4. *In vivo* pharmacodynamics study of composite coated dummy sensors

PLGA/PVA composite coated dummy sensors prepared in section 2.2.1.2 were implanted into the interscapular subcutaneous tissue of male Sprague-Dawley rats (weighing ~ 200 g, n=3) using 16-gauge thin wall needles. During the implantation procedure, the rats were under

anesthesia using 2% isoflurane in oxygen. Before implantation, the back of each animal was shaved and wiped with betadine solution. All of the procedures were conducted under aseptic conditions. One composite was implanted per rat on days 0, 35, 151 and 162 and the rats were sacrificed at day 165. Three rats were used for one group and each rat had a total of 4 composite implants at the end of the study. Accordingly, the duration of implantation for each of these implants is 165, 130, 14, and 3 days, respectively. The implants with surrounding subcutaneous tissue were harvested and fixed in 10% buffered formalin solution (Sigma-Aldrich Co. LLC.). All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) within the University of Connecticut prior to beginning the experiments. Paraffin blocks were prepared for tissue sections and a blinded histological evaluation was performed following hematoxylin and eosin (H&E) staining. Tissue samples were observed and digitally stored using an Olympus microscope (model BX51, Olympus America, Melville, NY).

2.3. Results and Discussion

2.3.1. *In vitro* characterization of microspheres

The microspheres were characterized for drug loading, particle size and thermal properties (Table 2.1). No significant differences were observed in the average particle size of all formulations investigated. For F-1, F-2 and F-3, the drug loading was approximately 8% (w/w) with a slight increase in loading with increase in the ratio of the more hydrophobic DLG7E polymer in the formulation. The drug loading of the F-4 formulation was significantly lower than that of the other formulations as determined by the paired student T-test ($p<0.05$ as significant). This increase hydrophobicity results in faster polymer solidification and therefore drug encapsulation may be enhanced. The glass transition temperatures for microspheres prepared with polymer blends are between the glass transition temperatures of DLG7E (52.97 °C) and

RG503H (42.1 °C). Although the rank order for the Tg(s) is consistent with the empirical Tg(s) calculated from Fox Equation for polymer blends [20], polymer segregation is still possible considering that the Tg(s) of the two polymers are very close. SEM examination will provide more detailed information regarding microsphere morphology. SEM micrographs (Figure 2.1) showed spherical microspheres with smooth morphology for most of the particles except for those of F-4. Large pores and crevasses were observed for the F-4 microspheres which can be explained by possible polymer phase separation in this formulation considering that equal amounts of the low and high Mw polymers were added in F-4. The peculiar structure of the F-4 formulation is an indication of phase separation. In addition, much higher burst release was observed for the F-4 formulation when compared to the other formulations.

2.3.2. "Real-time" *in vitro* drug release from composites

The release profiles obtained for the microsphere formulations under "real-time" conditions (37 ˚C) are shown in Figure 2.2 Dexamethasone release from PLGA microspheres typically has an initial burst phase, followed by a lag phase and then a secondary zero-order release phase. These three distinct phases were identified for all the formulations. The F-4 formulation exhibited the highest burst release (approximately 35%) and the shortest lag phase (approximately 10 days). The pores and crevasses observed in the surface of F-4 formulation (Figure 2.1.) are considered to be responsible for the high burst release associated with this formulation since easier drug diffusion from the surface is expected. The release of dexamethasone from the F-4 formulation reached a plateau within 1.5 months. Microspheres prepared using 100% RG503H have previously been shown to have a burst release of approximately 40% and a lag phase of approximately 7 days and the release was complete within 35 days. [18] The release profile of the F-4 formulation is closer to that of the microspheres prepared with 100% RG503H compared

to the other two polymer blend microspheres (F-2 and F-3). This may be a result of polymer phase separation in this microsphere formulation, as discussed above, as well as the relatively higher hydrophilicity of this formulation increasing water uptake and consequent polymer degradation. The F-1 formulation (prepared with 100% of the high Mw DLG7E polymer) has the lowest burst release (approximately 8%), the longest lag phase (approximately 4 months) and the entire release profile lasts for approximately 7 months. The DLG7E polymer has high hydrophobicity due to its high Mw and high lactic:glycolic acid ratio (90:10). Accordingly, polymer degradation is significantly slower compared to microspheres prepared with the polymer blends as the rate of water penetration and subsequent polymer hydrolysis are significantly reduced. The long lag phase of the F-1 formulation prohibits its use for the purpose of continuous drug release. The drug release profiles of the F-2 and F-3 formulations are in between those of the F-1 and F-4 formulations. These formulations have burst release phases of approximately 15%, lag phases of less than 2 weeks and their entire release profiles last for approximately 4.5 months. Water penetration and hence polymer degradation is more rapid compared to the F-1 formulation, since these two formulations were prepared by blending the more hydrophilic RG503H polymer with the DLG7E polymer. The relatively high hydrophilicity of the RG503H is attributed to three factors: *1)* a relatively small Mw of ~25 KDa; *2)* a lactic/glycolic acid ratio of 50/50; and *3)* the fact that it is capped with hydrophilic carboxylic groups [21]. In the blended polymers, the low Mw RG503H facilitates water penetration and the subsequent degradation of the RG503H polymer generates lactic/glycolic acid oligomers and monomers. The resultant decrease in pH catalyzes the degradation of the high Mw polymer (DLG7E). Therefore, the long lag phase associated with the high Mw polymer is eliminated and the microspheres prepared with polymer blends exhibit continuous release profiles following a

small burst and a short lag phase. Although the release profiles of the F-2 and F-3 formulations were similar, the F-3 formulation was selected for further studies.

2.3.3. Accelerated *in vitro* drug release from composites

Drug release profiles are important product performance indicators. A real-time release profile is typically a good indication for the *in vivo* performance of the formulation. Considering that real-time release tests for microsphere products can last for months, it is essential to develop accelerated release testing methods for quality control purposes. Maintaining the discriminatory ability of the method while shortening the testing duration is essential. Accelerated release tests can be conducted by adjusting parameters such as temperature, solvent, ionic strength, pH, and agitation rate as well as addition of enzymes and surfactants.[22] It has been shown that elevating temperature is an effective method of accelerating drug release from PLGA microsphere/PVA hydrogel composites [16, 18].

The F-1, F-3 and F-4 formulations were tested under elevated temperature conditions. Figure 2.3-A and B shows the release profiles for the three formulations at 45 ˚C and 53 ˚C, respectively. In order to investigate the effect of the glass transition temperature on the drug release profiles, two temperatures (one higher and another lower than the Tg of the formulations) were chosen for accelerated release testing method development. The release profiles for the individual formulations at real time (37 ˚C) as well as accelerated (45 and 53 ˚C) conditions are plotted in Figure 2.3-C, D and E for direct comparison. At 45 °C, although there were no lag phases, three distinct drug release phases were observed for all formulations. The burst release for F-1 and 3 is close to 10%, which is similar to that obtained at 37 °C. A slightly higher burst release was observed for the F-4 formulation with approximately 45% released after 24 hours compared to 35% at 37 °C. The middle phases for the F-3 and F-4 formulations at 45 °C lasted

for approximately 5 days and this can be compared to their lag phases at 37 °C which lasted for approximately 3 weeks. The middle phase for the F-1 formulation lasted approximately 1 month, which can be compared to the 4-month lag phase observed at 37 °C. It is speculated that the drug diffusion plays a significant role in the middle phase at 45 ˚C, while the third phase is both diffusion and erosion controlled. At 53 °C, drug release from all formulations was further accelerated and the typical three phase release profile was lost. Instead, formulations F-3 and F-4 showed two distinct release phases and the release was complete in less than 1 week. Formulation F-1 also showed two release phases with a slight inflection point around day 5 and release from this formulation was complete in less than 2 weeks. The glass transition temperatures of the F-1, F-3 and F-4 formulations are below 53 °C (51.31 °C, 50.25°C, and 48.06°C, respectively). In addition to acceleration of polymer degradation, polymer chain mobility increases greatly at temperatures above the Tg which facilitates drug diffusion from the polymer matrix. Both of these factors contribute to change in the drug release mechanism and hence the changes observed in the release profiles of all three formulations. Nevertheless, the accelerated tests at both 45 and 53 °C were able to distinguish the three formulations. Drug release testing performed at 53 °C may be more suitable for quality assurance purposes due to the significant reduction in the duration of release testing.

2.3.4. *In vivo* pharmacodynamics

From the *in vitro* release tests the F-3 formulation was selected as the most promising formulation to achieve long-term continuous dexamethasone release *in vivo*. The F-1 formulation is unsuitable due to the extremely long lag phase (5 months). The F-4 formulation is unsuitable due to the short duration of drug release (1.5 months). The F-3 formulation is superior to the F-2 formulation since it has a slightly higher daily dose. The F-3 formulation was able to control the

foreign body reaction for 4.5 months while the blank formulations triggered both acute and chronic inflammation. (Figure 2.4) The control group exhibited different stages of the foreign body reaction: *1)* inflammatory cell recruitment (mostly neutrophils as indicated by the red arrows) at day 3; *2)* fibroblast activation and alignment at day 14 (indicated by green arrows); and *3)* fibrous encapsulation (indicated by yellow arrows) at 4.5 and 5.5 months. The composites prepared using the F-3 formulation were able to prevent both acute inflammation (no inflammatory cell accumulation at day 3) and chronic inflammation (no activated fibroblast or formation of fibrous encapsulation) up to 4.5 months. At 5.5 months post implantation, fibrous encapsulation was observed for the F-3 formulation composites indicating that dexamethasone was depleted or was released at an insufficient dose at this point. The *in vivo* performance of the F-3 formulation is therefore consistent with its *in vitro* release profile. This continuously releasing (4.5-month) formulation has the longest duration of action of any dexamethasone microsphere formulation reported thus far. An even longer duration of action (approximately 7 months) could be achieved using a mixture of the F-1 and F-3 formulations.

2.4. Conclusions

The current study established the concept of blending different PLGA polymers to achieve continuous and long-term drug release from PLGA microspheres by integrating the benefits of both small and high Mw polymers. The low Mw PLGA increases water absorption into the polymer matrix and generates acidic oligomers/monomers which facilitates the autocatalysis of the high Mw polymer resulting in a smoother release profile. A polymer blend based dexamethasone microsphere/hydrogel formulation with continuous drug release and *in vivo* efficacy of 4.5 months was achieved. This formulation has the longest duration of action among any reported dexamethasone microsphere formulations thus far. In addition, it may be possible to

further extend the duration of action *in vivo* to approximately 7 months by mixing two of the microsphere formulations reported here. The composition of the polymer blends is critical to the release profile. One formulation blending the low and high Mw polymer at a 50:50 ratio exhibited high burst and short duration of drug release due to polymer phase separation during the microsphere formation process. In addition, accelerated drug release methods were developed under elevated temperature conditions to significantly shorten the testing period from months to days while retaining the ability to discriminate among the various formulations. The successful development of a long-term effective composite coating paves the way for the realization of long-term, totally implantable continuous glucose monitoring systems.

2.5. Tables and Figures

Table 2.1. Composition and physical characteristics of microsphere formulations using polymer blends

Formulation	Dexamethasone (mg)	PLGA (DLG7E, mg)	PLGA (RG503H, mg)	Drug Loading (w/w)	Particle Size (by number, µm)	Tg (°C)
F-1	50	500	-	8.34 ± 0.27	5.38 ± 5.27	51.3
F-2	50	375	125	8.09 ± 0.07	4.78 ± 4.62	51.3
F-3	50	333	167	8.11 ± 0.11	5.08 ± 4.88	50.3
F-4	50	250	250	7.86 ± 0.04	4.43 ± 4.42	48.1

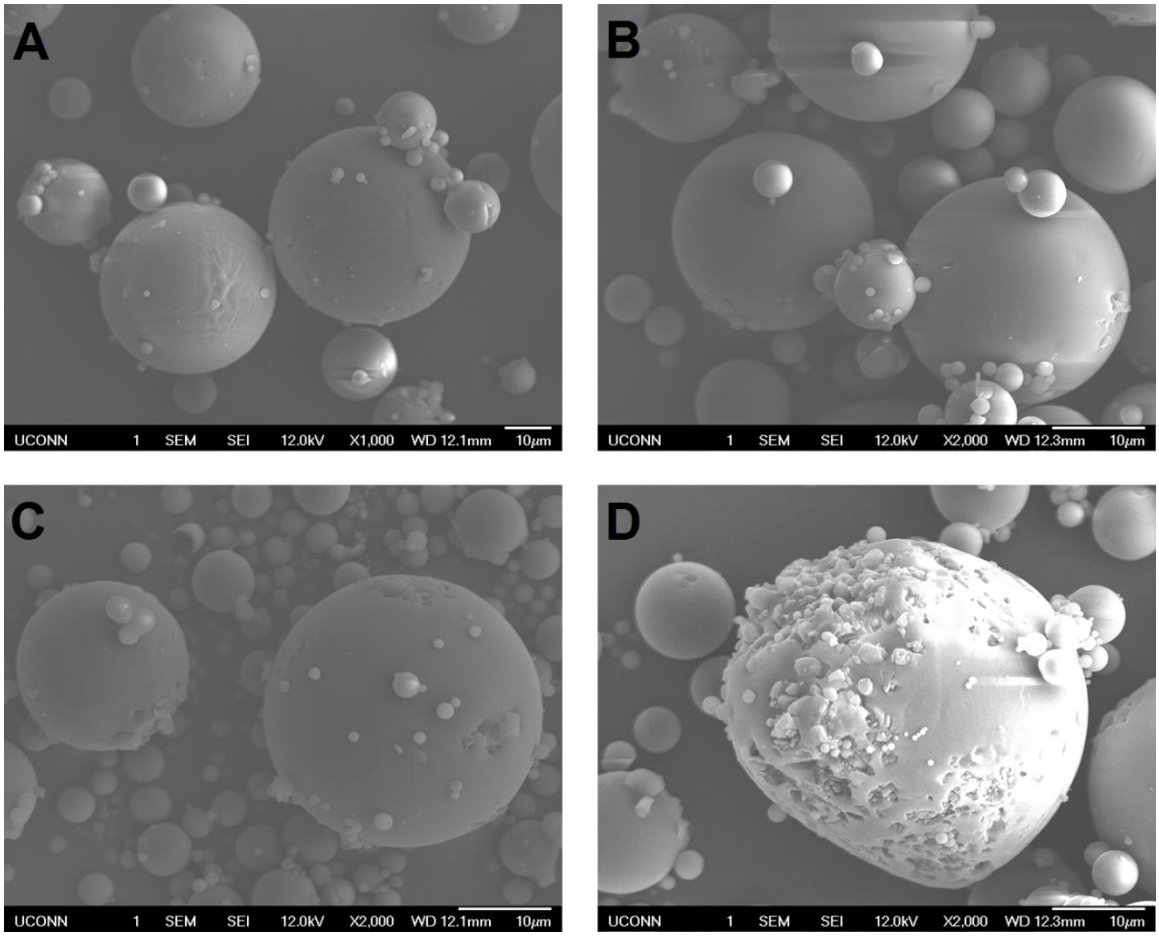

Figure 2.1. Scanning electron micrographs of microsphere formulations prepared using blends of PLGA polymers at various mass ratios: DLG7E/RG503H (w/w)=1/0, 3/1, 2/1 and 1/1 for F-1 (A), F-2 (B), F-3 (C) and F-4 (D), respectively.

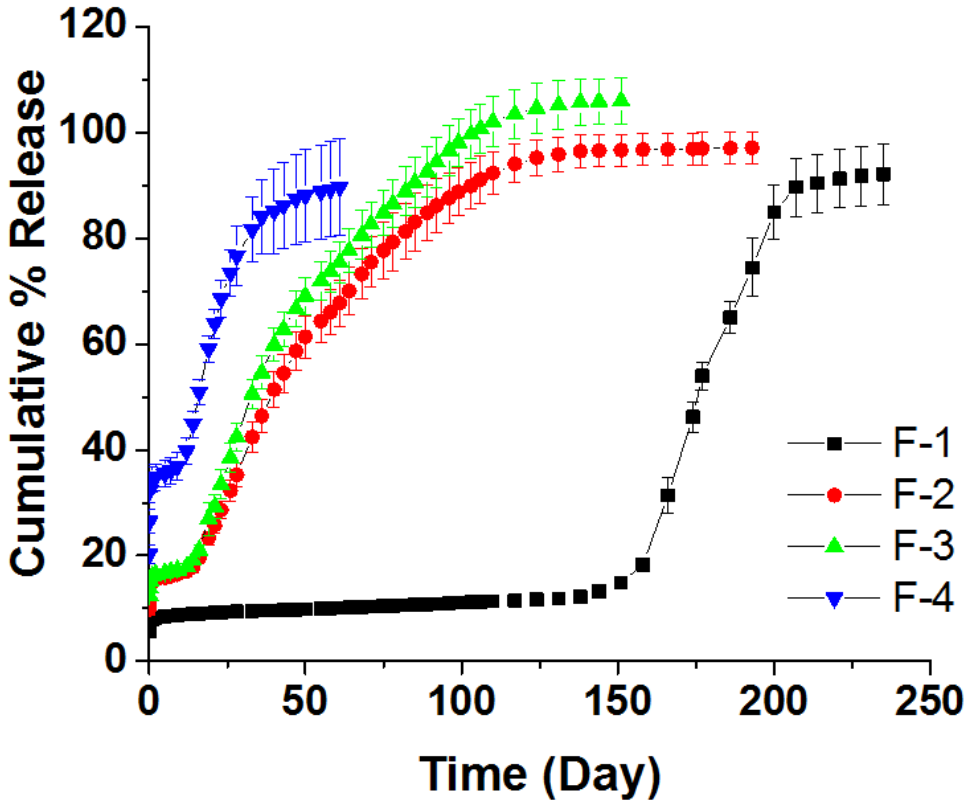

Figure 2.2. *In vitro* release profiles of microsphere/PVA hydrogel composite formulations at 37 °C. The cumulative % release was plotted as mean ± SD (n=3). Different ratios of polymers were used to prepare the microspheres: DLG7E/RG503H (w/w) = 1/0, 3/1, 2/1 and 1/1 for F-1, F-2, F-3 and F-4, respectively.

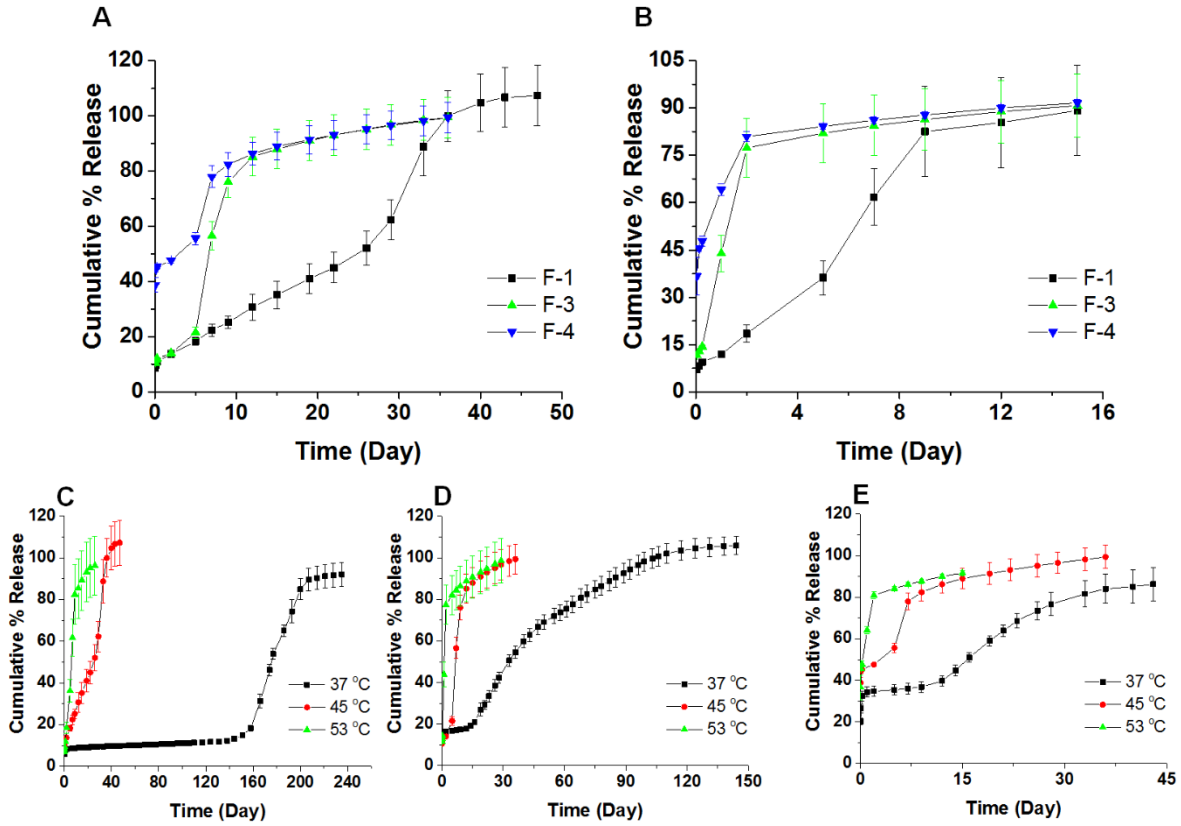

Figure 2.3. *In vitro* release profiles of microsphere formulations at elevated temperatures of 45 °C **(A)** and 53 °C **(B)**. Different ratios of polymers were used to prepare the microspheres: DLG7E/RG503H (w/w) = 1/0, 2/1, and 1/1 for F-1, F-3 and F-4, respectively. *In vitro* release profiles of microsphere formulations at both "real-time" and elevated temperatures (45 °C and 53 °C) of the F-1 **(C)**, F-3 **(D)** and F-4 **(E)** formulations.

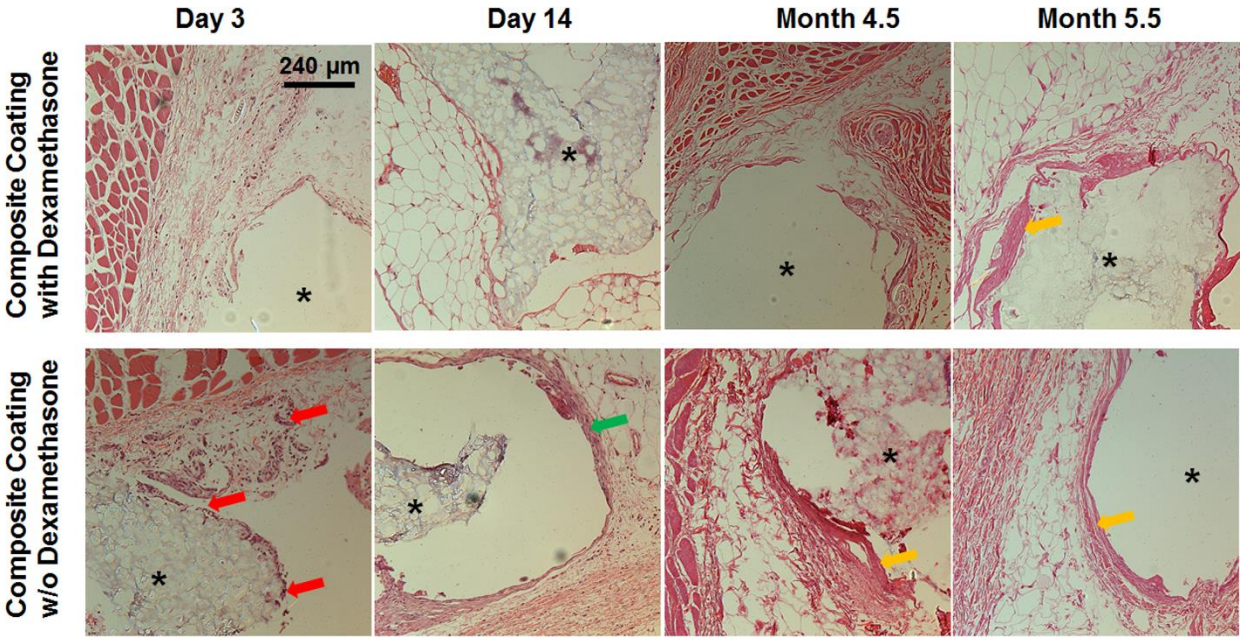

Figure 2.4. *In vivo* pharmacodynamics of implanted composite coated dummy sensors in rats. Stars indicate where the implants were located, the red arrows indicate inflammatory cell infiltration during the acute inflammatory phase, the green arrow indicates activated fibroblasts present during the transitional phase from acute to chronic inflammation, and the yellow arrows indicate a fibrous capsule formed around the implants.

2.6. References

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Chapter 3

Optimization of dexamethasone release from PLGA microspheres prepared with polymer blends using a design of experiment approach

Abstract

Hydrophobic drug release from poly (lactic-co-glycolic acid) (PLGA) microspheres typically exhibits a tri-phasic profile with a burst release phase followed by a lag phase and a secondary release phase. High burst release can be associated with adverse effects and the efficacy of the formulation cannot be ensured during a long lag phase. Accordingly, the development of a long-acting microsphere product requires optimization of all drug release phases. The purpose of the current study was to investigate whether a blend of low and high molecular weight polymers can be used to reduce the burst release and eliminate/minimize the lag phase. A single emulsion solvent evaporation method was used to prepare microspheres using blends of two PLGA polymers (PLGA5050 (25KDa) and PLGA9010 (113KDa)). A central composite design approach was applied to investigate the effect of formulation composition on dexamethasone release from these microspheres. Mathematical models obtained from this design of experiments study were utilized to generate a design space with maximized microsphere drug loading and reduced burst release. Specifically, a drug loading close to 15% can be achieved and a burst release less than 10% when a composition of 80% PLGA9010 and 90 mg of dexamethasone is used. In order to better describe the lag phase, a heat map was generated based on dexamethasone release from the PLGA microsphere/PVA hydrogel composite coatings. Using the heat map an optimized formulation with minimum lag phase was selected. The microspheres were also characterized for particle size/size distribution, thermal properties and morphology. The particle size was demonstrated to be related to the polymer concentration and the ratio of the two polymers but not to the dexamethasone concentration.

3.1 Introduction

Diabetes mellitus is a chronic metabolic disease affecting about 387 million people globally (2014 data) according to International Diabetes Federation [1]. Fluctuations in blood glucose levels is a typical symptom of diabetic patients due to underproduction or underutilization of insulin. It is critical for diabetic patients to closely monitor their blood glucose levels in order to control disease progression and prevent severe complications [2]. At present, most diabetic patients rely on glucose strips along with hand held glucose meters to measure blood glucose levels *via* finger pricking [3]. Continuous glucose monitoring provides the advantage of accurately monitoring the blood glucose trend for precise calculation of the insulin dose, therefore eliminating the possibility of hypo/hyperglycemic conditions [4]. Currently, commercially available continuous glucose monitoring devices can only function for up to 7 days with a glucose oxidase based transcutaneous amperometric sensor [5]. These sensors lose functionality after one week due to the foreign body reaction (FBR) which is a series of sequential events that ultimately rejects the implanted biomaterials [6]. The initial biofouling and sequential inflammatory cell attack can affect enzyme stability and reduce sensor sensitivity. Fibrous encapsulation, the final event of FBR, deprives the sensor of adequate analyte supply leading to a loss in sensor signal. Inhibition of local FBR is one of the most promising strategies to extend sensor lifetime [7].

In order to achieve long-term continuous glucose monitoring, biocompatible coatings composed of poly (lactic-co-glycolic acid) (PLGA) microspheres embedded in a polyvinyl alcohol (PVA) hydrogel have been developed [8-12] for a totally implantable, miniaturized (0.5mm x 0.5 mm x 5 mm) glucose biosensor (being developed in our laboratory). The PVA hydrogel acts as a hydrophilic base to support the microspheres and to allow glucose to readily diffuse to the biosensor. PLGA microspheres serve as drug reservoirs to continuously release dexamethasone to

inhibit local inflammation, therefore, preventing the foreign body reaction. However sustained release of dexamethasone is required over the entire sensor lifetime since a delayed tissue reaction can develop after exhaustion of the drug [9].

Dexamethasone loaded microspheres tend to have an initial burst phase, followed by a lag phase and then a secondary release phase [13]. A sufficient amount of dexamethasone should be released during the burst release phase to inhibit the acute inflammation that is caused by the trauma of implantation. However, too high an initial burst release may cause dose dumping, which can lead to severe side effects. High drug loading is desired as this can benefit both the daily dose and the duration of drug release. The lag phase, where the daily drug release is typically low, should be as short as possible in order to ensure efficacy during that period. It has always been a challenge to control the burst release and increase drug loading for PLGA based microspheres [14]. The burst release of drug from microspheres can be adjusted through process controls such as stabilizing the primary emulsion and changing the solvent evaporation rate by modifying the agitation type/speed [15]. The use of co-solvents is another effective strategy to reduce the initial burst release. For example, burst release of 10-hydroxycampetothecin from PLGA microspheres was reduced when dimethylformadide was used as a co-solvent with methylene chloride{Shenderova, 1997 #25}.

However, there has been a paucity of literature reports on how to control/eliminate the microsphere lag phase. Changing the composition of the formulation is the most straightforward and effective way to adjust microsphere release properties. Applying the concept of blending different polymers, it is possible to adjust the dexamethasone release profile by decreasing the burst release and shortening the lag phase to achieve sustained drug release for approximately 3 months [16]. For ganciclovir microspheres prepared by blending PLGA7525 and Resomer RG 502H, drug encapsulation and release parameters were shown to be altered significantly [17]. Modified

leuprolide release from PLGA microspheres was also reported by blending 8.6 KDa and 28.3 KDa polymers [18].

Based on the *in vivo* performance of previous formulations, further formulation optimization to increase the drug loading and control the burst release and lag phase is required to establish a long-term (6-month) effective microsphere formulation [11, 19]. Quality by design (QbD) and design of experiment (DoE) approaches have been widely used in the development of various pharmaceutical formulations [20-22]. Mathematical models generated from DoE studies can also be used for response prediction and formulation optimization purposes [23, 24]. In the current study, the drug loading and initial release phase of the dexamethasone microspheres were optimized using a DoE approach. In addition to burst release, the lag phase of the PLGA microspheres was also adjusted to achieve sufficient theoretical daily dose (0.17 µg/day from our previous study[19]) for *in vivo* efficacy. The release characteristics were correlated with the physico-chemical properties of the PLGA microspheres such as thermal properties, particle size as well as morphology.

3.2 Material and Methods

3.2.1 Materials

Dexamethasone was purchased from Cayman Chemical (Ann Arbor, MI), poly (vinyl alcohol) (PVA, Mw 30–70 KD), sodium chloride (NaCl, ACS grade), sodium azide (NaN_3), sodium phosphate dibasic dihydrate ($Na_2HPO_4 \cdot 2H_2O$), sodium phosphate monobasic (NaH_2PO_4) and dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich (St. Louis, MO). PVA (99% hydrolyzed, Mw 133 KD) was purchased from Polysciences, Inc. (Warrington, PA). PLGA Resomer® RG503H 5050 (RG503H, inherent viscosity 0.32–0.44 dl/g) was a gift from Boehringer-Ingelheim. PLGA 9010 DLG7E (DLG7E, inherent viscosity 0.6-0.8 dL/g) was

purchased from Lakeshore Biomaterials (Birmingham, AL). RG503H has carboxylic acid end groups and DLG7E is end-capped with a lauryl ester group. Methylene chloride (DCM), acetonitrile (ACN, HPLC grade), and tetrahydrofuran (THF, HPLC grade) were purchased from Fisher Scientific **(Pittsburgh, PA).** NanopureTM quality water (Barnstead, Dubuque, IA) was used for all studies.

3.2.2 Methods

3.2.2.1 Preparation of microspheres

Dexamethasone loaded microsphere formulations were prepared using an oil-in-water (o/w) emulsion solvent extraction/evaporation technique. The PLGA polymers (amounts and ratios as indicated in Table 3.1) were dissolved in 2 ml of methylene chloride and dexamethasone (amounts as indicated in Table 3.1) was dispersed in this solution and these dispersions were sonicated using a bath sonicator for 20 minutes. The dispersions were then further mixed using a T 25 digital ULTRA-TURRAX homogenizer (IKA Works, Inc., Wilmington, NC) at 10,000 rpm for 1 min. The organic phase was then slowly added to 10 ml of PVA solution (1% (w/v), average Mw 30–70 KDa) and homogenized at 10,000 rpm for 2.5 min. The emulsion was then transferred to 125 ml of an aqueous PVA solution (0.1% (w/v), Mw 30-70 KDa) and stirred at 600 rpm. A vacuum was applied to the aqueous phase for 2.5 hours to evaporate the methylene chloride and harden the microspheres. The hardened microspheres were transferred to 50 mL centrifuge tubes and collected *via* centrifugation at 1500 rpm for 2 minutes. The microspheres were then washed three times with deionized water (10 mL each time), collected using the same centrifugation procedure as before and dried using a freeze dryer. The prepared microspheres were stored at 4°C until further use.

3.2.2.2 Central composite design

A 3-factor 5-level central composite design was applied in order to optimize drug loading, burst release and lag phase by adjusting the total amount of PLGA, the percentage of DLG7E in the polymer blend and the amount of dexamethasone. The design involves preparation of 20 formulations including 6 center points (CCD-7, 9, 11, 12, 16, 20), 8 edge points (CCD-2, 4, 5, 10, 13, 14, 18, 19) and 6 star points (CCD-1, 3, 6, 8, 15, 17) as shown in Table 3. 1.

Table 3. 1. Variables and responses from the central composite design

		Variables			Responses		
Run #	Type of Design Point	Total Amount of PLGA (mg)	DLG7E Ratio (%)	Dexamethasone (mg)	Drug Loading (%)	Burst Release (%)	Encapsulation Efficiency (%)
CCD-1	Star	500.00	20.00	75.00	9.71	37.21	74.44
CCD-2	Edge	559.46	83.78	60.13	9.29	10.26	95.73
CCD-3	Star	500.00	60.00	100.00	13.36	13.71	80.16
CCD-4	Edge	559.46	83.78	89.87	13.18	10.33	95.23
CCD-5	Edge	559.46	36.22	89.87	11.19	30.13	80.85
CCD-6	Star	500.00	60.00	50.00	8.66	12.10	95.26
CCD-7	Center	500.00	60.00	75.00	12.12	18.92	92.92
CCD-8	Star	400.00	60.00	75.00	12.13	21.08	76.82
CCD-9	Center	500.00	60.00	75.00	11.47	17.93	87.94
CCD-10	Edge	440.54	83.78	60.13	11.25	9.75	93.67
CCD-11	Center	500.00	60.00	75.00	11.55	17.87	88.55
CCD-12	Center	500.00	60.00	75.00	11.21	15.17	85.94
CCD-13	Edge	440.54	36.22	89.87	14.32	35.20	84.52
CCD-14	Edge	559.46	36.22	60.13	8.77	15.20	90.37
CCD-15	Star	500.00	100.00	75.00	12.84	5.18	98.44
CCD-16	Center	500.00	60.00	75.00	11.32	14.28	86.79
CCD-17	Star	600.00	60.00	75.00	9.81	14.27	88.29
CCD-18	Edge	440.54	83.78	89.87	15.90	12.10	93.84
CCD-19	Edge	440.54	36.22	60.13	11.23	20.47	93.51
CCD-20	Center	500.00	60.00	75.00	12.41	15.38	95.14

3.2.2.3 Drug loading

Drug loading was determined by dissolving approximately 5 mg of dexamethasone-loaded PLGA microspheres in 10 ml THF. This solution was filtered (Millex® HV, PVDF 0.45 μm

syringe filter) and the dexamethasone concentration was determined using a Perkin Elmer series 200 HPLC system (Shelton, CT) equipped with a UV absorbance detector (240 nm wave length). The mobile phase consisted of acetonitrile/water/phosphoric acid (35/70/0.5, v/v/v). A Zobax C18 (4.6 mm × 15 cm, Agilent, Santa Clara, CA) analytical column was used with a flow rate of 1 ml/min. The injection volume used for drug loading determination was 5 µl. The chromatographs were analyzed by PeakSimple™ Chromatography System (SRI instruments, Torrance, CA).

Drug loading was determined as: % drug loading = (weight of drug loaded/weight of microspheres) × 100%.

3.2.2.4 Burst release

Burst release was determined by incubating approximately 5 mg of microspheres in 100 mL of phosphate buffered saline (PBS, 10 mM, pH 7.4) solution at 37 °C. After 24 hours incubation, 10 mL of PBS was filtrated through a 0.45 µm syringe filter and the dexamethasone concentration was determined using the HPLC method described above except that a 20 µl sample was injected.

Burst release was determined as: % burst release = (weight of drug released after 24 hours/weight of drug loaded in the microspheres) × 100%.

3.2.2.5 In vitro release testing

In vitro release testing was performed for the PLGA microsphere/PVA hydrogel (99% hydrolyzed, Mw 133 KD) composite formulations. The composites were prepared using a freeze-thaw method described previously [16]. Briefly, an appropriate amount of PLGA microspheres was dispersed into the PVA hydrogel (5% w/v) solution, then this suspension was filled into a plastic tubing mold (5 mm inner diameter and 2 cm in length) and subjected to three freeze-thaw

cycles consisting of 2 h freezing at −20°C followed by 1 h thawing at room temperature. The composites were air dried. Approximately 2 mg of each formulation was immersed in a 2 ml Eppendorf vial containing 1.8 ml of 10 mM PBS (pH 7.4) with 0.1% NaN_3 and incubated at 37 °C under constant agitation. At pre-determined time points, all the release media was removed and replenished with fresh media. Sink conditions were maintained throughout. The samples were filtered through 0.45 μm syringe filters and the concentration of dexamethasone in each sample was determined using the HPLC method as described above. Normalized daily drug release from each central composite design formulation was calculated for up to 21 days in order to determine the lag phase of the formulations.

Cumulative dexamethasone release was plotted *versus* time in the release profile.

Normalized daily drug release was determined as: amount of drug released between two time points/(initial sample weight*duration of two time points).

3.2.2.6 Particle size evaluation

An AccuSizer 780A autodiluter particle sizing system (Nicomp, Santa Barbara, CA) was used to determine the particle size of the prepared microspheres. Approximately 5 mg of microspheres were dispersed in 1 ml of 0.1% (w/v) PVA solution and 100 μl of the dispersion was injected into the system for particle size analysis. All measurements were conducted in triplicate and the results are reported as the volume based mean particle size ± SD. The standard deviation (indicating the distribution of the particle size) was also reported.

3.2.2.7 Thermal analysis

A TA Q1000 differential scanning calorimeter (DSC) (TA, New Castle, DE) was used to determine the glass transition temperature (Tg) of the prepared microspheres. Modulated DSC was performed with the cycle below: the samples were heated at a rate of 3 °C/min from 4°C to

80 °C at a modulating oscillatory frequency of 1°C/min. The thermograms were analyzed using Universal Analysis software (TA Instruments) to determine the glass transition temperatures.

3.2.2.8 Scanning electron microscopy (SEM)

A scanning electron microscope (FEI Nova™ NanoSEM 450) equipped with an ETD detector was used to evaluate the morphology of the prepared microspheres. Samples were mounted on carbon taped aluminum stubs and sputter coated with gold for 1.5 min at 6 mA. Images were taken with an accelerating voltage of 2.0 kV and a working distance of 4 mm.

3.2.2.9 Statistical analysis

Data collected for drug loading and burst release in each run were analyzed using Design Expert software (Version 9, StatEase, Minneapolis, MN, USA) and fitted into linear regression models. Analysis of variance (ANOVA) was used to determine the significance of the model and parameters. Model correctness was assessed by the lack of fit test. In the case of particle size and size distribution, paired Student's t-test was performed to determine whether there were statistically significant differences among the results.

3.3 Results and discussion

3.3.1 Central composite design

The drug loading and burst release for all DoE formulations are shown in Table 3. 1. CCD-18 has the highest drug loading (15.9%) among all the formulations. The encapsulation efficiency (EE, calculated by dividing the actual drug loading by the theoretical drug loading) is approximately 93.8%, indicating most of the dexamethasone added was loaded into the microspheres. CCD-6 has the lowest drug loading (8.66%) among all the formulations. This is due to the low theoretical drug loading of this formulation since the encapsulation efficiency is actually similar to that of CCD-18 (EE of CCD-6 is 95.3%). When comparing the encapsulation

efficiency of all formulations, CCD-1 has the lowest EE around 75%. This formulation (CCD-1) has the highest ratio of low molecular weight PLGA which is likely to cause slower polymer precipitation and consequently loss of drug to the aqueous phase. It has been reported that low drug encapsulation is associated with slow PLGA precipitation [25]. CCD-15, composed of only DLG7E (high molecular weight PLGA) and dexamethasone, was determined to have the lowest burst release of 5.18%. Thus, fast microsphere solidification is expected in this case which can lead to less surface associated or free dexamethasone present in the formulation [13]. The burst release of the CCD-1, 5 and 13 formulations are higher than 30%, which can be explained by lower DLG7E ratio and higher initial dexamethasone added into these formulations. A detailed statistical description for the DoE results regarding drug loading and burst release are discussed below.

3.3.2 Drug loading

A significant DoE model was obtained from the study according to the analysis of variance (ANOVA) table (Table 3.2) with a *p*-value less than 0.0001 and *Lack* of *Fit* value of 0.2026 (larger than 0.05). Four terms were included to generate a mathematical model for drug loading prediction as shown below (also included in Figure 3.1-C).

$$\boldsymbol{Drug\ Loading} = \mathbf{15.29} - \mathbf{0.017} \times \boldsymbol{PLGA} - \mathbf{0.051} \times \boldsymbol{DLG7E}\% + \mathbf{0.044} \times \boldsymbol{Dexamethasone} + \mathbf{1.071} \times \mathbf{10^{-3}} \times \boldsymbol{DLG7E}\% \times \boldsymbol{Dexamethasone}$$

The *p*-values for the effect of total PLGA amount, ratio of DLG7E and total dexamethasone amount are much smaller than 0.05, indicating that all these factors significantly affect microsphere drug loading. The *p*-value for the interaction term (BC, DLG7E ratio and dexamethasone amount) is 0.1133, which is slightly higher than 0.05. A small interaction may

exist between these two variables as increasing the amount of dexamethasone will leave excessive free drug to interact with the polymers, therefore affecting the drug loading of the formulation. The contour plots shown in Figures 3.1-A and 1-B indicate the effects of individual factors on the drug loading. Increase in the initial dexamethasone concentration or decrease in the total amount of PLGA increased the theoretical drug loading by adjusting the polymer/drug ratio and this is reflected in the actual drug loading. A higher ratio of the high molecular weight polymer (DLG7E) also leads to higher drug loading. This can be explained by fast precipitation of the high molecular weight polymer. When a higher ratio of DLG7E is used, fast solidification of the microspheres is expected which will lead to more dexamethasone encapsulation. The predicted *versus* actual drug loading values for all the CCD formulations are shown in Figure 3.1-D. A linear fit was obtained indicating that the model is valid.

Table 3.2 ANOVA table of the DoE model used to predict dexamethasone loading.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	57.58	4	14.40	35.49	< 0.0001	*Significant*
A-PLGA	*14.71*	*1*	*14.71*	*36.26*	*< 0.0001*	
B-DLG7E%	*6.43*	*1*	*6.43*	*15.86*	*0.0012*	
C-Dexamethasone	*35.29*	*1*	*35.29*	*87.01*	*< 0.0001*	
BC	*1.15*	*1*	*1.15*	*2.83*	*0.1133*	
Residual	6.08	15	0.41			
Lack of Fit	*4.95*	*10*	*0.49*	*2.17*	*0.2026*	*Not Significant*
Pure Error	*1.14*	*5*	*0.23*			
Correlation Total	63.67	19				

3.3.3 Burst release

A significant DoE model (indicated by a *p*-value less than 0.0001 and *Lack of Fit* value higher than 0.05) for burst release was obtained. The mathematical model for burst release prediction is shown below (also included in Figure 3.2-C):

$$\boldsymbol{Burst\ release}$$

$$= -0.37 - 0.077 \times PLGA - 0.39 \times DLG7E\% + 1.58 \times Dexamethasone$$
$$+ 8.03 \times 10^{-4} \times PLGA \times DLG7E\% - 9.63 \times 10^{-3} \times DLG7E\%$$
$$\times Dexamethasone + 3.02 \times 10^{-3} \times (DLG7E\%)^2 - 5.53 \times 10^{-3}$$
$$\times (Dexamethasone)^2$$

More terms were included in this mathematical model indicating a more complex interaction for burst release compared to drug loading. The initial burst release is a result of free dexamethasone or microsphere surface associated dexamethasone which can be related to the microsphere composition as well as the process parameters. In order to rule out the effect of process parameters on burst release, all processes were carried out following exactly the same procedures including the solvent evaporation time, volume and time for microsphere washing. The process is highly reproducible as indicated by the reproducible center points (CCD-7, 9, 11, 12, 16 and 20) of the DoE. Since dexamethasone is only slightly soluble in methylene chloride, the majority of the drug exists as crystalline particles in the formulation. With change in the DLG7E ratio in the polymer composition, the burst release varies significantly as shown in the contour plot (Figure 3.2-A). When 80% of DLG7E was added into the polymer blends, the burst release remains around 11% although it varies between 9 and 12% according to the total amount of polymer and dexamethasone (Figure 3.2-B). The composition of the polymer blends is the most important factor affecting the burst release which is also indicated in the ANOVA table (Table 3.3) where the DLG7E ratio term has the lowest *p*-value. A significant interaction between the DLG7E ratio and the dexamethasone amount was observed (*p*-value equal to 0.0032). This may be explained by dexamethasone having different interactions with different polymers. The square of the DLG7E ratio (B^2 in Table 3.3) is also significant. This is because the ratio of the two

polymers affects many formulations aspects that may be related to burst release (such as polymer precipitation rate during microsphere formation as well as the diffusion rate of drug from the surface of the microspheres).

Table 3.3. ANOVA table of the DoE model to predict dexamethasone burst release.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	1226.44	7	175.21	25.46	< 0.0001	*Significant*
A-PLGA	*38.91*	*1*	*38.91*	*5.65*	*0.0349*	
B-DLG7E%	*925.54*	*1*	*925.54*	*134.47*	*< 0.0001*	
C-Dexamethasone	*88.61*	*1*	*88.61*	*12.87*	*0.0037*	
AB	*10.31*	*1*	*10.31*	*1.50*	*0.2446*	
BC	*92.75*	*1*	*92.75*	*13.48*	*0.0032*	
B^2	*42.53*	*1*	*42.53*	*6.18*	*0.0286*	
C^2	*21.72*	*1*	*21.72*	*3.16*	*0.1010*	
Residual	82.60	12	6.88			
Lack of Fit	*64.92*	*7*	*9.27*	*2.62*	*0.1529*	*Not Significant*
Pure Error	*17.68*	*5*	*3.54*			
Correlation Total	1309.04	19				

3.3.4 Formulation optimization and model validation

Following the establishment of the models for drug loading and burst release prediction, the design space for microsphere composition was optimized to maximize drug loading and minimize burst release (in a range of 8 to 12%) based on a batch size of 500 mg PLGA total. Figure 3.3 shows the optimized design space using a contour plot of desirability. Desired formulations require both a large amount of dexamethasone and a high percentage of DLG7E in the total polymer.

Three points (marked as red crosses in Figure 3.3) in the design space were selected to verify the model. Three formulations (V-1, V-2 and V-3) were prepared according to the compositions listed in Table 3.4. Burst release and drug loading were analyzed for each formulation and compared to the predicted values for model validation. The burst release for all the formulations

was close to 10% and the drug loading ranges from approximately 10% to 14%. The actual drug loading and burst release for all the formulations tested are consistent with the predicted values as shown in Table 3.4, indicating that the models are very predictive.

Table 3.4. Verification of CCD models using microspheres prepared under conditions within the optimized design space **(n=3)**

	Composition			Drug Loading (%, w/w)		Burst release (%, w/w)	
#	Total PLGA (mg)	DLG7E ratio (%, w/w)	Dexamethasone (mg)	Predicted	Actual	Predicted	Actual
V-1	500	90	60	10.62	10.2 ± 0.09	9.52	9.4 ± 0.3
V-2	500	80	90	14.38	14.47 ± 0.18	9.45	9.4 ± 0.19
V-3	500	85	75	12.58	12.31 ± 0.26	9.93	10.27 ± 0.52

3.3.5 Duration of lag phase

Bulk degradation is the typical release mechanism for PLGA based microsphere formulations. For dexamethasone release from microspheres, the release profile can usually be divided into three phases: a burst release, a lag phase with limited drug release and a linear release phase. Our previous data has indicated that dexamethasone release during the lag phase (approximately 10 days) from a 1-month formulation is sufficient to inhibit the foreign body reaction [10, 11, 13]. However, it is possible that this may not be the case for microsphere formulations with an elongated lag phase that are prepared with high molecular weight PLGA. For example, for a microsphere formulation prepared using 75 KDa PLGA, the lag phase was approximately 1 month [10]. For higher Mw PLGA (such as 113 KDa used in the current study), the lag phase is expected to be even longer. Blending low and high molecular weight PLGA has been previously used in order to shorten the lag phase [16]. The lag phase was reduced from 1 month to approximately 15 days by blending 25 KDa PLGA with 75 KDa PLGA.

PLGA microsphere/PVA hydrogel composites were prepared for all the microsphere formulations in the DoE study considering that the composite coatings are intended for

application to biosensors for inhibition of the foreign body reaction. Figure 3.4-A shows the release profiles for the initial 21 days for the composite coatings. Release profiles plotted according to the different categories of design points (center, edge and star) is also available in supplementary data Figure 3.5**.** The lag phase for CCD-15 started from day 2 and lasted for the entire 21 days following a small burst release of less than 10 µg/mg. The low burst release for the CCD-15 composite coating is consistent with the low burst observed for the CCD-15 microspheres. CCD-2 and 6 showed slightly higher burst release (approximately 23 µg/mg) and reached a plateau at approximately day 2. The release profiles for the center points (CCD-7, 9, 11, 12, 16 and 20) are clustered in the middle of the graph with approximately 30 µg/mg burst at day 1 and the burst phase continues for these formulations till day 5 when the lag phase begins. The extended burst phase is probably due to a delayed effect from the PVA hydrogel. This is consistent with our previous observation that the release of dexamethasone from free drug embedded PVA hydrogels can last for up to 10 days [26]. CCD-3, 5, 8, and 13 (with high dexamethasone loading in the microspheres) have high burst release phases (more than 50 µg/mL) that also continue for approximately 5 days. The higher burst release exhibited by CCD-3, 5, 8, and 13 is considered to be due to the large amount of free/surface associated drug in the microspheres.

The burst and lag phases of all formulations were analyzed separately using various kinetics models. The models used for analyzing the burst release phase (till day 7) include: the first order release model; the Kosmeyer-Peppas model; and the Peppas-Sahlin model. The release kinetic parameters are shown in Table 3.5. The first order release model did not provide a good approximations since the R square values were small for most of the formulations. The n-value obtained using the Kosmeyer-Peppas model is an indication of whether drug release is

contributed by Fickian diffusion or polymer swelling (so called Case-II relaxation) [27]. However, the n-values obtained for these formulations were beyond the normal range of 0.43 to 0.85, indicating that a more complex mechanism is involved in the composite system. The microspheres are embedded in the PVA hydrogel which acts as an additional barrier for drug diffusion. Therefore, drug release was further analyzed using a more complex model, the Peppas-Sahlin model [28], which returned better R square values (higher than 0.95 for most formulations). The Peppas-Sahlin model is able to simulate both the Fickian diffusion of dexamethasone from the microsphere surface and Case-II relaxation from the PVA hydrogel. With respect to the lag phase (from day 7 to 21), zero order release kinetics did fit the data of all formulations (linear release trends were observed with R square values higher than 0.95). Zero order drug release indicates concentration independent drug release. This can be explained since the release rate limiting factor during the lag phase is mainly the polymer matrix structure instead of the drug concentration.

The lag phases for most of the formulations shown in Figure 3.4-A started at approximately 5 days. During the lag phase small amounts of drug are released and it is difficult to differentiate the various formulations based on their release profiles. Thus, in order to further analyze the lag phase, the amount of drug released per day was plotted as a heat map (Figure 3.4-B). The heat map allowed better differentiation of the formulations. Analyzing the heat map, it became apparent that CCD-15 has the lowest amount of drug released during the 21 day study. This can be explained since CCD-15 is composed of only the high molecular weight PLGA. CCD-2 and CCD-14 also have relatively lower amounts of drug released during the lag phase compared to the other formulations. However, these two formulations have the lowest initial dexamethasone loading which will contribute to lower release during the lag phase. It is also apparent from the

heat map that the daily drug release for CCD-18 is higher than that of the other formulations at most of the time points. The lowest daily dexamethasone release of CCD-18 is from day 7 to day 9 at approximately 0.15 µg/day, which is slightly less than the reported minimum effective daily dose (0.17 µg/day) to control the foreign body response in a rat model [19]. However, a lag phase of 2 days can be tolerated (at least in this animal model) without onset of the inflammatory response [10, 11, 13]. Considering the high drug loading (15.9%, w/w) along with the burst release (12.1%, w/w) and short lag phase of CCD-18, this formulation is optimal among those investigated. The short lag phase observed for CCD-18 is probably due to: *1)* the extended burst phase of this formulation; as well as *2)* the reduced lag phase from blending low and high molecular weight polymers. The lag phase for the low molecular weight polymer (RG503H) is approximately 10 days. With the accumulation of lactic/glycolic acid monomers from hydrolysis of the low molecular weight PLGA, the degradation of the high molecular weight PLGA is accelerated due to decreased local pH. Accordingly, a continuous release pattern is expected for this formulation after the onset of drug release [29].

3.3.6 Particle size and size distribution

Microsphere particle size has been shown to be affected by the formulation composition [30]. The star points (6 formulations) from the CCD design were divided into three groups. Each group has two formulations that were prepared under the same conditions except that one of the independent variables is changed. The three groups are: *1)* CCD-1 and 15 where the PLGA molecular weight ratio is varied; *2)* CCD-8 and 17 where the total amount of PLGA is varied; and *3)* CCD-3 and 6 where the amount of dexamethasone is varied. Therefore, by analyzing the star points in each group, the effect of each independent variable on the formulation characteristics was evaluated. The effect of each independent variable on the particle size and

size distribution is shown in Figure 3.6. CCD-15 has significantly larger particle size compared to CCD-1. Larger particle size is also observed for CCD-17 compared to CCD-8. These observations indicate that increasing the amount of high molecular weight polymer as well as the initial polymer concentration can lead to larger particle size. This particle size increase can possibly be explained by increase in the polymer solution viscosity that occurs under these conditions. With high polymer solution viscosity, the energy input required to generate the primary emulsion is greater. Therefore larger particle size emulsion droplets were generated for formulations with high viscosity compared to those with low viscosity at the same homogenization speed (same energy input). It has also been reported that higher particle size is associated with higher drug encapsulation which is consistent with observations on these formulations [31]. Particle size is also a major factor determining the diffusion rates of dexamethasone as the length of the diffusion pathways varies for different sized particles. However, no clear trend was observed when comparing the release rates of these formulations. This may be explained as the drug release is mainly controlled by the composition of these formulations. On comparing CCD-3 and CCD-6, no significant difference in particle size was observed since changing the amount of dexamethasone does not affect the viscosity. The standard deviation in the particle size for all formulations was approximately 15 µm indicating wide distributions with no significant difference between any of the groups. Wide particle size distribution is quite common for microspheres prepared *via* homogenization [32].

3.3.7 Modulated differential scanning calorimetry (mDSC)

Star points of the design were analyzed using mDSC to evaluate the thermal properties of the formulations as shown in Figure 3.7. Glass transition temperatures for all formulations except for CCD-15 were between 45-50 °C. CCD-15 has the highest glass transition temperature (Tg) of

approximately 52 °C. The Tg of CCD-15 (composed solely of DLG7E polymer) is similar to the Tg of the polymer (52.97 °C, data not shown) indicating that there was no plasticization in the microspheres. Only one Tg was detected for all the formulations, suggesting that the polymers are miscible in nature and no phase separation occurs. When decreasing the ratio of DLG7E, the Tg decreases. Overall, the Tg(s) of these formulations are higher than body temperature. Based on this, these formulations are expected to be relatively stable compared to formulations with Tg values close to the body temperature following subcutaneous implantation as composite coatings for the biosensors. Those formulations with Tg values lower than body temperature may experience dose dumping once implanted.

3.3.8 Microsphere morphology

In order to investigate the morphology of the prepared microspheres, the star points were also evaluated using scanning electron microscopy. As shown from Figure 3.8, most of the formulations investigated are spherical. However, some irregular shaped or broken microspheres were observed for formulations other than CCD-15. CCD-15 is the only formulation composed of a single polymer. Some polymer segregation may occur within the polymer blend microspheres and this could cause breakage during the microsphere solidification step. In addition, heterogeneous solvent evaporation may occur when polymer blends were used which also can explain the morphology observed including the large pores and crevasses. Incomplete dexamethasone encapsulation was also observed for CCD-1 which may be a result of slow microsphere hardening due to the high ratio of small molecular weight polymer. Incomplete drug encapsulation can be correlated with the low encapsulation efficiency (EE=74.44%) for this formulation. On the other hand, high encapsulation efficiency (EE=98.44%) was observed for the CCD-15 microspheres which were spherical in shape with smooth surfaces. Some non-

spherical particles were observed for CCD-3 (Figure 3.8-C) and CCD-8 (Figure 3.8-E), which appear to be free dexamethasone. This is in agreement with high burst release observed for these formulations. In addition, these formulations have a high drug/polymer ratio. On the other hand CCD-6 (Figure 3.8-D) and CCD-17 (Figure 3.8-F) which have low drug/polymer ratios, showed low burst release. The particle size for these formulations ranges from approximately 10 μm to 50 μm. A broad size distribution is observed for all the formulations. This is consistent with the particle size data shown in Figure 3.6.

3.4 Conclusions

The strategy of blending different MW polymers was shown to be effective in reducing the microsphere burst release to less than 10% and shortening the release lag phase to less than one week. The optimized formulation could potentially be used to prevent the foreign body reaction associated with long-term fully implantable glucose sensors. The current study also demonstrated that the design of experiments (DoE) principles are beneficial for optimizing PLGA microsphere products to achieve the desired release properties. A design space with appropriate formulation outcomes (e.g. high drug loading and low burst release) was obtained based on the highly predictive DoE models. The optimized design space was shown to be valuable in predicting and controlling both drug loading and encapsulation efficiency. According to the design space, approximately 85 mg of dexamethasone and 85% of high molecular weight PLGA were required in order to achieve a formulation with 15% dexamethasone loading and 10% burst release. The design space can also serve as a blueprint to design PLGA blend based microsphere formulations with defined release attributes, such as drug loading and burst release. In addition, controlling drug release using polymer blending broadens the application of PLGA polymers in long-term drug delivery systems. A fundamental understanding of the effect of microsphere composition on

the formulation performance was obtained by examining the formulation particle size, thermal properties as well as morphologies. Importantly, a novel heat map describing the daily drug release was developed to differentiate the various formulations during the lag phase where there is low/no drug release. Drug release properties are usually described using release profiles which may not be sufficiently detailed to describe the lag phase. In contrast, the drug release lag phase was illustrated clearly via the heat map and comparison between various formulations was easily made. Such heat maps are especially helpful for researchers who need to screen the release properties of a large number of formulations.

3.5 Tables and Figures

Table 3.1. Variables and responses from the central composite design

		Variables			Responses		
Run #	Type of Design Point	Total Amount of PLGA (mg)	DLG7E Ratio (%)	Dexamethasone (mg)	Drug Loading (%)	Burst Release (%)	Encapsulation Efficiency (%)
CCD-1	Star	500.00	20.00	75.00	9.71	37.21	74.44
CCD-2	Edge	559.46	83.78	60.13	9.29	10.26	95.73
CCD-3	Star	500.00	60.00	100.00	13.36	13.71	80.16
CCD-4	Edge	559.46	83.78	89.87	13.18	10.33	95.23
CCD-5	Edge	559.46	36.22	89.87	11.19	30.13	80.85
CCD-6	Star	500.00	60.00	50.00	8.66	12.10	95.26
CCD-7	Center	500.00	60.00	75.00	12.12	18.92	92.92
CCD-8	Star	400.00	60.00	75.00	12.13	21.08	76.82
CCD-9	Center	500.00	60.00	75.00	11.47	17.93	87.94
CCD-10	Edge	440.54	83.78	60.13	11.25	9.75	93.67
CCD-11	Center	500.00	60.00	75.00	11.55	17.87	88.55
CCD-12	Center	500.00	60.00	75.00	11.21	15.17	85.94
CCD-13	Edge	440.54	36.22	89.87	14.32	35.20	84.52
CCD-14	Edge	559.46	36.22	60.13	8.77	15.20	90.37
CCD-15	Star	500.00	100.00	75.00	12.84	5.18	98.44
CCD-16	Center	500.00	60.00	75.00	11.32	14.28	86.79
CCD-17	Star	600.00	60.00	75.00	9.81	14.27	88.29
CCD-18	Edge	440.54	83.78	89.87	15.90	12.10	93.84
CCD-19	Edge	440.54	36.22	60.13	11.23	20.47	93.51
CCD-20	Center	500.00	60.00	75.00	12.41	15.38	95.14

Table. 3.2 ANOVA table of the DoE model used to predict dexamethasone loading

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	57.58	4	14.40	35.49	< 0.0001	*Significant*
A-PLGA	*14.71*	*1*	*14.71*	*36.26*	*< 0.0001*	
B-DLG7E%	*6.43*	*1*	*6.43*	*15.86*	*0.0012*	
C-Dexamethasone	*35.29*	*1*	*35.29*	*87.01*	*< 0.0001*	
BC	*1.15*	*1*	*1.15*	*2.83*	*0.1133*	
Residual	6.08	15	0.41			
Lack of Fit	*4.95*	*10*	*0.49*	*2.17*	*0.2026*	*Not Significant*
Pure Error	*1.14*	*5*	*0.23*			
Correlation Total	63.67	19				

Table 3.3. ANOVA table of the DoE model to predict dexamethasone burst release

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	1226.44	7	175.21	25.46	< 0.0001	*Significant*
A-PLGA	*38.91*	*1*	*38.91*	*5.65*	*0.0349*	
B-DLG7E%	*925.54*	*1*	*925.54*	*134.47*	*< 0.0001*	
C-Dexamethasone	*88.61*	*1*	*88.61*	*12.87*	*0.0037*	
AB	*10.31*	*1*	*10.31*	*1.50*	*0.2446*	
BC	*92.75*	*1*	*92.75*	*13.48*	*0.0032*	
B^2	*42.53*	*1*	*42.53*	*6.18*	*0.0286*	
C^2	*21.72*	*1*	*21.72*	*3.16*	*0.1010*	
Residual	82.60	12	6.88			
Lack of Fit	*64.92*	*7*	*9.27*	*2.62*	*0.1529*	*Not Significant*
Pure Error	*17.68*	*5*	*3.54*			
Correlation Total	1309.04	19				

Table 3.4. Verification of CCD models using microspheres prepared under conditions within the optimized design space **(n=3)**

	Composition			Drug Loading (%, w/w)		Burst release (%, w/w)	
#	Total PLGA (mg)	DLG7E ratio (%, w/w)	Dexamethasone (mg)	Predicted	Actual	Predicted	Actual
V-1	500	90	60	10.62	10.2 ± 0.09	9.52	9.4 ± 0.3
V-2	500	80	90	14.38	14.47 ± 0.18	9.45	9.4 ± 0.19
V-3	500	85	75	12.58	12.31 ± 0.26	9.93	10.27 ± 0.52

Table 3.5. Release kinetics parameters for dexamethasone release data of DoE formulations

Burst release		CCD -1	CCD -2	CCD -3	CCD -4	CCD -5	CCD -6	CCD -7	CCD -8	CCD -9	CCD -10	CCD -11	CCD -12	CCD -13	CCD -14	CCD -15	CCD -16	CCD -17	CCD -18	CCD -19	CCD -20
first order *	k (d^{-1})	2.58	5.96	1.57	1.54	2.74	4.24	2.14	2.92	2.09	2.04	2.42	1.80	1.21	3.33	8.91	1.95	2.89	1.40	3.02	2.18
	Rsqr	0.91	0.86	0.94	0.94	0.97	0.66	0.89	0.98	0.94	0.95	0.93	0.91	0.91	0.98	0.91	0.87	0.90	0.63	0.95	0.87
Kosmeyer -Peppas *	kKP (d^{-1})	66.61	76.04	62.86	61.79	69.20	68.84	64.10	69.53	65.58	65.11	65.93	62.73	60.12	71.51	83.51	62.80	67.04	60.73	69.18	64.08
	n	0.23	0.15	0.27	0.27	0.23	0.17	0.23	0.22	0.24	0.25	0.23	0.24	0.29	0.22	0.10	0.23	0.21	0.22	0.22	0.23
	Rsqr	0.88	0.82	0.90	0.91	0.85	0.86	0.85	0.82	0.87	0.88	0.84	0.86	0.95	0.83	0.74	0.85	0.83	0.93	0.85	0.85
Peppas-Sahlin *	k1 (d^{-1})	101.1	124.1	91.4	89.3	106.1	110.4	98.6	108.5	98.8	97.0	101.9	94.9	83.1	112.0	142.8	95.9	105.6	89.3	107.3	98.9
	-k2 (d^{-1})	26.01	39.72	20.86	20.27	26.86	33.46	25.29	28.21	24.22	23.41	26.23	23.33	17.61	29.81	51.88	24.27	28.56	22.67	28.25	25.61
	m	0.45	0.33	0.51	0.50	0.49	0.37	0.48	0.48	0.49	0.49	0.48	0.49	0.49	0.47	0.26	0.48	0.46	0.41	0.46	0.47
	Rsqr	0.98	0.94	0.99	0.98	0.98	0.98	0.98	0.97	0.99	0.98	0.98	0.98	0.99	0.98	0.88	0.97	0.97	0.99	0.98	0.98
Lag phase																					
zero order	k0 (d^{-1})	0.32	0.44	0.25	0.22	0.12	0.86	0.67	0.23	0.32	0.21	0.53	0.64	0.17	0.12	0.28	0.72	0.64	0.87	0.27	0.71
	F0	92.90	90.74	94.61	95.18	97.49	81.02	85.09	94.97	92.86	95.42	88.40	86.02	96.34	97.39	94.14	83.94	85.87	80.96	94.06	84.08
	Rsqr	0.98	1.00	0.95	0.99	0.99	0.96	0.95	0.96	0.95	1.00	0.95	0.95	0.98	1.00	1.00	0.94	0.95	0.98	0.97	0.94

* The equations used for these models are: F=100*[1-Exp(-k1*t)] (First order), F=k1*t^m+k2*t^(2*m) (Peppas-Sahlin), F=kKP*t^n (Kosmeyer-Peppas), and F=F0+k0*t (Zero order)

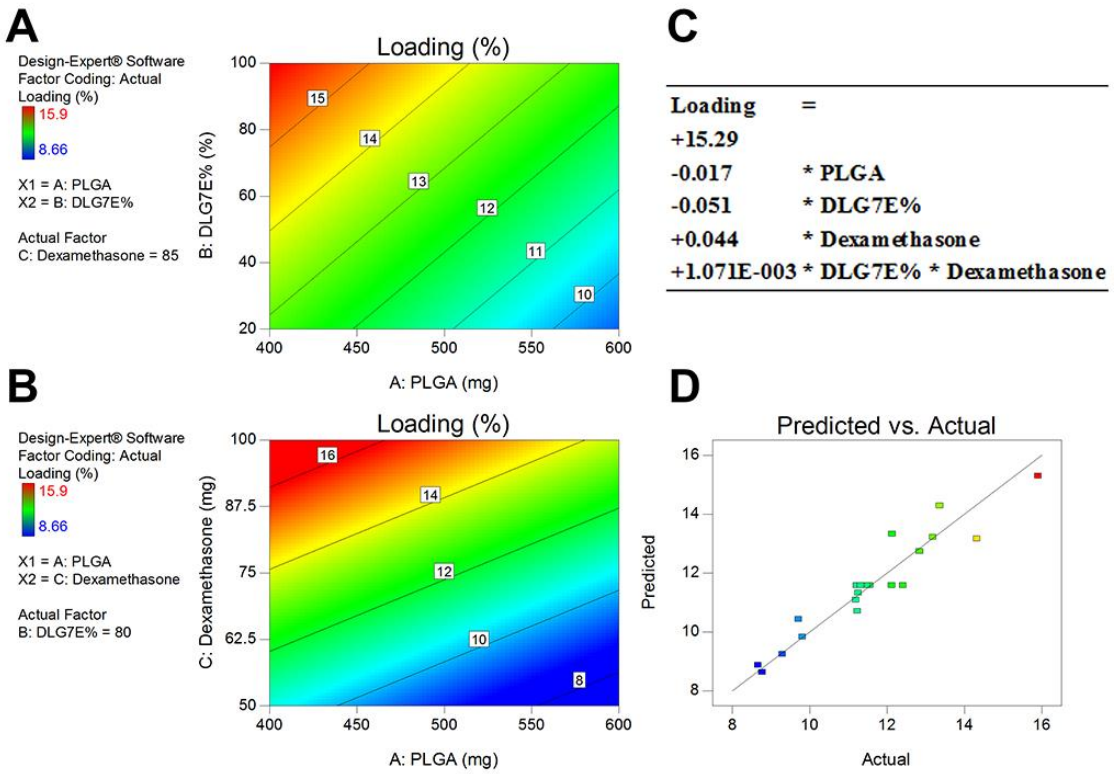

Figure 3.1. DoE model obtained for dexamethasone loading prediction of microspheres prepared using polymer blends. Contour plots (**A** and **B**) indicating correlation between drug loading and two independent variables. **A**: DLG7E ratio in the polymer blend and total amount of PLGA (the total amount of dexamethasone was set at 85 mg). **B**: Dexamethasone amount added and total amount of PLGA (DLG7E ratio was set at 80%). **C**: Mathematical equation used to predict dexamethasone loading. **D**: predicted *versus* actual experimental values based on the model, the line indicates a linear fit.

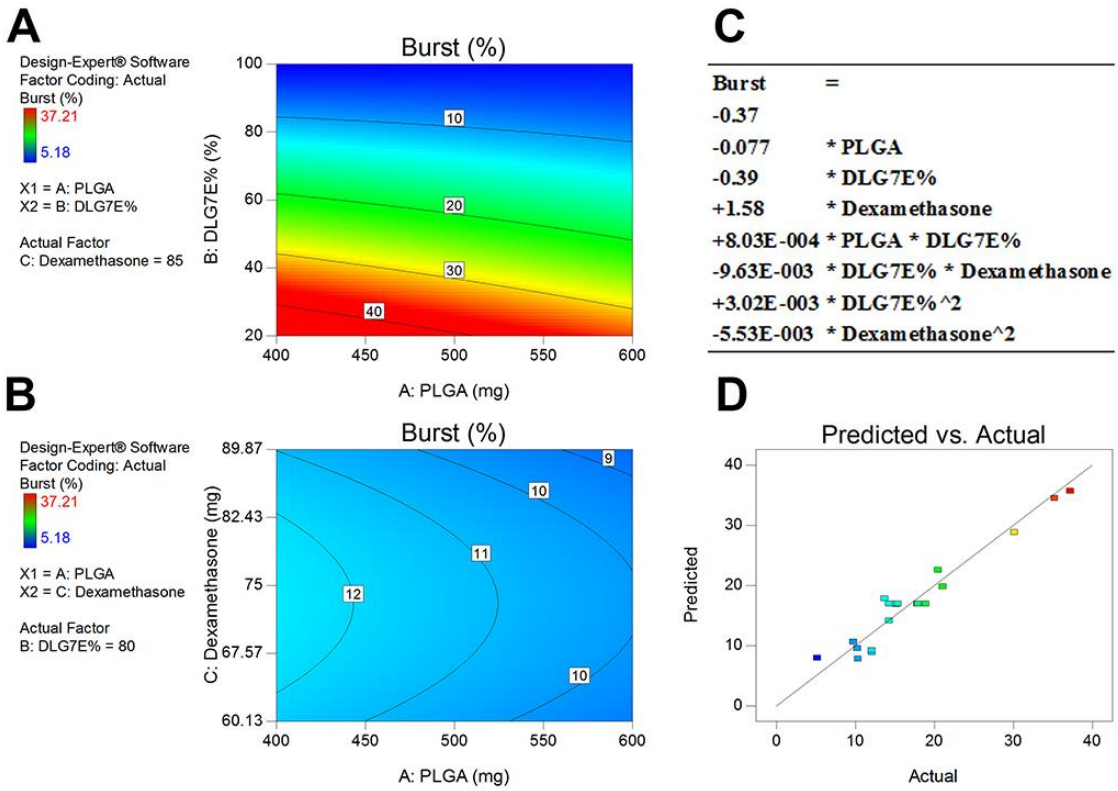

Figure 3.2. DoE model obtained for prediction of dexamethasone burst release from microspheres prepared using polymer blends. Contour plots (**A** and **B**) indicating correlation between burst release and two independent variables. **A:** DLG7E ratio in the polymer blend and total amount of PLGA (dexamethasone amount was set as 85 mg). **B:** Dexamethasone amount added and total amount of PLGA (DLG7E ratio was set as 80%)). **C:** Mathematical equation used to predict dexamethasone burst release. **D:** Predicted *versus* actual experimental values based on the model, the line indicates a linear fit.

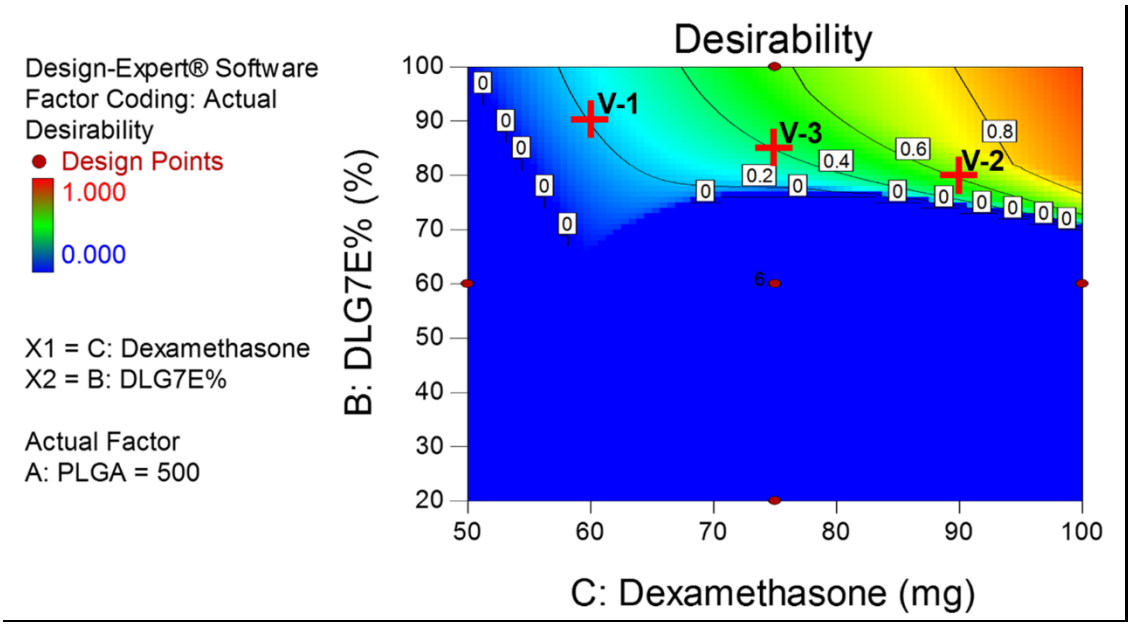

Figure 3.3. Optimized formulation composition to maximize drug loading and minimize burst release. The total amount of PLGA is set at 500 mg. Red crosses (V-1, V-2 and V-3) indicate conditions used for model validation.

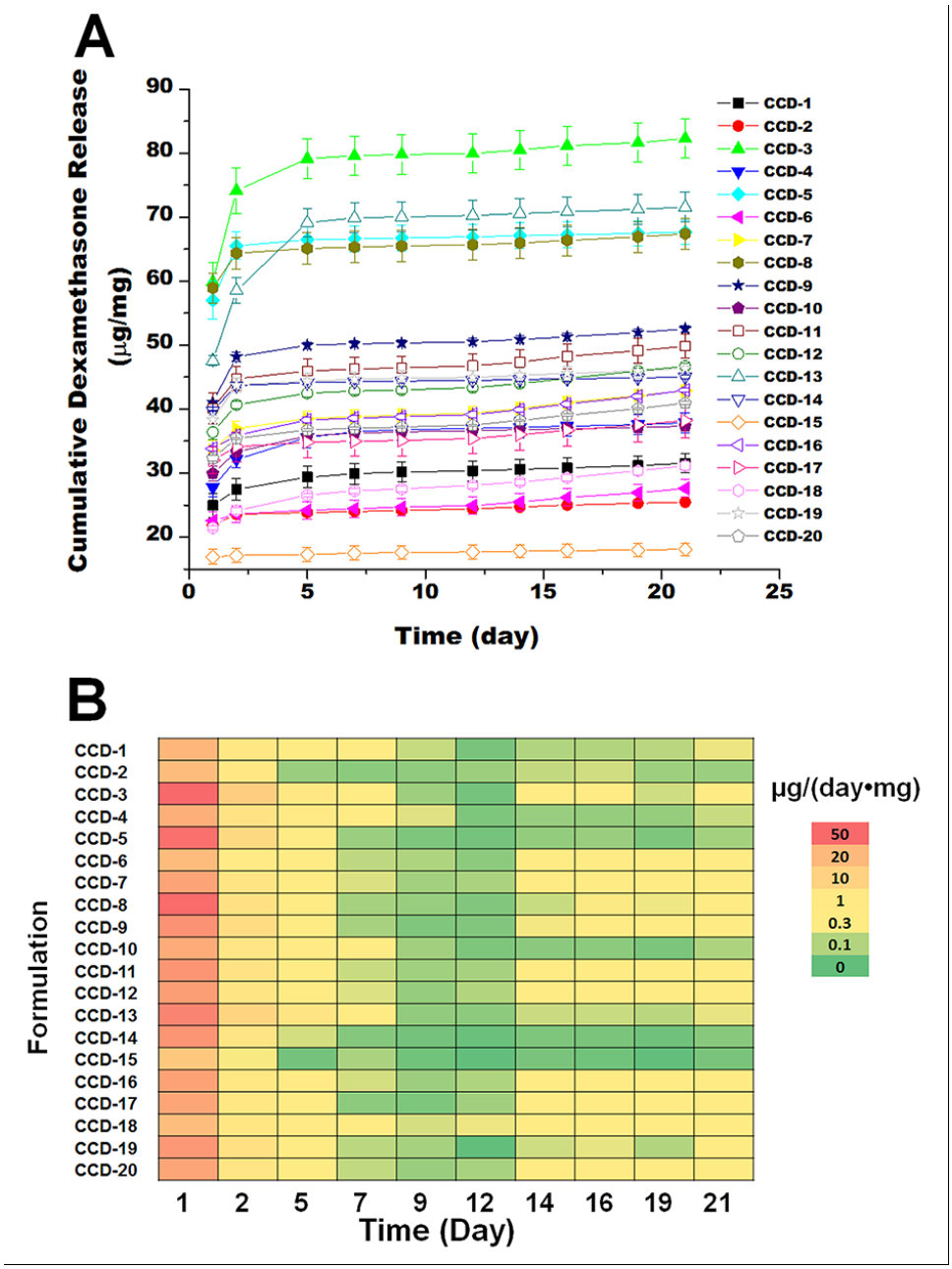

Figure 3.4. Release profile (**A**) and heat map (**B**) describing the initial drug release from the composite coatings prepared using the microspheres from the DoE study. **A**: Cumulative dexamethasone release was plotted *versus* time. **B**: The normalized daily dexamethasone release from the coatings was plotted in the heat map.

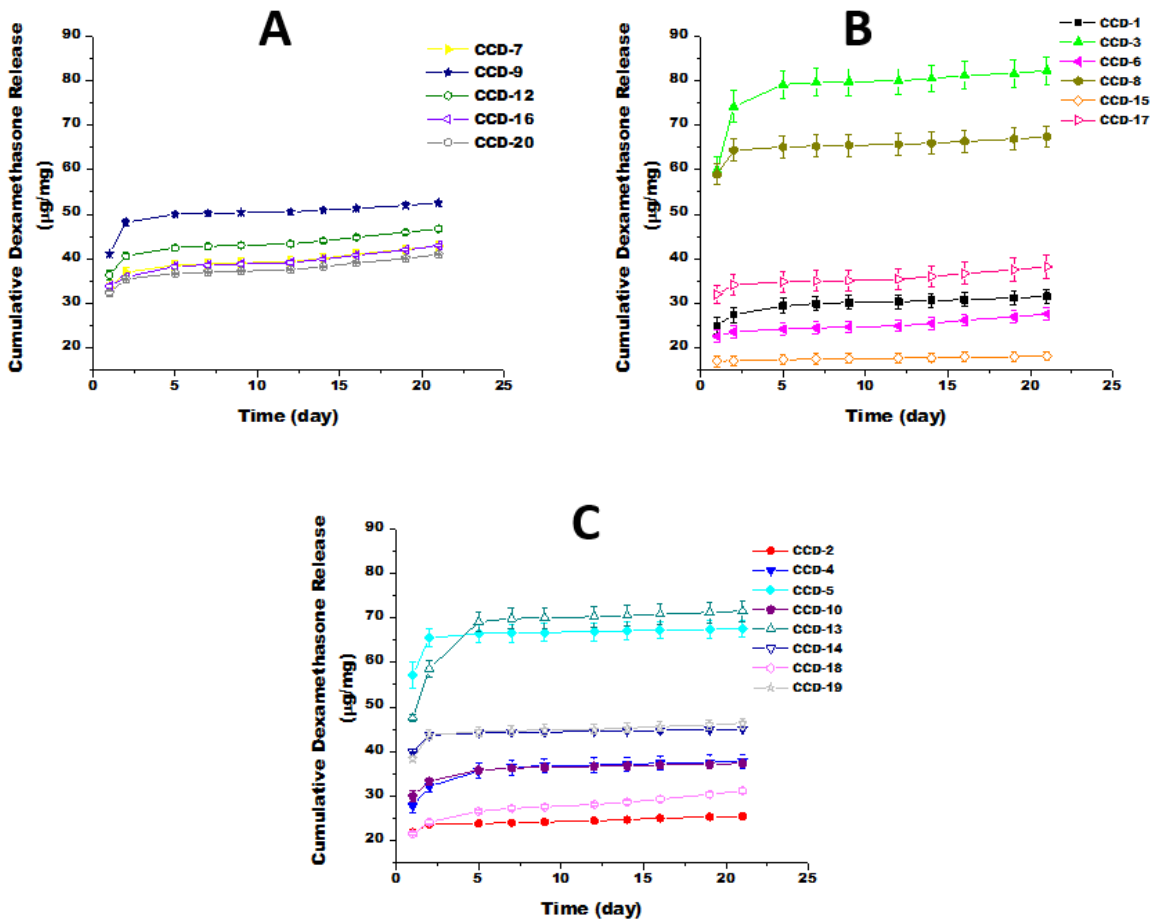

Figure 3.5. Dexamethasone release profiles of DoE formulations prepared at different design points: center point formulations (A), edge point formulations (B) and star point formulations (C)

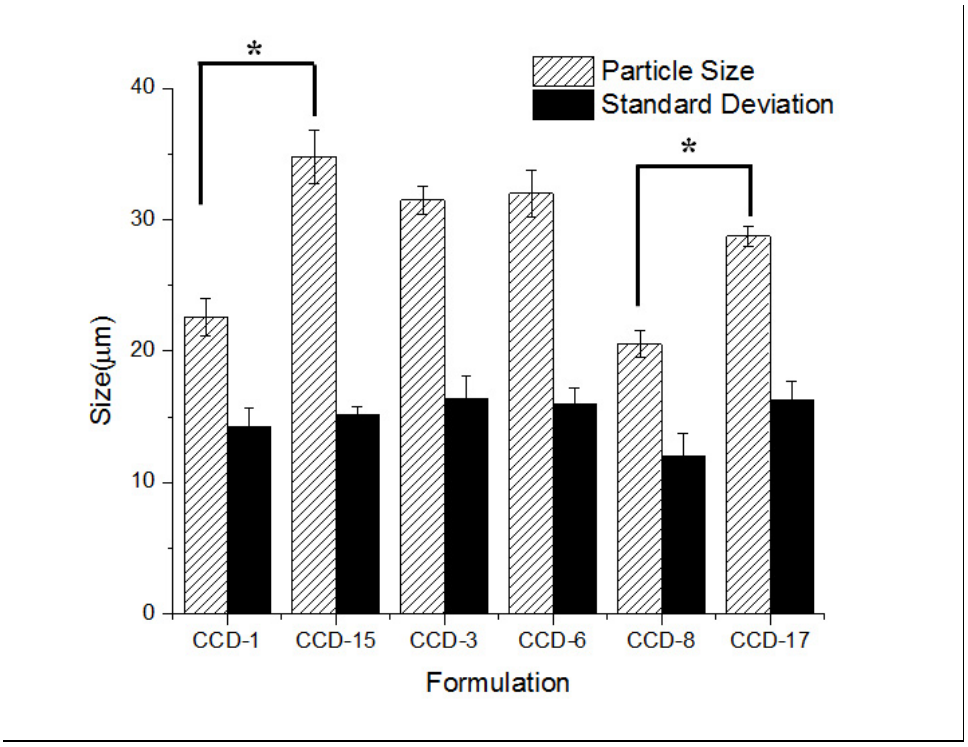

Figure 3.6. Particle size and size distribution (volume based) for the star points of the CCD microspheres (n=3). Center point parameters were used for these formulations except: *1)* the DLG7E ratio was different for CCD-1 (20%) and CCD-15 (100%); *2)* the dexamethasone amount was different for CCD-3 (50 mg) and CCD-6 (100 mg); and *3)* the total polymer amount was different for CCD-8 (400 mg) and CCD-17 (600 mg). The paired student's t-test was performed to determine whether there were any statistically significant differences. $P<0.05$ was considered as a significant difference.

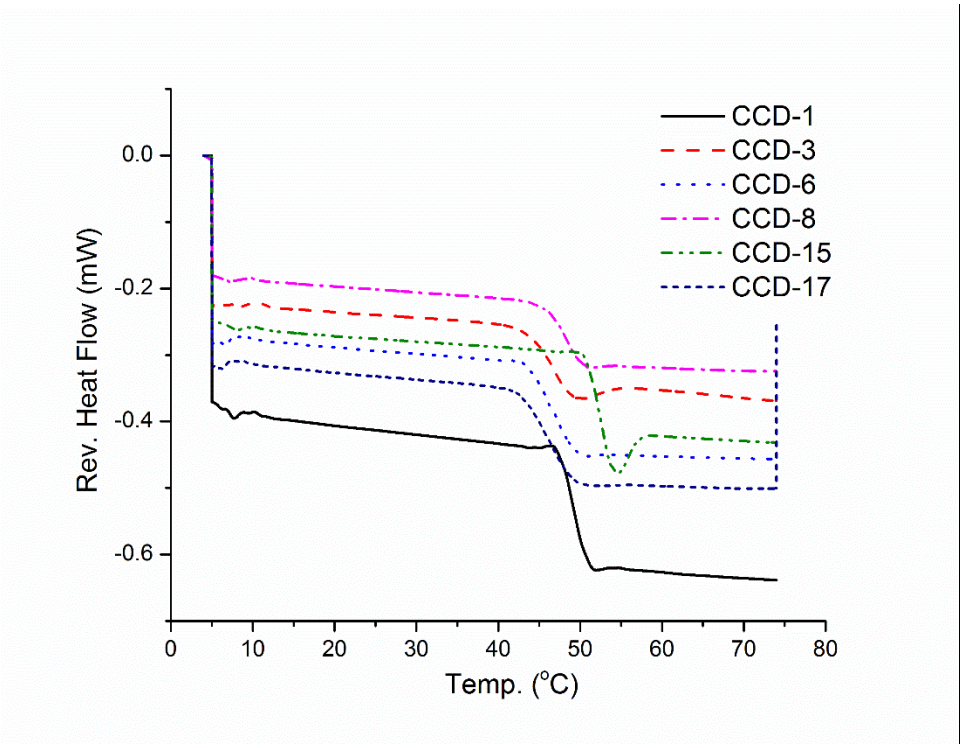

Figure 3.7. DSC thermograms of star points of the CCD microspheres. Center point parameters were used for these formulations except: *1)* the DLG7E ratio was different for CCD-1 (20%) and CCD-15 (100%); *2)* the dexamethasone amount was different for CCD-3 (50 mg) and CCD-6 (100 mg); and *3)* the total polymer amount was different for CCD-8 (400 mg) and CCD-17 (600 mg).

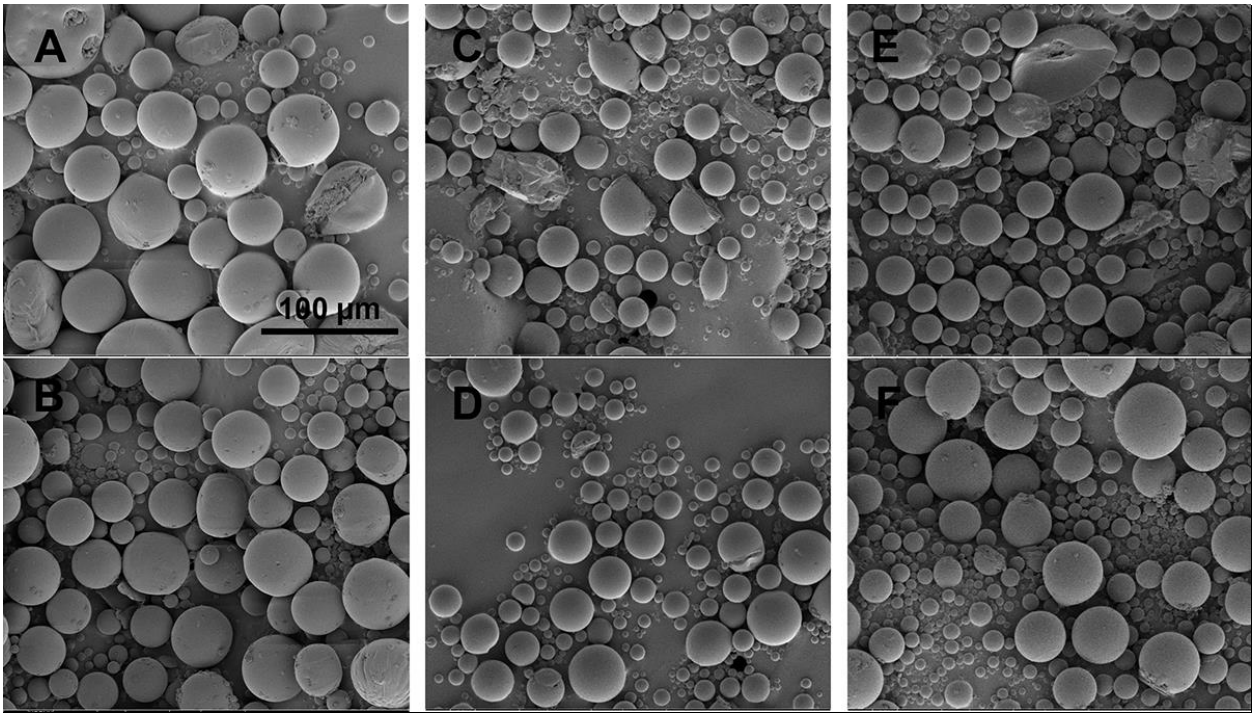

Figure 3.8. SEM images of star points of the CCD microspheres. Figures 3.7-A (top left panel), B (bottom left panel), C (top middle panel), D (bottom middle panel), E (top right panel), and F (bottom right panel) are corresponding to formulations CCD-1, 15, 3, 6, 8 and 17, respectively. Center point parameters were used for these formulations except: *1)* the DLG7E ratio was different for CCD-1 (20%) and CCD-15 (100%); *2)* the dexamethasone amount was different for CCD-3 (50 mg) and CCD-6 (100 mg); and *3)* the total polymer amount was different for CCD-8 (400 mg) and CCD-17 (600 mg).

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Chapter 4

6-month foreign body reaction suppression for glucose sensors using a composite coating

Abstract

The application of dexamethasone releasing poly (lactic-co-glycolic acid) (PLGA) microspheres embedded in a poly vinyl alcohol (PVA) hydrogel coatings have been successfully used in the suppression of the foreign body reaction to implantable glucose sensors. In the current study, dexamethasone loaded PLGA microspheres were prepared blending two types of PLGA polymers (RG503H and DLG7E with MW of approximately 25 kDa and 113 kDa, respectively) to achieve long-term (6 months) inhibition of the foreign body reaction. The microsphere composition was optimized according to the *in vitro* drug release profiles. The optimized microsphere formulation had a composition of DLG7E/RG503H/dexamethasone=21/4/5 (w/w/w) and released dexamethasone continuously for 6 months when embedded in a PVA hydrogel. Combining these microspheres with microspheres composed solely of the DLG7E polymer in the PVA hydrogel realized a coating with an even longer (greater than 7 months) drug release period *in vitro*. A heat map was constructed to precisely depict the *in vitro* daily dexamethasone dose released from the coatings in order to understand any lag phase that could affect the pharmacodynamic response. According to the *in vivo* study, the minimum effective daily dose to counter chronic inflammation was approximately 0.1 μg per mg of implant. The drug loaded implant coatings investigated *in vivo* showed inhibition of the foreign body reaction for 6 months. Such composite coatings composed of PLGA microspheres prepared using polymer blends could potentially be used to ensure long-term performance of glucose sensors.

4.1. Introduction

Diabetes mellitus is a chronic metabolic disease affecting approximately 10% of the U.S. population and is on a rapid growth trajectory [1]. Monitoring blood glucose levels is critical for diabetic patients to prevent hyper- and hypo- glycaemia through allowing enhanced accuracy in medication dose control, as well as through adjustment of diet and life style [2]. The advancement of biosensor technology provides new opportunities for diabetic patients to continuously monitor their blood glucose levels which cannot be achieved *via* the old-fashioned finger pricking method [3]. The foreign body reaction (FBR), is a major road block to the development of a long-term functional implantable glucose sensor [4]. The FBR, a series of immunological reactions, is marked by acute inflammation, chronic inflammation and the formation of fibrous capsule which results in the isolation of implants [5]. Currently, only semi-implantable sensors with a life-time of approximately 7 days are available on the market. Various coatings have been developed for glucose sensors to overcome the foreign body reaction to extend the sensor life-time including anti-biofouling coatings [6], porous coatings [7] [8], anti-inflammatory drug eluting coating [9-13], and angiogenic agent releasing coatings [14-16]. Among these coatings, dexamethasone-releasing coatings are the most promising. Although the other coatings were able to prevent the FBR for short periods of time, it is difficult to develop an effective long-term coating based on these approaches. Dexamethasone is a potent glucocorticoid which can suppress immune cell activation and promote up-regulation of anti-inflammatory cytokines [17, 18]. Dexamethasone loaded PLGA microsphere/PVA hydrogel composite coatings have been developed to prevent the FBR through continuous release of the drug [10, 13, 19, 20]. The depletion of dexamethasone can induce a delayed inflammatory reaction and therefore the efficacy of these coatings greatly depends on continuous drug release [13, 21]. 3-

month FBR inhibition was achieved by mixing two different populations of PLGA microspheres with complimentary drug release profiles [12]. Blending low and high molecular weight PLGA was further utilized to achieve a microsphere formulation with long-term drug release, and a 4.5-month efficacy was accomplished using this strategy [13]. The low Mw PLGA increases water absorption into the polymer matrix and generates acidic oligomers/monomers which facilitates the autocatalysis of the high Mw polymer resulting in a continuous release profile. Further extending drug release from the microspheres with sufficient efficacy requires fine tuning of the formulation composition to achieve appropriate dexamethasone release profiles [22].

In the current study, dexamethasone loaded PLGA microspheres were prepared *via* blending two PLGA polymers, RG503H and DLG7E, and the ratio of the two polymers was optimized to achieve a long-term drug release profile of at least 6 months. The optimized microspheres (with a composition of DLG7E/RG503H/dexamethasone=21/4/5, w/w/w) were characterized for morphology, particle size, and solid state properties. Three microsphere/PVA hydrogel composite coatings were prepared using different microsphere combinations for *in vitro* and *in vivo* testing: *1)* only the optimized microspheres; *2)* a mixture of the optimized microspheres and microspheres prepared solely with the DLG7E polymer to achieve a longer release profile; and *3)* a mixture of the optimized microspheres, the microspheres prepared solely with the DLG7E polymer (to achieve a longer release profile) and an amount free dexamethasone powder (to enhance the burst release to ensure the acute inflammatory phase was controlled). In addition, dexamethasone stability during a long-term drug release study was monitored using HPLC. These composite coatings were applied to dummy sensors and implanted into the subcutaneous tissue of rats for evaluation of long-term FBR suppression.

4.2. Material and Methods

4.2.1. Materials

Dexamethasone was purchased from Cayman Chemical (Ann Arbor, MI), poly (vinyl alcohol) (PVA, Mw 30–70 KD), sodium chloride (NaCl, ACS grade), sodium azide (NaN_3), sodium phosphate dibasic dihydrate ($Na_2HPO_4 \cdot 2H_2O$), sodium phosphate monobasic (NaH_2PO_4) and dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich (St. Louis, MO). PVA (99% hydrolyzed, Mw 133 KD) was purchased from Polysciences, Inc. (Warrington, PA). PLGA Resomer® RG503H 5050 (RG503H, inherent viscosity 0.32–0.44 dl/g) was a gift from Boehringer-Ingelheim. PLGA 9010 DLG7E (DLG7E, inherent viscosity 0.6-0.8 dL/g) was purchased from Lakeshore Biomaterials (Birmingham, AL). RG503H has carboxylic acid end groups and DLG7E is end-capped with a lauryl ester group. Methylene chloride (DCM), acetonitrile (ACN, HPLC grade), and tetrahydrofuran (THF, HPLC grade) were purchased from Fisher Scientific (Pittsburgh, PA). NanopureTM quality water (Barnstead, Dubuque, IA) was used for all studies.

4.2.2. Methods

4.2.2.1. Preparation and optimization of PLGA microspheres

Dexamethasone loaded microsphere formulations were prepared using an oil-in-water (o/w) emulsion solvent extraction/evaporation technique as previously reported. Briefly, both PLGA polymers at the specific ratio under investigation was dissolved in 2 ml of methylene chloride and dexamethasone was dispersed into this solution. Following a 20-minute sonication period in a bath sonicator, the dispersion was further mixed using a T 25 digital ULTRA-TURRAX homogenizer (IKA Works, Inc., Wilmington, NC) at 10,000 rpm for 1 min. In order to form an emulsion, the organic phase was homogenized into a 10-ml PVA solution (1% (w/v), average

Mw 30–70 KDa) at 10,000 rpm for 2.5 min. The emulsion was then transferred into a 125 ml aqueous PVA solution (0.1% (w/v), Mw 30-70 KDa) and stirred at 600 rpm under vacuum. After 2.5 hours, hardened microspheres were transferred to 50 mL centrifuge tubes and collected *via* centrifugation at 1500 rpm for 2 minutes. The microspheres were then washed three times with deionized water (10 mL each time), recollected using the same centrifugation procedure and dried *via* freeze drying. The prepared microspheres were stored at 4°C until further use. Blank microspheres were prepared following the same procedure except that no dexamethasone was added. In order to achieve a formulation with long-term drug release of 6 months, microsphere composition optimization was conducted using a central composite design (3 factors and 5 levels) composed of 20 formulations as previously reported [22].

4.2.2.2. Characterization of optimized PLGA microspheres

4.2.2.2.1. Particle size and morphology

The particle size and size distribution was determined using (Nicomp, Santa Barbara, CA). Approximately 5 mg of microspheres were dispersed in 1 ml of 0.1% (w/v) PVA solution (30-70 KDa) and 100 µl of the dispersion were injected into the system for particle size analysis. The morphology of the microspheres were determined using a scanning electron microscopy (a FEI Nova NanoSEM 450 unit). Samples were mounted on carbon taped aluminum stubs and sputter coated with gold for 1.5 min at 6 mA before imaging.

4.2.2.2.2. Thermal analysis

A TA Q1000 differential scanning calorimeter (DSC) (TA, New Castle, DE) was used to determine the glass transition temperature (Tg) of the prepared microspheres. Modulated DSC was performed with the cycle below: the samples were heated at a rate of 2 °C/min from 4°C to 80 °C at a modulating oscillatory frequency of 1°C per 50 seconds. The thermograms were

analyzed using Universal Analysis software (TA Instruments) to determine the glass transition temperature.

4.2.2.2.3. Powder X-ray diffraction (PXRD)

The crystallinity of the optimized microsphere formulation was determined using PXRD. X-ray diffraction patterns were obtained using an X-ray diffractometer (Model D5005, Bruker AXS Inc., Madison, WI) with Cu-Kα radiation, a voltage of 40 kV, and a current of 40 mA. All the scans were performed with a scanning rate of 2°/min with steps of 0.02° from 5° to 40° at 2θ ranges. The PLGA polymers, a physical mixture of polymer and dexamethasone, and the dexamethasone loaded PLGA microspheres were evaluated using the diffractometer.

4.2.2.3. Preparation of PLGA microsphere/PVA hydrogel composites

Cylindrical implants were prepared using a two-piece grooved mold based method after three freeze-thaw cycles [23]. Three formulations were prepared (Table 4.1) with different microsphere and dexamethasone combinations to achieve various release profiles.

Table 4.**1** Coatings prepared with different microsphere and dexamethasone combinations

	Microsphere 1 *	Microsphere 2 **	Additional Dexamethasone Powder
Formulation-A	150 mg	-	-
Formulation-B	120 mg	30 mg	-
Formulation-C	120 mg	30 mg	3 mg
Formulation-D		BLANK formulation	

* Microsphere 1 was prepared with DLG7E/RG503H/dexamethasone = 21/4/5 (w/w/w)
** Microsphere 2 was prepared with DLG7E/dexamethasone = 20/3 (w/w)

Briefly, the microspheres were suspended using 1 ml of 5% w/w PVA solution (133 KDa) and filled in a 1-mL syringe. The PVA solution was pre-filtrated using 0.22-µm sterile filters to ensure sterility. The suspension was sonicated in a bath sonicator for 10 seconds followed by one freeze–thaw cycle (2 hour at - 20 °C and 1 hour at ambient temperature). The partially thickened suspension was fed into a 2-piece mold with 1.5-mm grooves. The dummy sensors (silicon chips

with dimension of 5 × 0.5 × 0.5 mm) were sandwiched between the two mold pieces and were then subjected to additional two freeze thaw cycles. The coated dummy sensors were air dried and cut into 7-mm length implants. The grooves in the mold are 1.5-mm diameter. To ensure size consistency of each implant, dummy sensors are placed in the center of the groove between the two pieces of the mold. Sterile tools (*e.g.* vials, tubes, twizzles and containers, *etc*.) were used in the coating process. All the procedures were conducted in a laminar flow hood under sterile conditions. Blank formulations were also prepared using blank PLGA microspheres without dexamethasone.

4.2.2.4. Drug loading

Approximately 5 mg of dexamethasone-loaded PLGA microsphere/PVA hydrogel composites were dissolved in 10 ml DMSO to determine the drug loading. These solutions were filtered (Millex® HV, PVDF 0.45 μm syringe filter) and subjected to HPLC analysis with 5 μl of injection volume. A Perkin Elmer series 200 HPLC system (Shelton, CT) was equipped with a UV absorbance detector (240 nm wave length for dexamethasone analysis). Acetonitrile/water/phosphoric acid (35/70/0.5, v/v/v) was used as the mobile phase. A Zobax C18 (4.6 mm × 15 cm, Agilent, Santa Clara, CA) analytical column was used with a flow rate of 1 ml/min. The chromatographs were analyzed using a PeakSimple™ Chromatography System (SRI instruments, Torrance, CA).

Drug loading was determined as: % drug loading = (weight of drug loaded/weight of microspheres) × 100%.

4.2.2.5. *In vitro* drug release

In vitro release testing was performed on the PLGA microsphere/PVA hydrogel (99% hydrolyzed, Mw 133 KD) composites. The composites were prepared using a freeze-thaw

method described above except that no dummy sensors were used. Approximately 5 mg composite samples were immersed in 5 ml of 10 mM PBS (pH 7.4) with 0.1% NaN_3 and incubated at room temperature (37 °C) under constant agitation. At pre-determined time points, all the release media was removed and replenished with the same volume of fresh media. After 5 days, the volume of release media was changed to 1 ml and sink conditions were maintained throughout. The samples were filtrated through 0.45 μm syringe filters and the concentration of dexamethasone in each sample was determined using the HPLC method described above. Cumulative percent release and the normalized daily drug release were calculated during the release period in order to obtain the release profile and a heat map, respectively.

Cumulative percent release = (total amount released at sampling time/total amount loaded) × 100.

Normalized daily drug release = amount of drug released between two time points/(initial sample weight*duration of the two time points).

4.2.2.6. *In vivo* pharmacodynamics study of composite coated dummy sensors

PLGA/PVA composite coated dummy sensors prepared in section 4.2.2.3.1 were implanted into the interscapular subcutaneous tissue of male Sprague-Dawley rats (weighing ~ 200 g, n=3) using 16-gauge thin wall needles. During the implantation procedure, the rats were under anesthesia (2% isoflurane in oxygen). Before implantation, the back of each animal was shaved and wiped with betadine solution. All of the procedures were conducted under aseptic conditions. Four composites were implanted per rat and the implants were kept for 7 days, 14 days and 1, 2, 3, 4, 5, 6 months. At the end of the 6 months, rats were sacrificed. The subcutaneous tissue surrounding the implants were harvested and fixed in 10% buffered formalin solution. Paraffin blocks were prepared for the tissue sections and a blinded histological

evaluation was performed following hematoxylin and eosin (H&E) staining. Tissue samples were observed and digitally stored using an Olympus microscope (model BX51, Olympus America, Melville, NY). All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Connecticut prior to any animal studies.

4.3. Results and discussion

4.3.1. Microsphere optimization

In order to achieve an optimized microsphere composition, 20 formulations were prepared according to a central composite design. The composition of each formulation is listed in Table 4.1. DoE models were previously obtained for the prediction of drug loading and burst release.[24] Figure 4.1 shows the heat map for drug release from the 20 formulations over approximately 7 months. Formulation 18, with a composition of DLG7E/RG503H/dexamethasone of approximately 21/4/5 (w/w/w), resulted in the overall highest drug release on a daily basis compared to the other formulations, making it the optimal formulation to carry out 6-month *in vivo* testing. Formulation 15, with a composition of DLG7E/dexamethasone of approximately 20/3 (w/w), resulted in the longest lag phase of more than 4 months followed by rapid dexamethasone release starting from approximately 4.5 months. By the end of 7 months, the daily drug release from this formulation is still approximately 1 μg per mg of implant. Therefore, it was considered that a combination of Formulation-15 and Formulation-18 in the composite coating may lead to a formulation with a continuous drug release period longer than 7 months. All other formulations presented a very low daily dose (< 0.05 μg per mg of implant) of dexamethasone starting from approximately 41 days. Several

formulations (*e.g.* formulations-2, 5, 10, 13, and 14) presented a very low daily dexamethasone dose after approximately 7 days.

4.3.2. Particle size and morphology

The composition of formulation-18 was determined to be suitable to achieve 6-months continuous drug release. Accordingly, the morphology and particle size distribution of this formulation was obtained (Figure 4.2). As shown from Figure 4.2-A and B, the microspheres are spherical with smooth surfaces. Their smooth surfaces indicate that no or little dexamethasone is present on the surface of these microspheres and this contributes to the low burst release observed for these formulations. The volume based mean particle size of this formulation is 34.26 ± 13.44 μm with a range of 2 to 75 μm as shown in Figure 2-C. The polydispersity of this formulation is typical of microspheres prepared using the emulsion-solvent evaporation method [13].

4.3.3. Solid state characterization of microspheres

The optimized microspheres were characterized for their thermal properties using TGA (Figure 4.3-A), and mDSC (Figure 4.3-B, C). From the TGA thermogram, both the DLG7E and RG503H polymers started to lose weight at approximately 270 ˚C and the weight loss continued until less than 5% of the weight was left at approximately 350 and 360 ˚C, respectively. Two phases were observed for dexamethasone weight loss with a first phase from approximately 270 ˚C to 340 ˚C where approximately 70% of the weight was lost and a second phase from approximately 340 ˚C to 450 ˚C where the remaining 25% of the weight was lost. The optimized microsphere formulation and the physical mixture presented similar 2-phase patterns except that approximately 90% of the weight was lost during the first phase. This pattern is between that of the pure dexamethasone and the PLGA polymer. In addition, no significant weight loss was

observed for this microsphere formulation within 100 ˚C, indicating that there was no or an insignificant amount of residual solvent in the formulation and the microspheres were completely dried during the freeze drying process.

The total heat flow from the DSC thermogram is shown in Figure 4.3-B. Crystalline dexamethasone has a melting temperature (T_m) of approximately 262 ˚C which is very difficult to observe *via* DSC as this temperature is very close to its decomposition temperature. Therefore mDSC was performed in the temperature range (4-80 ˚C) to detect the thermal transition of PLGA. Endothermal peaks at approximately 50 ˚C were observed for the polymers, the physical mixture and the microspheres. The endothermic event observed is a result of both the PLGA polymer glass transition and the enthalpy of relaxation. Both the physical mixture and the microsphere formulation presented broader endothermic peaks (with non-smooth transitions circled in Figure 4.3-B-d2 and Figure 4.3-B-e2) compared to individual polymers. This is probably due to the presence of two polymer phases.

The reverse heat flow was analyzed to determine the glass transition temperatures (Tg) of the polymers, the physical mixture and the microspheres (Figure 4.3-C). The RG503H polymer presented a Tg of approximately 49.2 ˚C and DLG7E had a slightly higher Tg (approximately 53.1 ˚C) due to its higher molecular weight and hydrophobicity. In spite of the non-smooth transition observed for the polymer physical mixture, only one glass transition was observed from the reversible heat flow (Figure 4.3-C-d3). In contrast, a non-smooth transition was observed during the glass transition region of the microsphere formulation (Figure 4.3-C-e3). From the non-smooth transition observed in the reversible heat flow, two Tgs were determined at approximately 48.3 ˚C and 52.3 ˚C, indicating that two polymer phases exist in the formulation. It is interesting that two Tgs were observed for the microsphere formulation but not in the

polymer physical mixture. This is possibly due to the low concentration of one polymer (RG503H) and inefficient heat transfer between the two polymer phases in the physical mixture which can reduce modulated heat flow signals. Good heat transfer between the different phases in the microsphere formulation is expected. Although the Fox equation can be used to determine the composition of each polymer phase in the microspheres according to the Tg values [25], it is difficult to perform such estimation here as the glass transition temperatures of these two polymers are very close. The overall Tg of the microsphere formulation is relatively high when compared to the body temperature (37˚C), and this is beneficial for the microspheres to maintain stability during the drug release phase *in vivo*.

Dexamethasone encapsulated in PLGA microspheres prepared using the solvent evaporation method have been shown to be crystalline using polarized light microscopy [26]. However, the PXRD pattern has never been obtained for such microspheres. PXRD diffraction profiles of the PLGA polymers, dexamethasone, the physical mixture and the microspheres are shown in Figure 4.4. Both the DLG7E and RG503H polymers showed broad bumps without diffraction peaks indicating their amorphous nature. Typical dexamethasone diffraction peaks were observed for both the physical mixture (Figure 4.4-D) and the microsphere formulation (Figure 4.4-E) indicating that dexamethasone is crystalline in the microspheres. The dexamethasone peak observed from the physical mixture is not as sharp as from the microspheres. This is probably because the DLG7E polymer is in the form of large particles (1-3 mm) rather than fine powder which affects its packing in the sample holder. The microspheres can pack nicely in the holder and a sharper diffraction pattern was therefore observed. Considering that PXRD has a fairly high (2-3%) detection limit for mixed materials, the clear dexamethasone diffraction peaks observed from the microspheres can also be attributed to their high dexamethasone loading

(approximately 15%, w/w). The microsphere preparation process preserved the crystalline nature of dexamethasone, which is beneficial to achieve long-term stability during drug release both *in vitro* and *in vivo*.

4.3.4. *In vitro* release

Three composite formulations were prepared using different combination of microsphere and dexamethasone to achieve various release profiles. The drug loadings in the composite coatings for formulation A, B and C are 6.91%, 6.6% and 9.7%, respectively. The cumulative % release of dexamethasone from the three formulations is shown in Figure 4.5-A. All the formulations presented very similar release patterns with an initial burst release phase, an approximate10-day low dose release phase, and multiple distinct linear drug release phases. Within 24 hours, both formulations A and B had approximately 13% burst release while formulation C had a burst release of approximately 30% due to free dexamethasone loaded in the composites. The 1st linear release phase was observed starting from approximately day 15 and lasted for approximately 1.5 months. This phase is probably due to the initiation of RG503H polymer degradation. Starting from approximately 2 months, the drug release was reduced and a 2nd linear release phase was observed lasting for approximately 2 months. After approximately 4.5 months, drug release started to increase and a 3rd linear drug release phase was observed from 4.5-months to approximately 5.5-months followed by a 4th drug release phase when drug release gradually reduced and complete release was reached. By end of the 8-month testing period, all formulations reached approximately 85% cumulative release.

Although the release profiles gave a general idea of drug release patterns from the composites, it is difficult to obtain the actual daily dose of dexamethasone released from each formulation and therefore distinguish between the different formulations. This information is critical to evaluate

the efficacy of the coatings to counter chronic inflammation when implanted. Therefore, a heat map was generated to depict the dose of drug released per day for these formulations (Figure 4.5-B). Dexamethasone was continuously released during the entire incubation period for all formulations and seven drug release phases were observed for each formulation. Burst release was observed during the first day with approximately 10 μg dexamethasone released per mg of implant for formulations A and B. For formulation C, the dose for day 1 release was approximately 30 μg/mg (red region of the heat map) due to additional free dexamethasone added into the implant. Following the burst release, dexamethasone daily release was maintained at approximately 0.4 μg per mg of implant (yellow regions in the heat map) except for two periods when the daily dose was approximately 0.1 μg per mg of implant (green regions in the heat map). Dexamethasone release duration for formulations B and C are approximately 1 month longer than formulation A due to the addition of Microsphere 2, which was prepared using only DLG7E polymer. Formulations prepared using only DLG7E polymer had been previously reported to present a very long lag phase of approximately 4.5 months, making it unsuitable to achieve continuous dexamethasone release [13]. However, by mixing the two microsphere formulations discussed in this study a coating was achieved with the longest reported dexamethasone release (more than 7 months), which successfully eliminated the long lag phase associated with the DLG7E based microspheres.

4.3.5. Stability of dexamethasone

In order to achieve optimal conditions for *in vitro* release testing, dexamethasone stability in different phosphate buffers (10 and 100 mM) at pH 7.4 was investigated at 37˚C. According to the HPLC method developed, dexamethasone is eluted with a retention time of approximately 5.2 min. The buffer concentration had a significant effect on dexamethasone degradation (Figure

4.7). Approximately 98% of dexamethasone remain stable after 3-days incubation at 37˚C in 10 mM phosphate buffer and no degradation peaks were observed. In contrast, in 100 mM phosphate buffer, approximately 20% of dexamethasone degraded within 3 days and two major degradation peaks were observed following the dexamethasone peak (data not shown). Therefore, release testing was conducted using 10 mM phosphate buffer and the media was replaced twice per week to ensure dexamethasone stability for accurate quantification.

The HPLC diagrams of dexamethasone released at various time points (up to 7 months) are shown in Figure 4.6 No major degradation peaks were observed at any of the time points, indicating that dexamethasone is stable inside the microspheres over the 7-months period. It is interesting that dexamethasone remains stable when loaded in the microspheres under incubation, considering that the microspheres are fully hydrated internally. It is possible that dexamethasone presents as both crystalline and solution state inside the microspheres. It is likely that the solution state exists within the microspheres only transiently, and therefore the dissolved dexamethasone is released from the microspheres before degradation may occur.

4.3.6. *In vivo* pharmacodynamics

The coated dummy sensors were implanted into the subcutaneous tissue of normal rats to determine the *in vivo* efficacy of the coatings (Figure 4.8 and 4.9). Development of acute inflammation (day 7), chronic inflammation (day 14), formation and maintenance of a fibrous capsule (from 1 to 7 month) was observed for Formulation-D, which had no dexamethasone loaded. All three formulations loaded with dexamethasone were able to successfully inhibit the foreign body reaction for 6 months. During the burst phase, no inflammatory cell infiltration was observed within 7 days for all three formulations (Formulation-A, B, and C). The lowest 24-h burst dose was observed for Formulation-B (no additional free dexamethasone loaded) at

approximately 8.3 μg per mg of implant. This dose was sufficient to inhibit acute inflammation. Following burst phase, no inflammatory cell infiltration or fibrous encapsulation was observed at all time points, with the exception of Formulation-C at the 3 month time point where a mild inflammatory cell infiltration was observed (neutrophils, macrophages, and lymphocytes). The inflammatory cell infiltration at this time point is possibly due to the low dose of dexamethasone release from this formulation during the 2-3 month period (less than 0.1 μg per mg of implant per day). However, with the continuous dexamethasone release from the implant, the mild inflammatory cell infiltration was not able to develop to the formation of foreign body capsule. In contrast, the daily dose was above 0.1 μg/mg for the other two formulations (Formulation-A and B).

Dexamethasone has long been used to counter foreign body reactions to implanted devices [27-29]. Due to the high potency of dexamethasone, very low doses are needed to exert a local effect. The US FDA approved a 0.7 mg dexamethasone implant (Ozurdex®) in PLGA for the treatment of macular edema following retinal vein occlusion in 2009 and for the treatment of noninfectious intermediate and posterior uveitis in 2010 after a series of successful clinical trials [30-33]. The weight of each piece of Ozurdex® is approximately 1 mg and the efficacy of Ozurdex® can last for 3-6 months with approximately 90% of dexamethasone released within 1 month [34]. Therefore, the average daily maintaining dose for the remaining 5 months is approximately 0.47μg per mg of implant, which is higher than the daily dose (0.1-0.4 μg) from the implants investigated in this study, indicating the potential safety of these formulations.

4.4. Conclusions

The current study established a 6-month dexamethasone releasing coating to counter FBR to implantable sensors. This formulation has the longest duration of action among any reported

dexamethasone implant thus far. Long-term continuous drug release was achieved from PLGA microspheres prepared with polymer blends, indicating that blending different MW PLGAs is a promising strategy to adjust drug release profiles. In order to obtain detailed information regarding the entire drug release phase, a heat map was used and shown to be advantageous over traditional release profiles to distinguish formulations with optimal daily dose and release duration. The heat map was helpful to understand dose-response correlations during each release phase for long-term drug release formulations. Importantly, the minimum effective dexamethasone daily dose during the acute and chronic inflammatory phases will provide guidance for future coating design for long-term biosensors. The successful development of a long-term effective composite coating paves the way towards the realization of long-term, totally implantable continuous glucose monitoring systems.

4.5. Tables and Figures

Table 4.1 Coatings prepared with different microsphere and dexamethasone combinations

	Microsphere 1 *	Microsphere 2 **	Additional Dexamethasone Powder
Formulation-A	150 mg	-	-
Formulation-B	120 mg	30 mg	-
Formulation-C	120 mg	30 mg	3 mg
Formulation-D		BLANK formulation	

* Microsphere 1 was prepared with DLG7E/RG503H/dexamethasone = 21/4/5 (w/w/w)
** Microsphere 2 was prepared with DLG7E/dexamethasone = 20/3 (w/w)

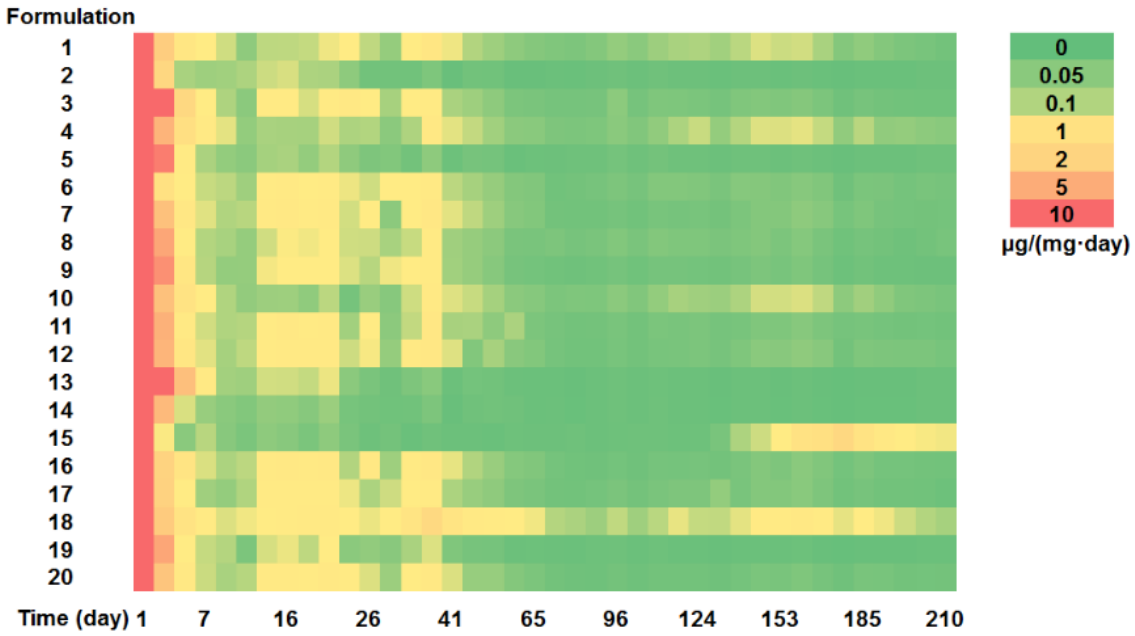

Figure 4.1. Heat map illustrating dexamethasone release from composites prepared with microspheres according to the central composite design. (n=2 for each time point)

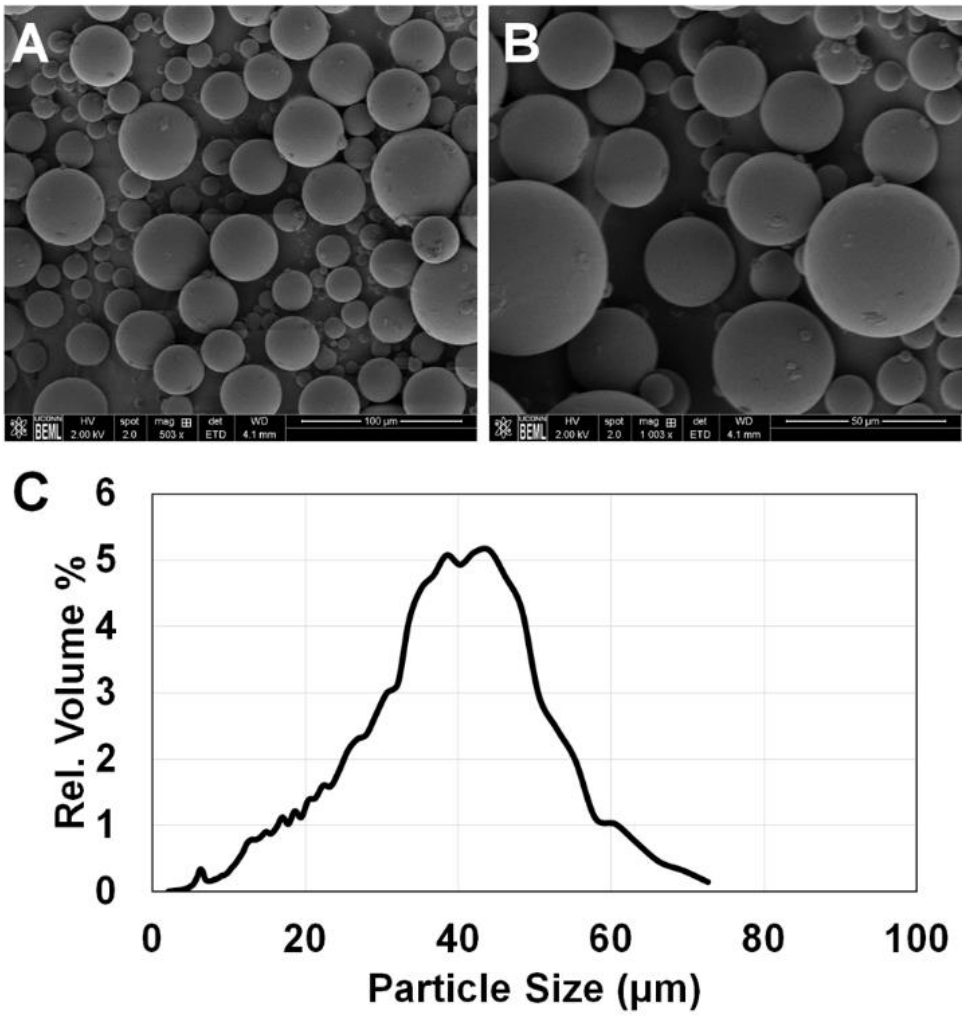

Figure 4.2. SEM micrographs of the optimized PLGA microspheres (prepared using a composition of DLG7E/RG503H/dexamethasone = 21/4/5, w/w/w) at 500X (A) and 1000X (B) magnification. Volume based particle size distribution of the microspheres (C) obtained using an AccuSizer 780A autodiluter particle sizing system. Approximately 140,000 particles were analyzed using the particle sizer.

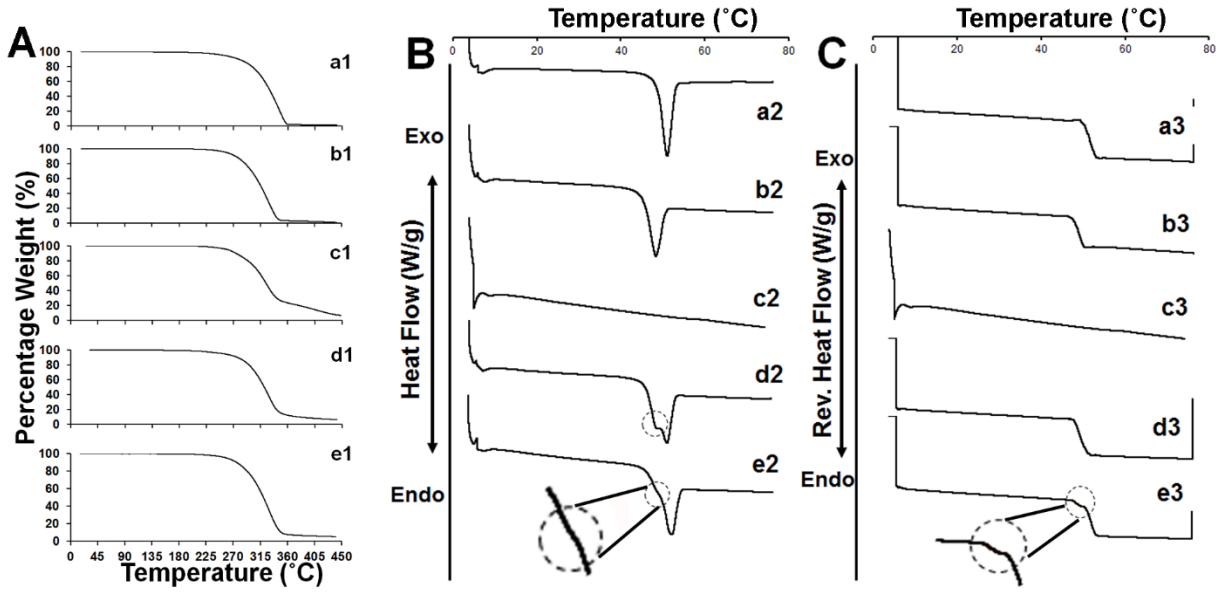

Figure 4.3. TGA thermograms of: RG503H polymer (a1); DLG7E polymer (b1); dexamethasone (c1); physical mixture of RG503H:DLG7E:dexamethasone = 4:21:5 (w/w/w) (d1); and microsphere 1 (e1). DSC thermogram of: RG503H polymer (a2); DLG7E polymer (b2) dexamethasone (c2); physical mixture of RG503H:DLG7E:dexamethasone = 4:21:5 (w/w/w) (d2); and microsphere 1 (e2). Reverse heat flow thermograms of: RG503H polymer (a3); DLG7E polymer (b3); dexamethasone (c3); physical mixture of RG503H:DLG7E:dexamethasone = 4:21:5 (w/w/w) (d3); and microsphere 1 (e3). The nonsmooth transitions of heat flow were circled in B and C.

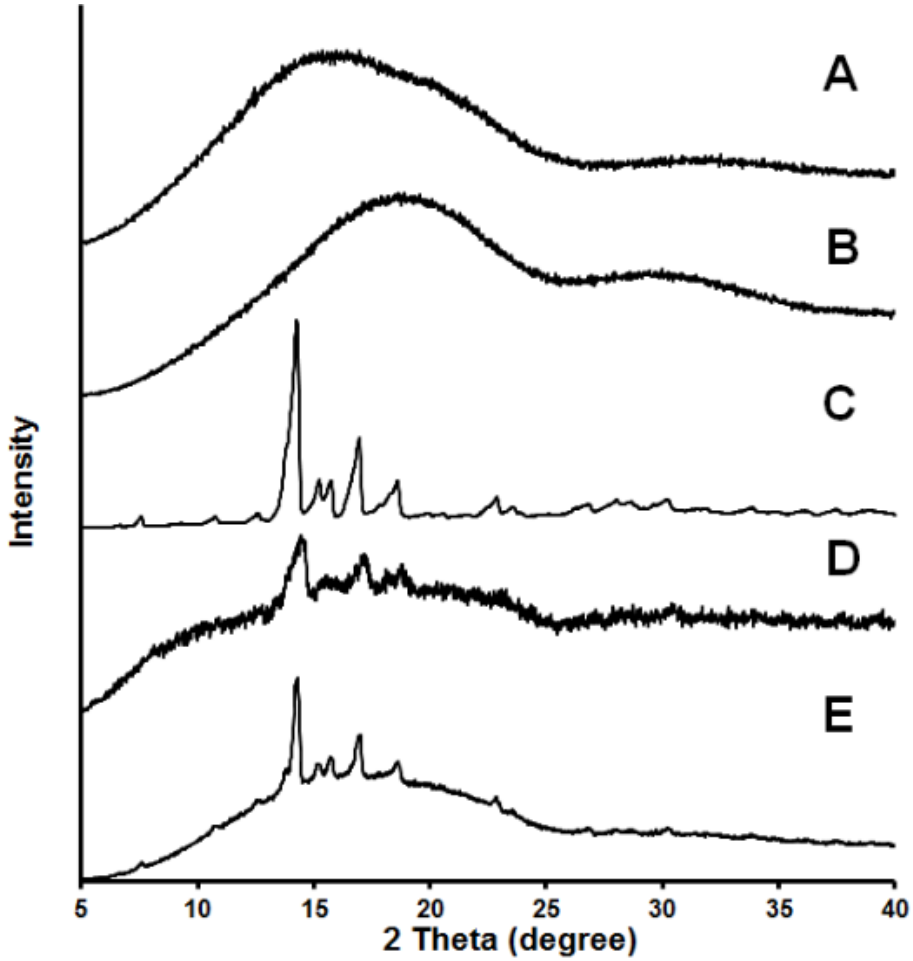

Figure 4.4. PXRD diffraction profiles of: RG503H polymer (A); DLG7E polymer (B); dexamethasone (C); physical mixture of DLG7E:RG503H:dexamethasone = 21:4:5 (w/w/w) (D); and microsphere 1 (E)

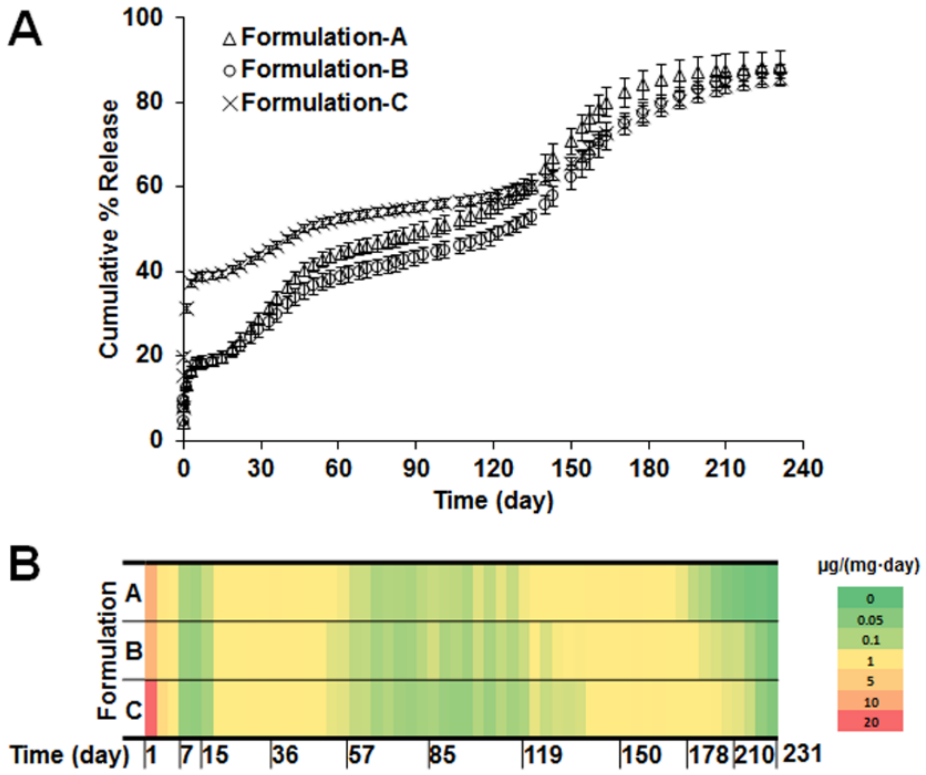

Figure 4.5. *In vitro* dexamethasone release profiles (A) and heat map (B) of composite coatings prepared using different combinations of microspheres. (n=3 for each time point)

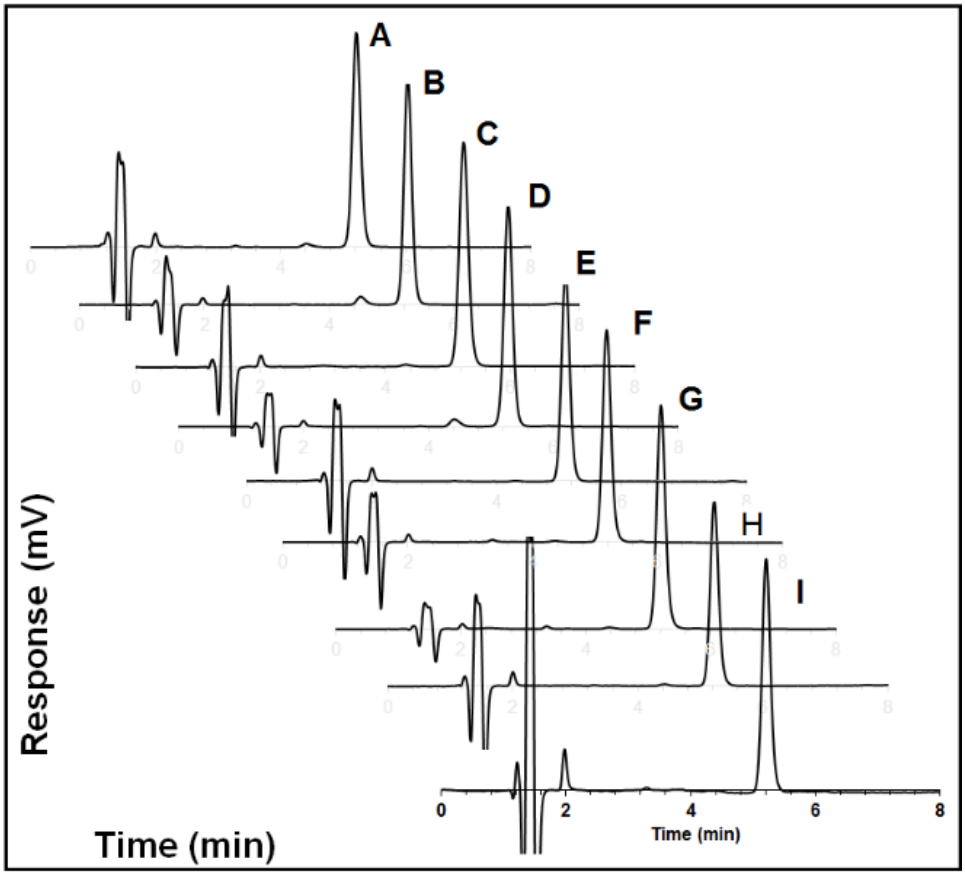

Figure 4.6. HPLC diagram of: dexamethasone standard (A); and dexamethasone released from composite coatings following incubation for 7-days (B); 1-months (C); 2-months (D); 3-months (E); 4-months (F); 5-months (G); 6-months (H); and 7-months (I).

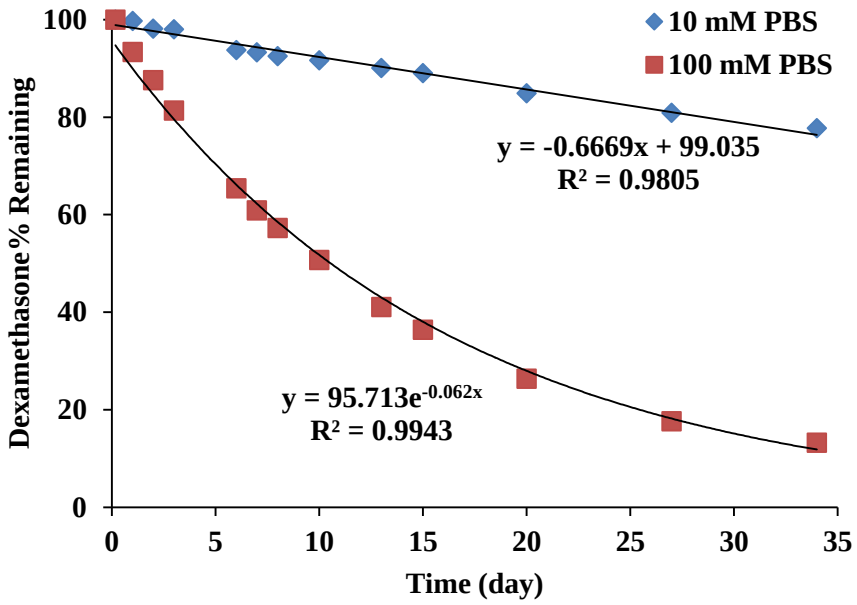

Figure 4.7. Dexamethasone degradation kinetics in 10 mM and 100 mM phosphate buffer. Approximately 20 mL dexamethasone solution (25 μg/mL) was incubated at 37˚C and percentage remaining dexamethasone was quantified using HPLC.

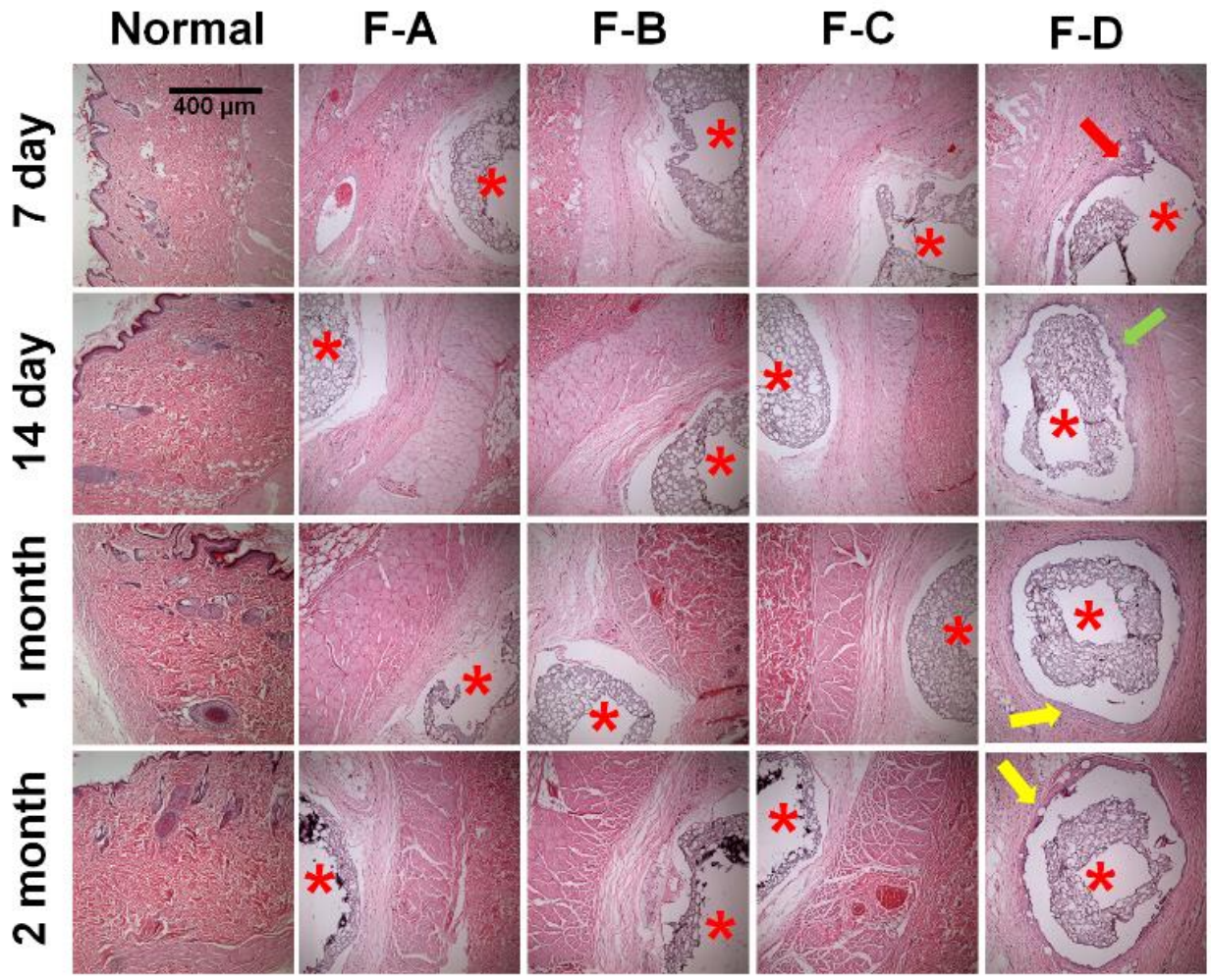

Figure 4.8. *In vivo* pharmacodynamics of implanted composite coated dummy sensors in rats following 7-day, 14-day, 1-month and 2-month implantation (top to bottom). From left column to right column are normal tissue, formulation-A, B, C and D. Stars indicate where the implants were located, the red arrows indicate infiltrated inflammatory cells, the green arrow indicates activated fibroblasts present during the transitional phase from acute to chronic inflammation, and the yellow arrows indicate a fibrous capsule formed around the implants.

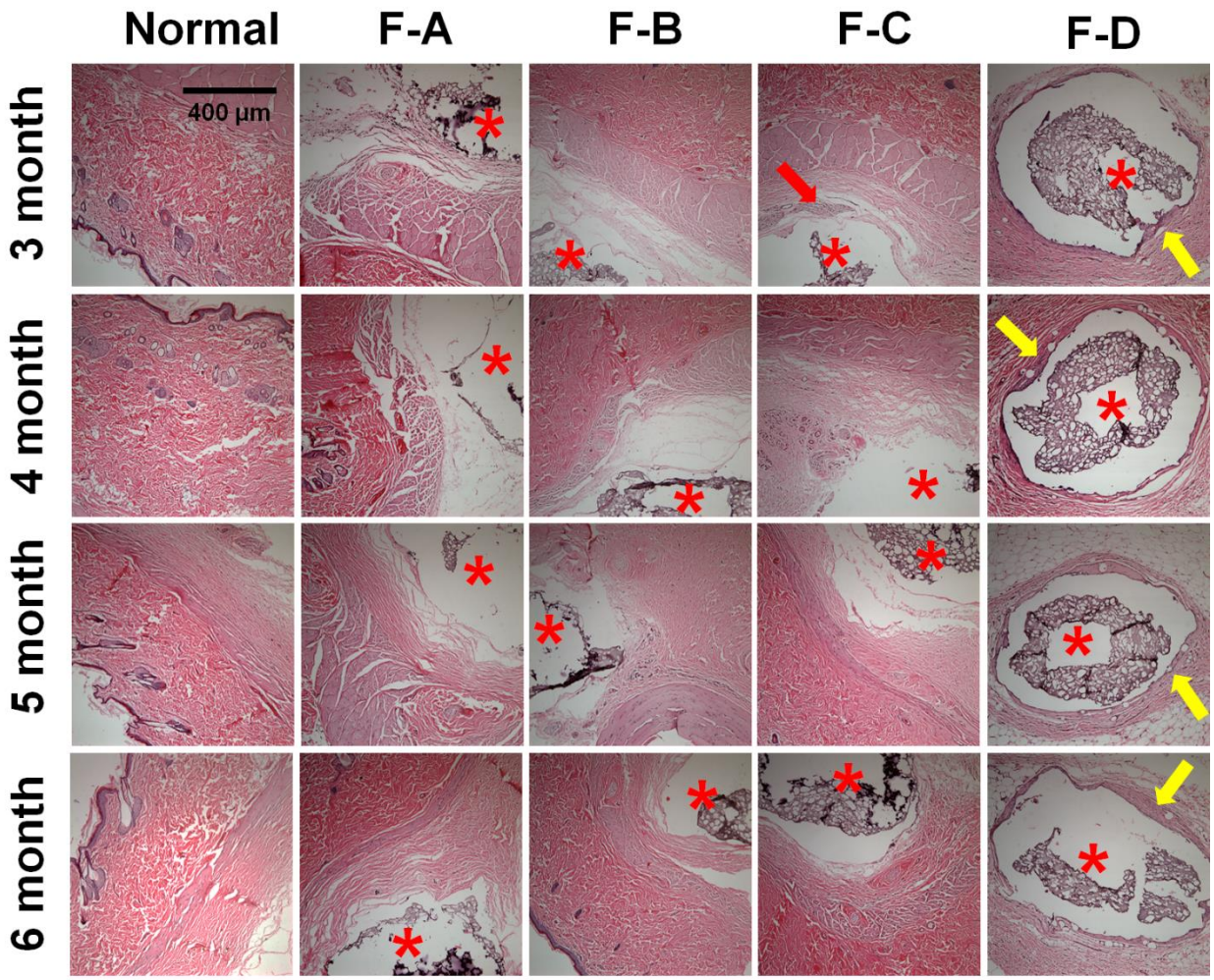

Figure 4.9. *In vivo* pharmacodynamics of implanted composite coated dummy sensors in rats following 3-, 4-, 5-, and 6-month implantation (top to bottom). From left column to right column are normal tissue, formulation-A, B, C and D. Stars indicate where the implants were located, the red arrows indicate infiltrated inflammatory cells and the yellow arrows indicate a fibrous capsule formed around the implants.

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Chapter 5

Effect of PLGA microsphere degradation on glucose transport through composite coatings

Abstract

The aim of this study was to understand the polymer degradation and drug release mechanism from PLGA microspheres embedded in a PVA hydrogel. Two types of microspheres were prepared with different molecular weight PLGA polymers (approximately 25 and 7 kDa) to achieve different drug release profiles, with a 9-day lag phase and without a lag phase, respectively. The kinetics of water uptake into the microspheres coincided with the drug release profiles for both formulations. For the 25 kDa microspheres, minimal water uptake was observed in the early part of the lag phase followed by substantial water uptake at the later stages and in the drug release phase. For the 7 kDa microspheres, water uptake occurred simultaneously with drug release. Water uptake was approximately 2-3 times that of the initial microsphere weight for both formulations. The internal structure of the PLGA microspheres was evaluated using low temperature scanning electron microscopy (cryo-SEM). Burst drug release occurred followed by pore forming from the exterior to the core of both microspheres. A well-defined hydrogel/microsphere interface was observed. For the 25 kDa microspheres, internal pore formation and swelling occurred before the second drug release phase. The surface layer of the microspheres remained intact whereas swelling, and degradation of the core continued throughout the drug release period. In addition, microsphere swelling reduced glucose transport through the coatings in PBS media and this was considered to be a as a consequence of the increased thickness of the coatings. The combination of the swelling and microdialysis results provides a fresh understanding on the competing processes affecting molecular transport of bioanalytes (*i.e.* glucose) through these composite coatings during prolonged exposure in PBS.

5.1 Introduction

Poly (lactic-co-glycolic acid) (PLGA) based parenteral formulations are widely used for sustained delivery of various therapeutic entities such as small molecules, peptides as well as proteins [1-3]. There are currently twelve PLGA/PLA based microsphere products available on the market, including Risperdal® Consta®, Sandostatin® LAR, Zoladex®, and Lupron Depot®. This type of formulation is suitable for achieving long-term efficacy with reduced dosing frequency and is typically administered *via* the intramascular (I.M.) and subcutaneous (S.C.) routes. It is critical to understand the drug release mechanism from microspheres in order to design and develop formulations with controlled release kinetics. Diffusion and degradation/erosion are two main release mechanisms associated with this type of formulation. The initial phase of drug release is usually considered to be controlled by drug diffusion from the surface and the later stage of drug release is associated with degradation and erosion[4]. Hydration takes place with great speed relative to erosion when the microspheres are immersed in an aqueous buffer. Microsphere hydration is followed by polymer chain degradation which occurs throughout the polymer matrix in the form of random hydrolysis and leads to the formation of internal pores [5].

However, the detailed underlying mechanisms/processes of drug release from PLGA microspheres are not yet fully understood. Complex mechanisms/processes have been proposed by researchers regarding degradation and drug release from various types of PLGA microspheres [5, 6]. Heterogeneous bulk degradation was proposed as two distinctive glass transition temperatures were observed in different PLGA microspheres with varied copolymer compositions [7]. Such heterogeneity can be partly attributed to a pH gradient from the interior (low pH) to the surface (high pH) of the microspheres, due to accumulation of hydrolyzed

lactic/glycolic acid monomers and oligomers within the microspheres [8, 9]. This occurs as a result of the slower diffusion of lactic/glycolic acid monomers and oligomers compared to buffer components [8]. An often neglected fact is that water diffusion from the outside to the inside (swelling) due to the increased osmotic pressure can lead to a higher degree of dilution of the acidic components close to the surface. Swelling is an important process during PLGA microsphere degradation. Initial microsphere swelling has been reported to form a skin layer on the surface as a result of pore-closing [10] and therefore delay the initial drug release which may cause an apparent lag phase. Initial microsphere swelling has also been reported to cause burst release [11]. Swelling and water uptake in clonidine-loaded PLGA microspheres during the second drug release phase was reported by Gaignaux *et al*[12]. However, the method used by these researchers which involved measuring filtered wet microspheres may have resulted in significant error due to adsorbed water on the microsphere surfaces. More recent findings have indicated that microsphere swelling with significant volume increase coincides with the onset of the second drug burst release phase [13, 14]. Microsphere swelling is possibly a result of polymer chain relaxation due to elevated temperature and increased osmotic pressure resulting from accumulation of dissolved drug and degradation species [15]. Unfortunately, the water uptake into these microspheres was not reported in these studies.

The microsphere internal structure may significantly affect drug release. In protein loaded microspheres prepared using the double emulsion-solvent evaporation method, pore closing and opening events were observed to affect drug release [10, 16]. In a multi-layer reservoir type microsphere formulation, rupture of the outer layer caused by inner layer swelling was observed and shown to govern drug release [17]. Microsphere structure collapse and particle agglomeration have been reported during the later stages of drug release [18, 19]. While pore

formation is generally believed to happen internally, detailed pore morphology has yet to be revealed. Microsphere morphology change during drug release is usually determined *via* scanning electron microscopy (SEM). To evaluate the internal structure of microspheres, samples are usually incubated for a period of time, then collected, dried and cut/crushed/fractured before SEM imaging [10, 16, 19, 20]. This sample preparation process may create defects which may alter the internal structure of the particles. Accordingly, low temperature scanning electron microscopy (cryo-SEM) may be a better technique to investigate microsphere internal structure since the samples are flash-frozen in liquid nitrogen and then freeze-fractured to maintain the structural characteristics for imaging. This technique has been successfully used for biological samples [21]. To the best of our knowledge, cryo-SEM has never been used to investigate PLGA microsphere internal structure changes.

Dexamethasone containing PLGA microspheres embedded in poly vinyl alcohol (PVA) hydrogels have been developed as composite coatings for subcutaneous implants to inhibit the foreign body reaction [22-27]. The efficacy of these coatings was shown to be dependent upon the drug release profile from the PLGA microspheres [26, 28]. Dexamethasone release from PLGA microspheres can typically be divided into three phases, a burst release phase, a lag phase and a second drug release phase following a bulk degradation mechanism. When embedded in PVA hydrogels, the burst release phase has been shown to be slightly extended due to the diffusional resistance caused by the hydrogel [29]. The lag phase and second drug release phase are mainly controlled by drug release from the microspheres [30]. The PVA hydrogels maintain a neutral pH and are permeable to water and other small molecules. The PVA hydrogel also provides a protective layer to maintain microsphere structure during drug release [20]. Therefore,

investigating the composite coating will provide valuable information regarding drug release from PLGA microspheres.

In this study, two different PLGA microsphere formulations with different drug release profiles were prepared. PLGA microsphere/PVA hydrogel composite coatings were evaluated from three aspects: *1)* the swelling properties (water uptake) determined as the swelling ratio, *2)* internal structure change evaluated using cryo-SEM, and *3)* glucose diffusion through the coating investigated using microdialysis. The swelling and internal structure change of the coatings may facilitate understanding of the physicochemical properties of the composites for glucose sensors coating design.

5.2 Material and Methods

5.2.1 Materials

Dexamethasone was purchased from Cayman Chemical (Ann Arbor, MI), poly (vinyl alcohol) (PVA, Mw 30–70 KD), sodium chloride (NaCl, ACS grade), sodium azide (NaN_3), sodium phosphate dibasic dihydrate ($Na_2HPO_4 \cdot 2H_2O$), sodium phosphate monobasic (NaH_2PO_4) and dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich (St. Louis, MO). PVA (99% hydrolyzed, Mw 133 KD) was purchased from Polysciences, Inc. (Warrington, PA). PLGA Resomer® RG503H 5050 (RG503H, with molecular weight approximately 25 kDa) was a gift from Boehringer-Ingelheim. PLGA 5050 DLG1A (DLG1A, with molecular weight approximately 7 kDa) was purchased from Lakeshore Biomaterials (Birmingham, AL). Methylene chloride (DCM), acetonitrile (ACN, HPLC grade), and tetrahydrofuran (THF, HPLC grade) were purchased from Fisher Scientific (Pittsburgh, PA). NanopureTM quality water (Barnstead, Dubuque, IA) was used for all studies.

5.2.2 Methods

5.2.2.1 Preparation of PLGA microspheres

Dexamethasone loaded microspheres were prepared using an oil-in-water (o/w) emulsion solvent extraction/evaporation technique. Two microsphere formulations were prepared using either RG503H or DLG1A PLGA. 500 mg of PLGA were dissolved in 2 ml of methylene chloride and 50 mg of dexamethasone were dispersed in this solution and these dispersions were sonicated using a bath sonicator for 20 minutes. The dispersions were then further mixed using a bench top homogenizer (T25, IKA Works, Inc., Wilmington, NC) at 10,000 rpm for 1 min. The organic phase was then slowly added to 10 ml of PVA solution (1% (w/v), average Mw 30–70 KDa) and homogenized at 10,000 rpm for 2.5 min. The emulsions were then transferred to 125 ml of an aqueous PVA solution (0.1% (w/v), Mw 30-70 KDa) and stirred at 600 rpm. A vacuum was applied to the aqueous phase for 2.5 hours to evaporate the methylene chloride and harden the microspheres. The hardened microspheres were transferred to 50 mL centrifuge tubes and collected *via* centrifugation at 1500 rpm for 2 minutes. The microspheres were then washed thrice with deionized water (10 mL each time), collected using the same centrifugation procedure as before and dried using a freeze dryer. The prepared microspheres were stored at 4°C until further use.

5.2.2.2 Characterization of PLGA microspheres

The microspheres were characterized for drug loading, glass transition temperatures and morphology. The drug loading was evaluated by dissolving approximately 5 mg of microspheres in 10 mL THF and analyzed using HPLC. Briefly, the solution was filtered (Millex® HV, PTFE 0.45 μm syringe filter) and 5 μl samples were injected into the HPLC column. A Perkin Elmer series 200 HPLC system (Shelton, CT) equipped with a UV absorbance detector (240 nm wave length for dexamethasone analysis) was used. Acetonitrile/water/phosphoric acid (35/70/0.5,

v/v/v) was used as the mobile phase. A Zobax C18 (4.6 mm × 15 cm, Agilent, Santa Clara, CA) analytical column was used with a flow rate of 1 ml/min. The chromatographs were analyzed by PeakSimple™ Chromatography System (SRI instruments, Torrance, CA). A TA Q1000 differential scanning calorimeter (DSC) (TA, New Castle, DE) was used to determine the glass transition temperature (Tg) of the prepared microspheres. Modulated DSC was performed with the following cycle: the samples were heated at a rate of 3 °C/min from 4°C to 80 °C at a modulating oscillatory frequency of 1°C/min. The thermograms were analyzed using Universal Analysis software (TA Instruments) to determine the glass transition temperature. The morphology of the microspheres was determined using a scanning electron microscopy (a FEI Nova NanoSEM 450 unit). Samples were mounted on carbon taped aluminum stubs and sputter coated with gold for 1.5 min at 6 mA before imaging.

5.2.2.3 PVA hydrogel composite coatings

The PLGA microsphere/PVA hydrogel composites were prepared using a previously developed freeze-thaw method. Different amounts of PLGA microspheres (0, 50, 75, and 100 mg) were dispersed in 1 mL 5% (w/w) PVA solutions (133 KDa) and the dispersions were filled into 1-mL syringes (BD precision glide). Three freeze–thaw cycles (2 hour at - 20 °C and 1 hour at ambient temperature for each cycle) were applied to the suspension to form the crosslinked PVA hydrogels with PLGA microspheres embedded. The crosslinked composites were then removed from the syringes, air dried and stored in 4 ˚C for further use.

5.2.2.4 Coating of microdialysis probes

CMA20 microdialysis probes (Harvard Apparatus, Holliston, MA) with 20,000 molecular weight cutoff, 10 mm polyethersulfone (PES) membranes, were used for microdialysis testing. For coating the probes, the microspheres were dispersed in 5% (w/w) PVA solution (133 KDa) and

one freeze-thaw cycle was applied to the dispersion to thicken the gel before coating the microdialysis probes. Teflon tubing (0.047 inch inner diameter) was used to provide a cylindrical mold for coating. Using a syringe, the thickened gel solution was quickly dispensed into the tubing. The microdialysis probe tips (0.5 mm diameter) were inserted in the tubing and 2 additional freeze-thaw cycles were applied. The tubing was removed from the hydrogel-coated microdialysis probes and the coatings were allowed to be air dried and stored in 4 ˚C for further use. Microdialysis probes coated with PVA hydrogel only were prepared using the same method except that microspheres were not added into the PVA solution.

5.2.2.5 *In vitro* release testing

In vitro release testing was performed for the PLGA microsphere/PVA hydrogel (99% hydrolyzed, Mw 133 KD) composite coatings. Approximately 2 mg of each formulation was immersed in a 2 ml Eppendorf vial containing 1.8 ml of 10 mM PBS (pH 7.4) with 0.1% NaN_3 and incubated at 37 °C under constant agitation. At pre-determined time points, all the release media was removed and replenished with fresh media. Sink conditions were maintained throughout. The samples were filtered through 0.45 μm syringe filters and the concentration of dexamethasone in each sample was determined using the HPLC method as described above.

5.2.2.6 Swelling of composite coatings

The swelling characteristics of the composite coatings were obtained by measuring their initial and swollen weights in phosphate buffered solution (10 mM, pH 7.4, PBS). Approximately 5 mg of completely dried samples were weighed (W_d) and immersed into 1.8 mL PBS solutions incubated at 37 ˚C. At predetermined time points, the samples were then weighed again to obtain the swollen weight (Ws (t)) after being removed from the solutions and carefully dried

using kimwipes to absorb any surface water. The degree of swelling was calculated as the swelling ratio using the equation below.

$$Swelling\ Ratio = \frac{W_s(t) - W_d}{W_d}$$

where W_d is the initial weight of dried coating and $W_s(t)$ is the weight of swollen coating measured at the specified incubation time interval (t).

5.2.2.7 Glucose diffusion through composite coatings

All the microdialysis probes (without coating, with PVA coatings, with PVA/PLGA composite coatings) were incubated in the Franz cell apparatus with 5 mL PBS maintained at 37 °C for 3 hours prior to the experiments to ensure complete hydration of the coatings. The probes were then connected to a syringe pump equipped with a 3-mL syringe filled with PBS. The pumping rate was set at 5 µL/min. After a 30 min equilibration period, 2 mL of 6 mg/mL glucose solution was added into the Franz cells and the perfusion fluid was collected every 6 mins for 30 min. The same test was repeated at pre-determined time points and the microdialysis probes were incubated in PBS maintained at 37 °C between each test. The glucose concentration in the outlet dialysate (C_d) and medium external to the dialysis probe (C_e) were measured using a YSI 2300 Stat Plus Glucose & Lactate analyzer (YSI Inc., Yellow Spring, Ohio). The permeability was determined as relative recovery (RR), which was calculated using the following equation:

$$RR = \frac{C_d(t)}{C_e(t)}$$

where $C_d(t)$ is the glucose concentration in the dialysate and $C_e(t)$ is the glucose concentration in the medium external to the dialysis probe at the specified time points (t).

5.2.2.8 Low temperature scanning electron microscopy (cryo-SEM)

In order to investigate the internal structure change of composite coatings, cryo-SEM was performed to evaluate the samples after incubation in PBS at 37 ˚C after specified time points. Samples of ~ 1 mm^3 size were mounted onto standard specimen carriers (for the EM VCT100 16BU012098-T holder, Leica Microsystems) surrounded by buffer, and plunge-frozen in liquid nitrogen slush. The samples were freeze-fractured at -140 °C, etched for 2 min at -95°C, and sputter coated with 7 nm thickness of platinum in the cryo-SEM sample prep station (EM MED 020, Leica Microsystems). Samples were then transferred under vacuum to the FEG-SEM (Nova NanoSEM 450, FEI) and imaged at -140 °C (EM VCT100 cryo shuttle and cryo stage, Leica Microsystems).

5.3 Results

5.3.1 Characterization of PLGA microspheres

Two types of microspheres were prepared using different polymers as shown in Table 1. DLG1A polymer has lower intrinsic viscosity and is more hydrophilic. The Tg of DLG1A microspheres was approximately 43.3 ˚C and had a molecular weight of approximately 7 kDa. RG503H has higher intrinsic viscosity and is more hydrophobic. The molecular weight of this polymer is approximately 25 kDa which is higher than that of DLG1A, leading to a higher Tg of approximately 48.2 ˚C. The Similar drug loading was obtained for both microsphere formulations, around 7.6% (w/w).

Table 1 Physicochemical properties of the PLGA microsphere formulations

Formulation	Polymer type	Polymer intrinsic viscosity (dl/g) *	Tg (˚C)	Drug loading (w/w)
I	5050DLG1A	~0.08	43.3	7.72 ± 0.33%
II	5050RG503H	~ 0.4	48.2	7.63 ± 0.28 %

* Information provided in Analytical Report from Lakeshore Biomaterials

Microsphere morphology was evaluated using SEM (Figure 5.1). Both formulations presented spherical shaped particles. Some irregular shaped particles were observed on the surface of the DLG1A (low MW) formulations while the surface of microspheres prepared using the RG503H

(high MW) was smooth. These particles are considered to be crystalline dexamethasone, which was not encapsulated inside the microspheres. The crystalline nature of these particles was confirmed using polarized light microscopy (data not shown). Surface dexamethasone may lead to higher drug burst release from this formulation.

5.3.2 *In vitro* drug release from composite coatings

Release profiles were obtained for composite coatings prepared using the two microsphere formulations (Figure 5.2). DLG1A based composite coatings exhibited a burst release of approximately 50% at 3 hours, more than 70% of the dexamethasone was released within 24 hours, and drug release was complete within 10 days. For composite coatings prepared using RG503H based microspheres, three release phases were observed including an initial burst release phase, followed by a lag phase with minimal drug release and a secondary zero-order release phase. Approximately 35% of the drug was released during the burst phase within 24 hours. The lag phase lasted for approximately 9 days and the drug release plateaued at approximately 1 month with more than 90% of the loaded drug released.

5.3.3 Swelling of composite coatings

Swelling properties of the composite coatings prepared with RG503H (high MW) microspheres are shown in Figure 5.3. The weight change of the samples is plotted in Figure 5.3-A. There was significant swelling within first few hours. After the initial swelling on the first day, the coatings without microspheres retained similar weight throughout the 45-day testing period. For the coatings with embedded microspheres, following the initial 1-day swelling period the coating weight was maintained for approximately 6 days following which the weight increased significantly until approximately day 24 and then decreased. From the swelling ratio plot shown in Figure 5.3-B, the composite coatings gained approximately 60% to 100% of their initial weight while the PVA hydrogel alone (no microspheres) gained more than 170% of the initial

weight during the first 24 hours. A negative correlation with the amount of microspheres embedded in the hydrogel was observed during the first 24 hours. While the swelling ratio of the PVA hydrogel alone was maintained at around 170% for 45 days, the swelling ratio of composite coatings increased starting from day 9 and reached a maximum after approximately 24 days. The maximum swelling ratio for the composite coatings ranged from 220% to 280% with a positive correlation with the amount of microspheres embedded.

The swelling properties of the composite coatings prepared with DLG1A microspheres (low MW) are shown in Figure 5.4. The coatings loaded with the DLG1A microspheres continued to gain weight after incubation and reached a maximum at around 13 days. After day 13, the weight started to decrease during the testing period. As was the case for the coatings loaded with RG503H microspheres, these composite coatings gained approximately 60% to 100% of their initial weight within the first 24 hours. The maximum swelling ratio of these formulations occurred at day 13 and ranged from approximately 200% to 250% for the various microsphere concentrations. A positive correlation between the maximum swelling ratio and the concentration of microspheres embedded in the coating was observed.

5.3.4 Glucose diffusion through composite coatings

RR of glucose from the microdialysis probes is an indication of glucose permeability through various coatings (as shown in Figure 5.5). The RR of glucose through uncoated microdialysis probes and PVA hydrogel coated probes were maintained at approximately 37% and 21%, respectively over the testing period. When coatings containing embedded microspheres (prepared with the RG503H polymer) were applied to the probes, the RR decreased with increase in the microsphere concentration. For those coatings loaded with PLGA microspheres, the RR decreased initially following incubation and reached a minimum at approximately 24 days and

then started to increase. The lowest RR (~5%) was observed for the 100 mg MS/ml PVA formulation at day 24.

In order to further investigate the effect of microsphere swelling and degradation on glucose diffusion through the composite coating, glucose RR was also tested for coatings prepared using DLG1A microspheres (MS) following different incubation periods. Similar results to those for the coatings embedded with the RG503H microspheres were obtained, except that the time scale was faster, as shown in Figure 5.6. The RR decreased initially after incubation and reached the lowest point (~5%) at day 16 before it started to increase. When compared to the RR obtained for the composite coatings prepared using the RG503H microspheres, the initial RR decrease for the composites prepared with the DLG1A formulation was more abrupt and the minimum RR was reached earlier (approximately 16 days compared to 24 days).

5.3.5 Internal pore formation

Composite swelling and glucose diffusion characteristic changes are associated with internal structural change in the coatings. Cryo-SEM was performed on DLG1A microsphere based composite coatings after incubation for different time periods. Figure 7 shows the internal structure of the composite coatings following 2-h, 1-day, 3 days and 7 days. Two distinct layers, an external layer with small pores and an internal layer without pores, were observed in the microspheres following 2 hours of incubation (Figure 5.7-A2). From the highest magnification (~26000X) image (Figure 5.7-A3), it was determined qualitatively that the pores formed at 2 hours are similar in size to the pores of the PVA hydrogel matrix. Interestingly, a transitional phase was observed between the microspheres and the PVA hydrogel indicating some possible interaction between the two phases (Figure 5.7-A2). Following 24 hours incubation, pores were observed throughout these microspheres (Figure 5.7-B2) and the pore size increased with time (comparing Figure 5.7-B3 with Figure 5.7-A3). After 3 and 7 days of incubation, increase in particle size was observed in the low magnification images (comparing Figure 5.7-C1, 5.7-D1 to Figure 5.7-A1). It can be observed from the high magnification images that the size of the internal pores continued to increase (Figure 5.7-C3, 5.8-D3). The transitional phase between the

microspheres and the hydrogel disappeared in the later time points (Figure 5.7-C3, 5.8-D3). It is also worth noting that these microspheres rapidly lost their spherical shape even after 2 hours of incubation. During the whole incubation period, no significant changes were observed in the PVA hydrogel structure other than the transitional phase.

Cryo-SEM was also performed on PLGA microsphere/PVA hydrogel composites prepared using the RG503H polymer (Figure 5.8). Pores started to present close to the surface of the microspheres following 5 hours incubation. The pores grew in a pattern from the outside to the inside with small pores located close to the exterior and larger pores in the center of the microspheres. By day 6, the interior of the microspheres was filled with pores. The pore size increased from day 6 to day 9 (from approximately 240±138 nm to approximately 367±197 nm). Minor internal structure deformation was observed at day 9 (Figure 5.8-F). Starting from day 12, internal structure collapse was observed from the interior to the exterior as shown in Figures 5.8-G and 5.8-H. Microsphere particle size was analyzed and the particle size increased significantly from day 6 to day 15 (Figure 5.8-I), which coincides with the swelling ratio change shown in Figure 5.3. The transitional layer between the microspheres and the PVA hydrogel was also observed for this formulation as shown in Figures 5.8-A and 5.8-B. It is worth noting that these microspheres maintained their spherical shape until day 6 following which shape changes were observed.

5.4 Discussion

5.4.1 Amount of microsphere water uptake during swelling

PVA hydrogels have been widely used as biocompatible materials[31]. The short term swelling property of these hydrogels has been thoroughly investigated [32, 33]. They usually reach swelling equilibrium within a few hours which is consistent with the results reported here for the hydrogels alone and those loaded with the RG503H microspheres (Figure 5.3). In the case of the

hydrogels containing the DLG1A microspheres, a fast hydrogel equilibration period was not observed and this is probably due to masking by the rapid swelling of these microspheres.

The long-term swelling properties of the PLGA microsphere/PVA hydrogel composites has not been previously reported. A positively correlated microsphere concentration dependent long-term swelling pattern indicates that the microspheres were absorbing significant amounts of water. The onset of microsphere swelling coincides with the onset of the second drug release phase of RG503H microspheres and persisted during the remainder of the drug release period. Although a significant particle size change has been reported for PLGA microspheres at the onset of the second rapid drug release phase, the water uptake was not quantified in this study [14]. In the current study, we were able to quantify the amount of water uptake by the microspheres as they were embedded in the PVA hydrogels and the hydrogels only absorbed water during the initial fast equilibrium phase. The approximate maximum amount of water taken up by the microspheres was calculated using the equation below:

$$Maximum\ Swelling\ Ratio_{MS} = \frac{W_s(t_{max}) - W_s(3)}{W_d \times (C_{MS} \div (C_{MS} + 50))}$$

where W_d is the initial weight of the dry coating and $W_s(t_{max})$ is the weight of the swollen coating measured at the maximum swelling time (t), $W_s(3)$ is the weight of the swollen coating following 3 hours incubation, C_{MS} is the amount (mg) of microspheres in one mL of 5% (w/w) PVA solution.

For both microspheres, the maximum amount of water uptake ranges from 2 to 3 times their weight (Table 5.2).

Table 2 Calculated maximum swelling ratio of microspheres embedded in the composite coatings according to Figures 3 and 4

Microsphere Concentration	50 mg/mL PVA	75 mg/mL PVA	100 mg/mL PVA
DLG1A MS	2.49 ± 0.03	2.33 ± 0.27	2.62 ± 0.12
RG503H MS	2.15 ± 0.18	2.55 ± 0.11	2.97 ± 0.12

5.4.2 Drug release mechanism from microspheres

Different release profiles were observed for the composites prepared with the two different microsphere formulations (Figure 5.2). The high burst release observed for the DLG1A microsphere/PVA hydrogel composites is consistent with the low PLGA molecular weight, the hydrophilicity of these microspheres and the observation of surface associated dexamethasone (SEM images in Figure 5.1). In contrast, dexamethasone release from the RG503H formulation followed the typical tri-phasic PLGA microsphere release profile. The burst release, duration of the lag phase and the period for complete drug release is very similar to previously reported results obtained for RG503H PLGA microsphere/PVA hydrogel composites [20]. The tri-phasic release profile indicates that the RG503H microspheres follow a bulk erosion mechanism.

The burst release of drug from microspheres is usually considered to be a result of surface drug diffusion. From our cryo-SEM results, the formation of transitional layers in the PVA hydrogel at early time points (Figures 5.7-A2, 5.8-A and 5.8-B) may be due to large amounts of drug diffusing out of the microspheres and disturbing the intrinsic gel structure during the burst release phase. The coincidence between the drug release profile and water uptake for both microsphere formulations indicates swelling as a possible contributing factor to drug release from both formulations. Increased osmotic pressure due to accumulation of degradation products as well as polymer matrix plasticization have been suggested as contributing factors to

microsphere swelling [14]. From the current investigation, the internal structure changes (*i.e.* development of a pore structure) also play a significant role affecting microsphere swelling. The onset of microsphere swelling was shown to correlate with the development of an internal porous structure for both types microspheres investigated (day 1 and day 6 for DLG1A and RG503H, respectively). During the lag phase, the following sequence of events occurs: *1)* pores build up internally until the entire microsphere structure is porous, *2)* the microspheres swell following pore formation, and *3)* the significant influx of water leads to internal structure deformation followed by the onset of the second drug release phase. For hydrophobic drugs, the microspheres form a natural "osmotic pump" to maintain osmotic pressure from two aspects: 1) the internal microsphere space is filled with saturated drug solution providing consistent osmotic pressure; 2) osmotic pressure contributed by degraded oligomers and monomers is compromised from the microsphere swelling, and accordingly the second drug release phase is pseudo zero order. The surface layer of the microspheres remained intact during the drug release phase. This may be the reason that the microspheres continued to swell during the entire drug release phase.

For both microsphere formulations, small pores formed early close to the exterior and large pores formed later within the microspheres. This pattern can be explained by a local pH gradient from the interior (high acidity) to the surface (low acidity) and the water diffusion kinetics into the microspheres. As the microspheres are in contact with the aqueous phase, water diffusion into the microspheres can result in a concentration gradient of drug and acidic degradation species. The surface layer of the microspheres is exposed to water earlier and therefore starts to degrade faster. However, the acidic degradation products do not build up close to the surface and therefore large pores do not form in this region. In contrast, the microsphere cores are exposed to water later compared to the surface and consequently pore formation occurs slower. However,

due to the pH effect from the accumulated acidic degradation products, those pores tend to grow large quickly. The pore morphology is interesting in that the larger pores are spherical in shape indicating that they are not formed as a result of connecting with neighboring pores. The uniform wall thickness between neighboring pores may originate from less viscous acid terminated PLGA oligomers that prefer to segregate their carboxyl ends toward pore surface.

5.4.3 Effect of swelling on glucose diffusion

Glucose diffusion through the composite coatings is another important characteristic with respect to their application as a coating for glucose biosensors. Glucose diffusion through the composite coatings is affected by the hydrophilicity and porosity of the coatings. When coated with the composites, microdialysis probes can provide insight into coating permeability to a specific analyte (*i.e.* glucose) in the form of RR. Employing the steady-state mass balance theory, a model has been proposed by Bungay *et al*. attributing RR to the perfusate flow rate (Qd) and a series of mass transport resistances [34]. The correlation is presented using the equation below:

$$RR = 1 - \exp(-\frac{1}{Q_d \times (R_d + R_z + R_e)})$$

where R_d, R_z, and R_e are transport resistances of the dialysate, dialysis membrane, and external medium, respectively. Under well stirred conditions, the external medium resistance (R_e) can be considered as zero. Norton, *et al* further separated R_z into the resistance contributed by the dialysis membrane (R_m), and that contributed by the hydrogel coating (R_h) using the equation below [35].

$$R_z = R_m + R_h$$

R_h can be further defined using the following equation:

$$R_h = \frac{\ln(r_h/r_o)}{2\pi L D_h \varphi_h}$$

where r_o is the outer radius of dialysis probe, r_h is the outer radius of the coating, L is the length of the microdialysis membrane, D_h is the glucose diffusion coefficient in the coating and φ_h is the fraction of glucose in the coating. Therefore, the apparent RR should be negatively related to the thickness of the coating (affecting r_h) and positively related to the glucose diffusion coefficient (D_h) in the coating.

In the current study, a positive correlation was observed using a plot between the RR and swelling ratio at 3 hours while a negative correlation was observed at maximum swelling date (day 24) for coatings prepared with RG503H microspheres (Figure 5.9). For coatings prepared using DLG1A microspheres, although the positive correlation at 3 hours was not observed due to the masking effect from microsphere swelling, at maximum swelling date (day 13), such pattern of negative correlation was also observed. The positive correlation at 3 hours can be attributed to a negative dependence of water uptake into the PVA hydrogel on the microsphere concentration (prepared using the RG503H polymer). During this initial phase, more glucose mobility (increase of D_h) is expected with increase in the hydrophilicity of the coating. Further swelling of the coating was observed and water uptake reached a maximum at day 24. As the majority of the coating is composed of water by this time point, the effect of the coating thickness change (change of r_h) dominates the contribution to RR leading to a negative correlation between swelling and RR. Glucose diffusion through the microsphere is limited as the microsphere shell was observed throughout the drug release phase (shown in Figure 5.8) in addition to the high internal osmotic pressure. However, the increase of RR post the second drug release phase can possibly be explained by the disappearance of the microsphere shell and therefore glucose is able to freely pass through the holes that were earlier occupied by the microspheres. In addition,

decrease in water uptake was observed post the second drug release phase indicating that the coating was shrinking (decreasing in thickness) with the disappearance of the microsphere shells.

5.5 Conclusion

The present study demonstrated new insights into PGLA microsphere drug release mechanisms through investigation of swelling, internal pore formation and glucose diffusion for two types of PLGA microspheres (with and without lag phase) embedded in PVA hydrogels. For the first time, detailed internal structure of PLGA microspheres during drug release was revealed with the assistance of cryo-SEM. The results suggest that both types of microspheres undergo heterogeneous erosion, and swelling. The outside-in pattern of porosity progression in the microspheres explains the lag phase observed in some PLGA microsphere products. The length of the lag phase is determined by the time required for the entire microsphere to become porous which is controlled by the molecular weight and hydrophobicity of the polymer. The onset of drug release post the lag phase appears to be a consequence of microsphere swelling following pore formation. Continuous microsphere swelling during the second drug release phase may also affect the drug release kinetics. In addition, the timing and amount of water absorption measured during the swelling process will be useful for researchers who are interested in building mechanistic mathematical models depicting drug release from microspheres. This information can be used by researchers to develop microspheres with specific release patterns for different applications. The information generated in this study on the dynamic internal changes in the microspheres will also be useful in understanding drug stability during the release process. Furthermore, the correlation between microsphere swelling and glucose permeation through the coatings will facilitate coating design for glucose sensors and other similar implantable devices.

5.6 Tables and Figures

Table 5.1 Physicochemical properties of the PLGA microsphere formulations

Formulation	Polymer type	Polymer intrinsic viscosity (dl/g) *	Tg (˚C)	Drug loading (w/w)
I	5050DLG1A	~0.08	43.3	7.72 ± 0.33%
II	5050RG503H	~ 0.4	48.2	7.63 ± 0.28 %

* Information provided in Analytical Report from Lakeshore Biomaterials

Table 5.2 Calculated maximum swelling ratio of microspheres embedded in the composite coatings according to Figures 5.3 and 5.4

Microsphere Concentration	50 mg/mL PVA	75 mg/mL PVA	100 mg/mL PVA
DLG1A MS	2.49 ± 0.03	2.33 ± 0.27	2.62 ± 0.12
RG503H MS	2.15 ± 0.18	2.55 ± 0.11	2.97 ± 0.12

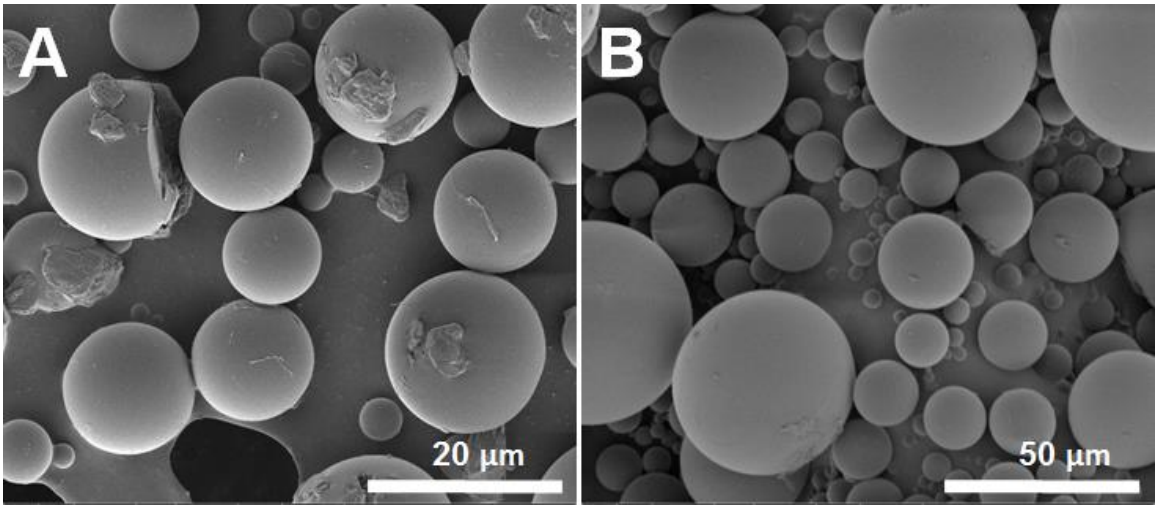

Figure 5.1. SEM images of PLGA microspheres prepared using DLG1A (A) and RG503H (B)

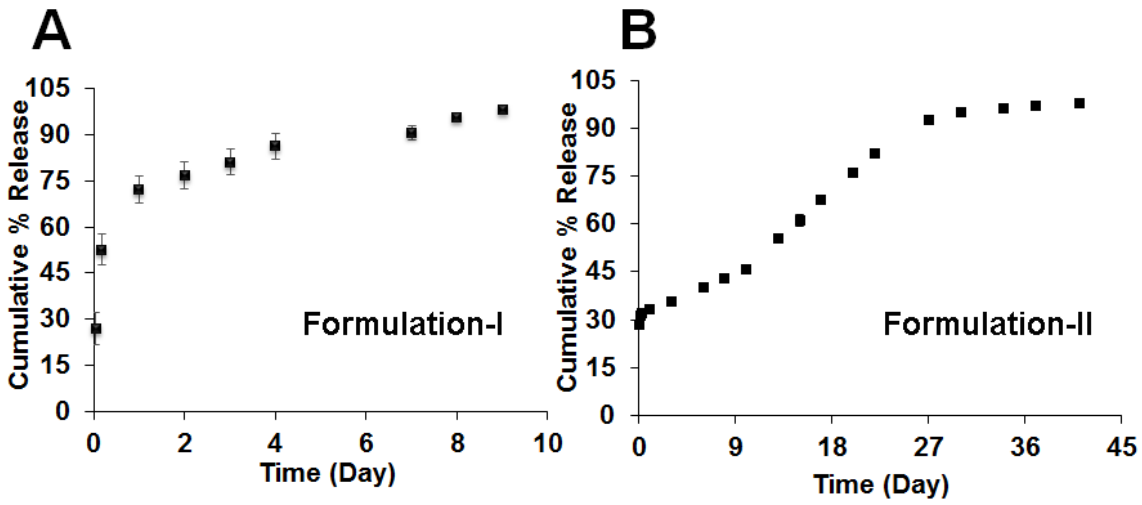

Figure 5.2. *In vitro* release profiles of composite coatings prepared using DLG1A (A) and RG503H (B) based microspheres

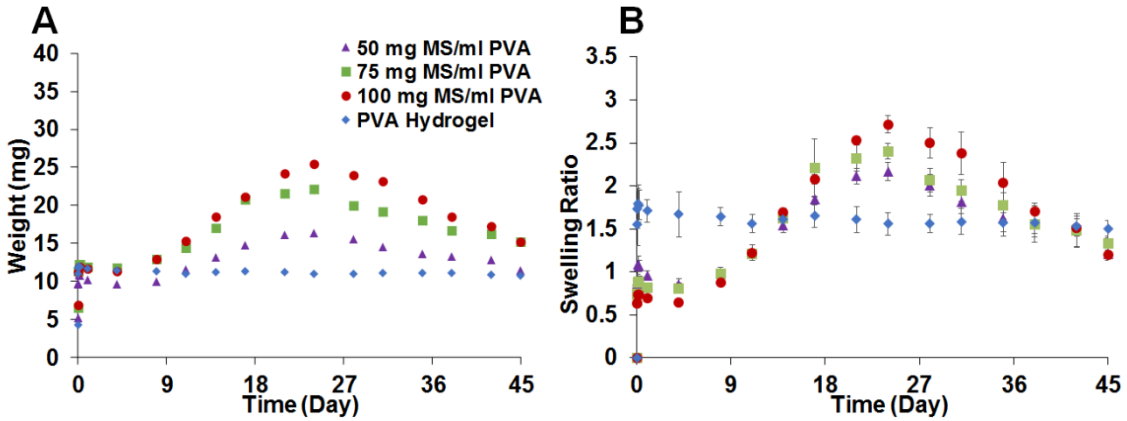

Figure 5.3. Swelling of PLGA microsphere/PVA hydrogel composite coatings prepared using RG503H based microspheres. The weight change of the samples is shown in A (n=3) and the swelling ratio change is shown in B (n=3). The data is presented as average ± SD for the swelling ratio.

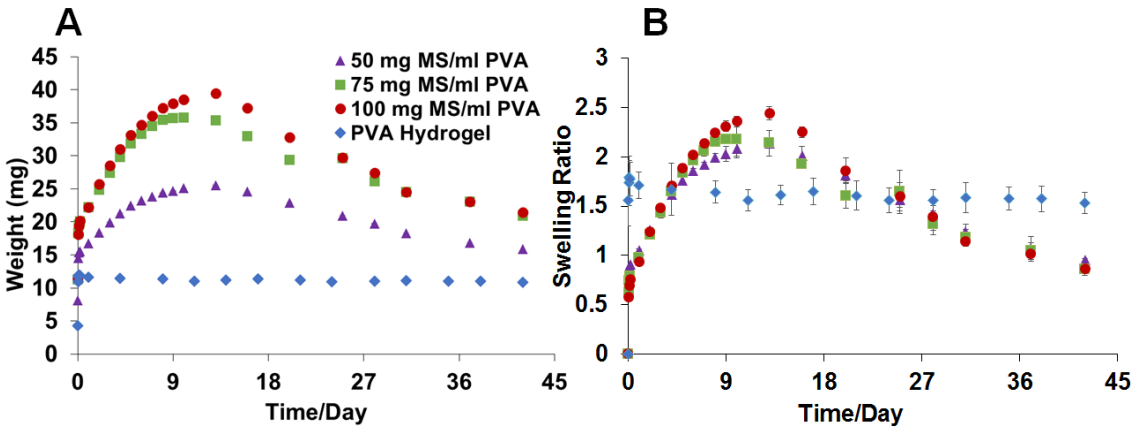

Figure 5.4. Swelling of PLGA microsphere/PVA hydrogel composite coatings prepared using DLG1A based microspheres. The weight change of the samples is shown in 4 (n=3) and swelling ratio change is shown in 4 (n=3). The data is presented as average ± SD for the swelling ratio.

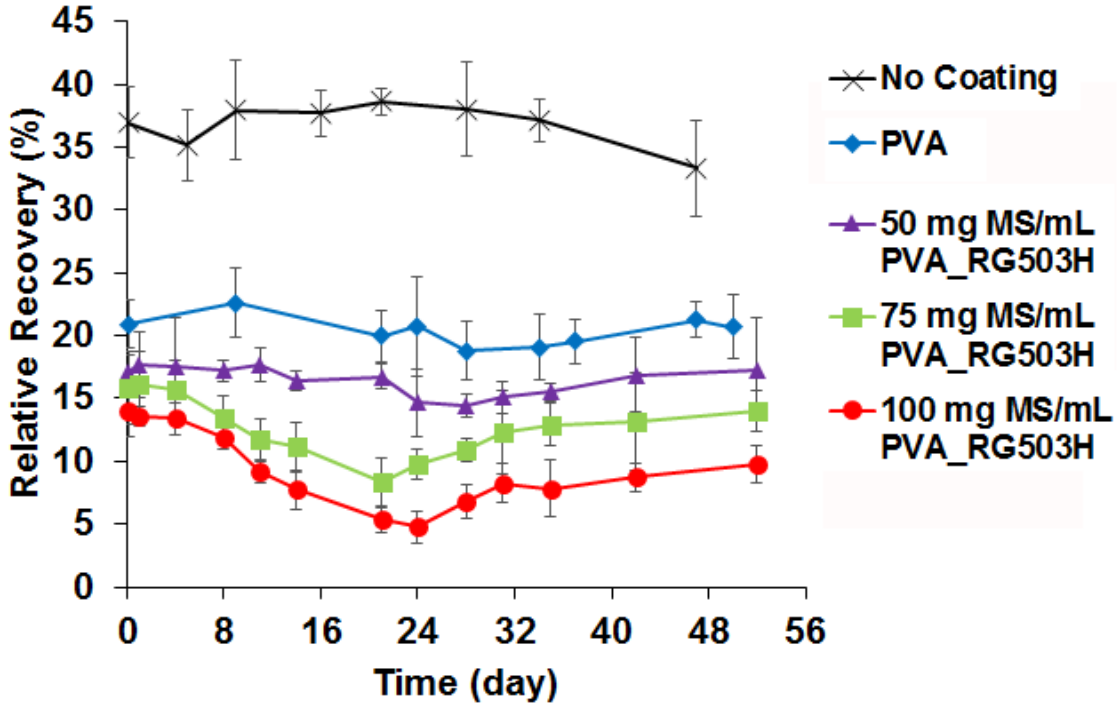

Figure 5.5. Effect of different types of coating and incubation time on glucose relative recovery obtained using microdialysis probes (n=3 for each time point). Microspheres used were prepared using the RG503H polymer.

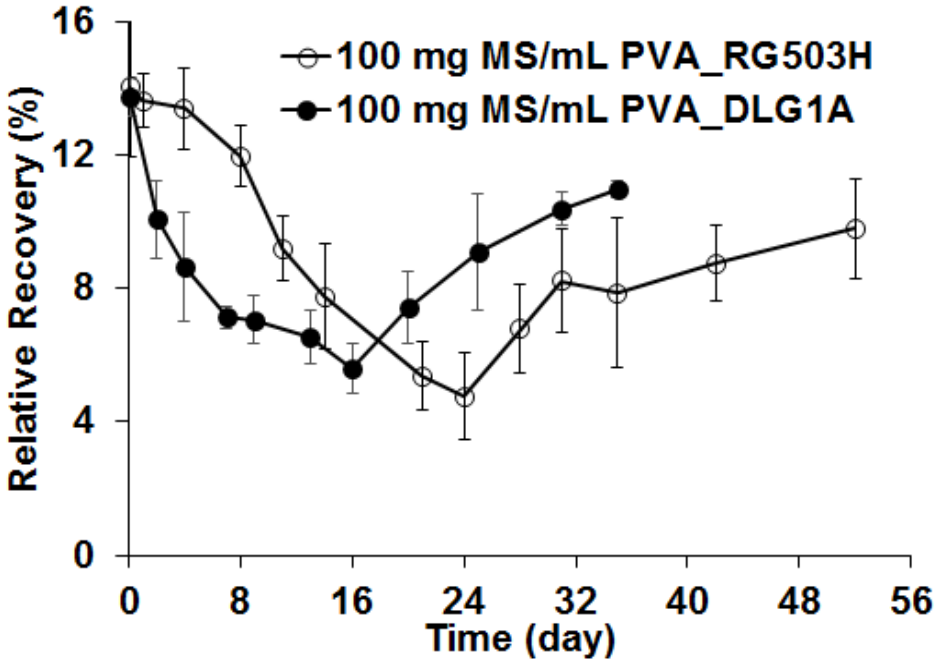

Figure 5.6. Glucose relative recovery from composite coatings embedded with microspheres (100 mg MS/ml PVA) prepared using RG503H and DLG1A polymers

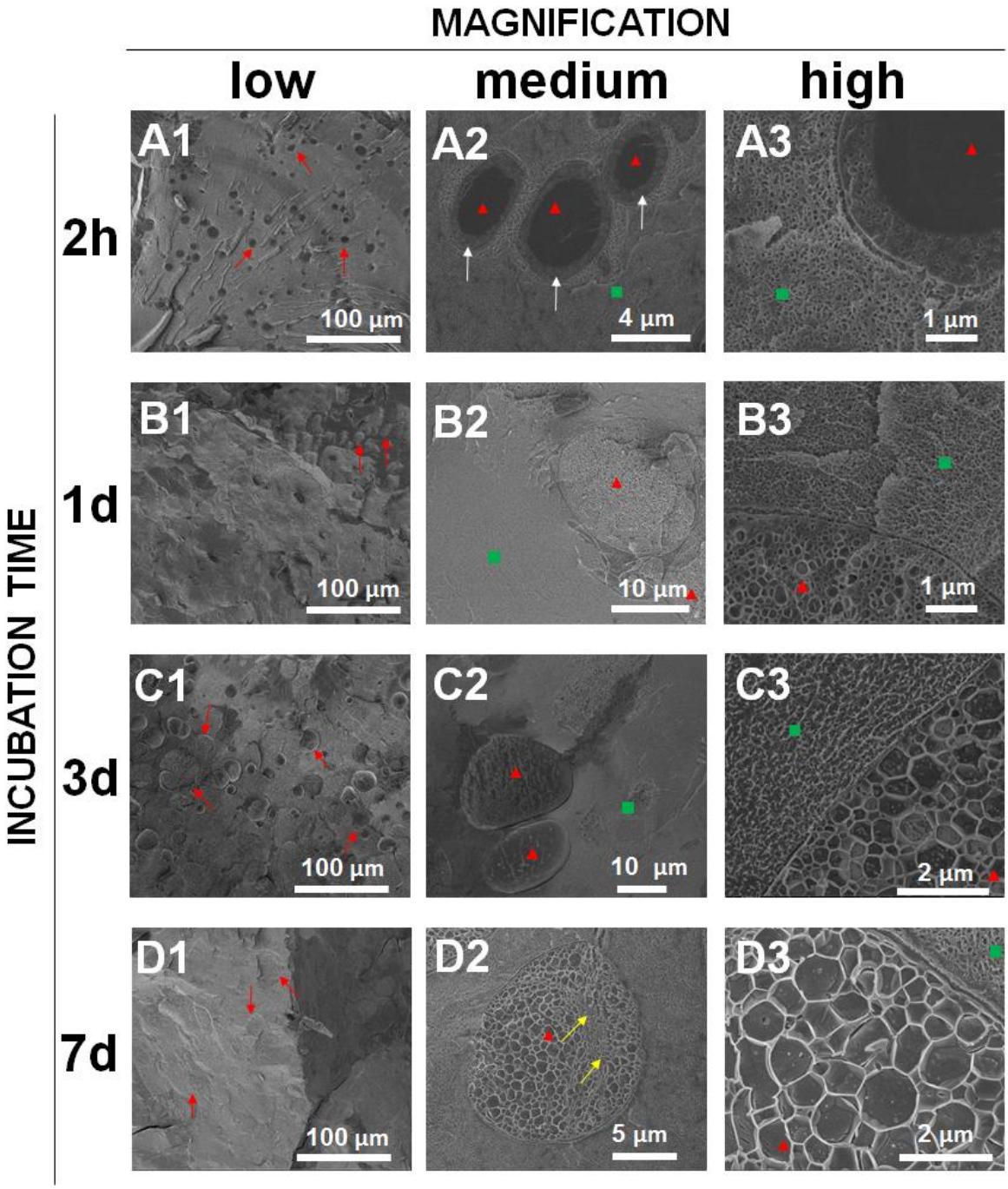

Figure 5.7. Cryo-SEM images showing the internal structure of the PLGA microsphere/PVA hydrogel composites (prepared using DLG1A polymer) after incubation in phosphate buffer for 2 hours (A1, A2, A3), 1 day (B1, B2, B3), 3 days (C1, C2, C3) and 7 days (D1, D2, D3). Images are provided at low magnification (A1, B1, C1, D1), medium magnification (A2, B2, C2, D2) and high magnification (A3, B3, C3, D3). Red arrows point at the microspheres at low magnification, red triangles point at the microspheres at medium/high magnification, green squares point at the hydrogel at medium/high magnification and white arrows point at the interphase between the microsphere and the PVA hydrogel.

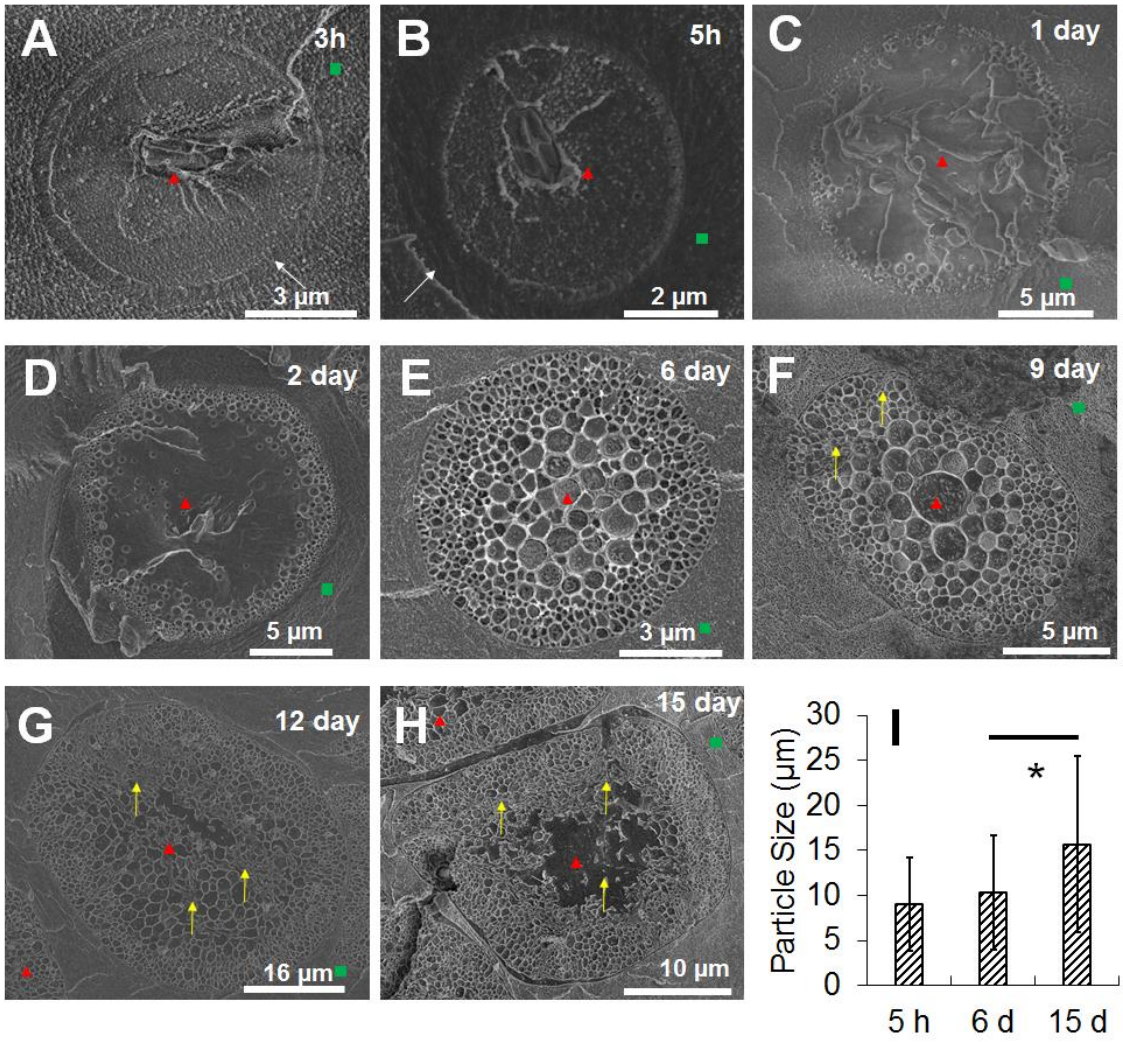

Figure 5.8. Cryo-SEM images showing the internal structure of the PLGA microsphere/PVA hydrogel composites (prepared using the RG503H polymer) following incubation in phosphate buffer for 3h, 5h, 1 day, 2 days, 4 days, 9 days, 12 days and 15 days (corresponding to A, B, C, D, E, F, G, H, respectively). The red triangles point at the microspheres, the green squares point at the hydrogel, the white arrows point at the interphase between microsphere and PVA hydrogel, and the yellow arrows point at the deformation/collapse of the porous structure. I shows particle size change over time obtained by analyzing low magnification images (approximately 1000X, data not shown) following 5h, 6 day and 15 day incubation. Approximately 100 particles were analyzed for each image.* indicates statistical significance analyzed using paired student t-test ($p<0.001$)

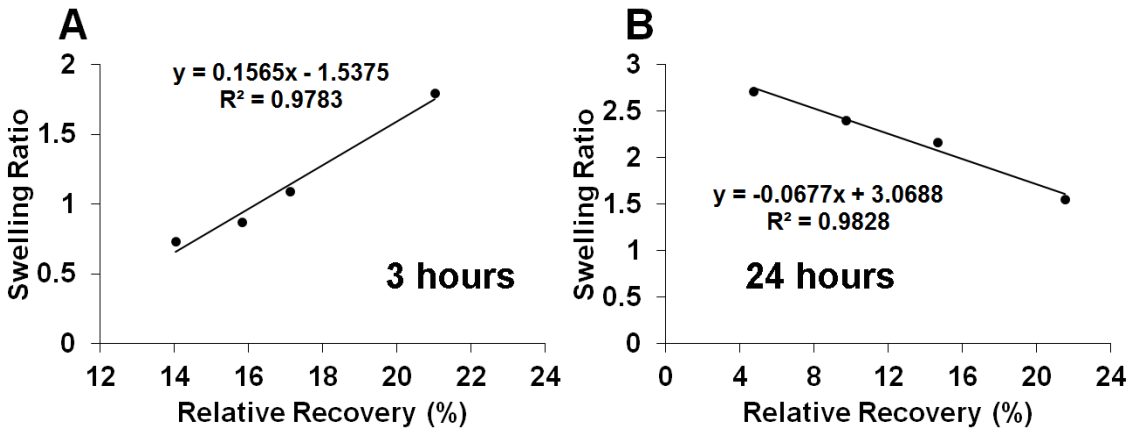

Figure 5.9. Correlation between coating swelling ratio and RR of glucose through the coatings prepared with RG503H microspheres following 3 hours (A) and 24 days (B) incubation.

5.7 References

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Chapter 6

Conclusions and Future Studies

6.1. Summary and conclusions

Continuous glucose monitoring using implantable glucose sensors will significantly improve the quality of life for diabetic patients. There are currently several semi-implantable glucose sensors available on the market. However, these sensors suffer from short life-time of approximately 7 days which limits their application. The sensors lose functionality rapidly due to a series of immune reactions including acute inflammation, chronic inflammation and fibrous encapsulation. PLGA microsphere/PVA hydrogel composites have been developed to act as biocompatible coatings for implantable glucose sensors to increase sensor life-time and enhance sensor performance. The sensor life-time can be extended with the continuous release of anti-inflammatory drug (*i.e.* dexamethasone) to counter those reactions and the duration of drug release has a significant impact on sensor life-time. The sensor performance is related to glucose diffusion through the coating and therefore is related to the microscopic dynamic changes (*i.e.* hydrophilicity and porosity) in the coating during drug release. Accordingly, the main scope of this work was to develop a coating to maximize sensor life-time through long-term (6 months) drug release, and understanding of dynamic changes in the coating.

Blending different PLGA polymers for microsphere manufacturing was shown to be an effective strategy modulating drug release properties. The low Mw PLGA increases water absorption into the polymer matrix and generates acidic oligomers/monomers which facilitate the autocatalysis of the high Mw polymer resulting in a smoother release profile. A polymer blend based dexamethasone microsphere/hydrogel formulation with continuous drug release and *in vivo* efficacy of 4.5 months was achieved in a preliminary testing. The composition of the polymer blends is critical to the release profile. One formulation blending the low and high Mw polymer at a 50:50 ratio exhibited high burst and short duration of drug release due to polymer

phase separation during the microsphere formation process. In addition, accelerated drug release methods were developed under elevated temperature conditions to significantly shorten the testing period from months to days while retaining the ability to discriminate among the various formulations. The discriminatory accelerated testing method can be used to evaluate long-term drug releasing coatings in the future for quality control purposes.

Further optimization of the composition was achieved following a DoE approach using a central composite design. A design space with appropriate formulation outcomes (*e.g.* high drug loading and low burst release) was obtained based on the developed highly predictive DoE models. The optimized design space was shown to be valuable in predicting and controlling both burst release and drug loading, and it can serve as a blueprint to design PLGA blend based microsphere formulations with defined release attributes. According to the design space, approximately 85 mg of dexamethasone and 85% of high molecular weight PLGA were required in order to achieve a formulation with approximately 15% (w/w) dexamethasone loading and 10% (w/w) burst release. In addition, a fundamental understanding of the effect of microsphere composition on the formulation performance was obtained by examining the formulation particle size, thermal properties as well as morphologies. Importantly, a novel heat map describing the daily drug release was developed to differentiate the various formulations during the lag phase where there is low/no drug release. Drug release properties are usually described using release profiles which may not be sufficiently detailed to describe the lag phase. In contrast, the drug release lag phase was illustrated clearly *via* the heat map and comparison between various formulations was easily made. Such heat maps are especially helpful for researchers who need to screen the release properties of a large number of formulations.

The optimized microsphere formulation was shown to continuously release dexamethasone at a sufficient dose to inhibit the FBR for 6 months. Further mixing the optimized microspheres with a single polymer based formulation demonstrated *in vitro* drug release of more 7 months, achieving the longest dexamethasone releasing formulation reported by far. Meanwhile, the heat map obtained using the formulations evaluated *in vivo* suggested that the minimum dexamethasone daily dose required to suppress chronicle inflammation is approximately 0.1 μg/mg of implant in rats, which can provide guidance for future coating design of glucose sensors. The heat map can potentially serve as a useful tool for establishing a clinically relevant dose of such long-term drug release formulations. The successful development of a long-term effective composite coating paves the way for the realization of long-term, totally implantable continuous glucose monitoring systems.

Lastly, new insights into PGLA microsphere drug release mechanisms was obtained from the investigation of swelling, internal pore formation and glucose transport for two types of PLGA microspheres (with and without lag phase) embedded in PVA hydrogels. Detailed internal structure of PLGA microspheres during drug release was revealed with the assistance of cryo-SEM. The results suggest that both types of microspheres undergo heterogeneous erosion, and swelling. The outside-in pattern of porosity progression in the microspheres explains the lag phase observed in some PLGA microsphere products. The length of the lag phase is determined by the time required for the entire microsphere to become porous which is controlled by the molecular weight and hydrophobicity of the polymer. The onset of drug release post the lag phase appears to be a consequence of microsphere swelling following pore formation. Continuous microsphere swelling during the second drug release phase lead to an increase of coating thickness which negatively affected glucose diffusion. This correlation between

microsphere swelling and glucose permeation through the coatings will facilitate coating design for glucose sensors and other similar implantable devices in the future.

The significant and novel contributions resulting from this work include: *i)* expanding the application of PLGA polymers by proving that drug release properties from PLGA microspheres can be modulated *via* blending different types of PLGA polymers; *ii)* generating predictive models to achieve appropriate microsphere composition for desired release profiles; *iii)* developing accelerated drug release methods for such long-term drug release formulations; *iv)* establishing a novel method (*i.e.* a heat map of daily drug release *in vitro*) for depicting drug release from long-term drug release formulations; *v)* achieving a 6-month *in vivo* effective dexamethasone eluting coating for implantable glucose sensors; *vi)* determining the minimum dexamethasone dose required to suppress chronic inflammation in a rat model; *vii)* revealing the drug release mechanisms from PLGA microspheres embedded in PVA hydrogel coatings; *viii)* demonstrating the long-term effect of microsphere degradation on glucose transport through the composite coatings. In conclusion, the results of this research have advanced the implementation of PLGA microsphere/PVA hydrogel composites as a drug-eluting coatings for long-term functional glucose biosensors. This research also adds knowledge to the fundamental understanding of PLGA microspheres which can further their applications as drug delivery systems.

6.2. Future studies

The continuous drug release profile obtained from polymer blend based microspheres is composed of multiple distinct phases which are not a simple combination of the release profiles obtained from mixing two individual microspheres prepared each with one polymer. Further investigation into the drug release mechanism from these microspheres can be conducted by

evaluating changes in molecular weight, internal structure and pH distribution, as well as swelling properties. A more detailed physical state (phase separation and polymer miscibility) of blended PLGA in these microspheres can be evaluated using solid state NMR, small-angle X-ray scattering, and small-angle neutron scattering techniques.

The current study demonstrated that the *in vivo* efficacy of the optimized formulation lasted for approximately 6 months, which approximately corresponds to the *in vitro* drug release duration from the coatings. Development of a detailed *in vitro-in vivo* correlation (IVIVC) can be an interesting future topic. In addition, it will be helpful to develop more detailed dose-response correlations during different inflammatory phases for sensors with different size/geometry. PVA hydrogels loaded with different amounts of free dexamethasone can be used to determine the dose required during the acute inflammation phase. During the chronic inflammation phase, with the assistance from the heat map, the effect of dexamethasone dose on specific immune reactions such as cytokine expression, macrophage polarization, timing of neutrophil recruitment and fibroblast activation can also be evaluated using immuno-histological staining and western blot analysis, *etc*. In addition, the dexamethasone dose obtained from animal studies should be carefully investigated to satisfy both efficacy and safety when transitioning the coating to clinical studies considering differences between species.

So far, these long-term coatings have not been tested with functioning glucose sensors. It is possible that long-term dexamethasone release may suppress the growth of blood vessels near the sensor therefore affecting sensor response. Different types of coatings can be evaluated to promote angiogenesis including co-delivery of vascular growth factors (*e.g.* VEGF and PDGF) and changing the coating morphology (*e.g.* different degrees of porosity).

The dynamic changes within the PLGA microspheres embedded in the PVA hydrogel were revealed using CryoSEM. This technique may be further used to evaluate individual microspheres to explore how/when the microsphere swelling/internal pore formation takes place. The process of microsphere structure collapse may be different without the supporting of a PVA hydrogel. In addition, microdialysis was used in the study as a tool to evaluate the effect of the coating on *in vitro* glucose transport. Microdialysis can also be performed *in vivo* to determine the effect of different phases of the FBR on glucose transport.