Controlling Acute Inflammation with Fast Releasing Dexamethasone-PLGA Microsphere/PVA Hydrogel Composites for Implantable Devices

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Abstract

Background:
Continuous release of dexamethasone from PLGA microsphere/PVA hydrogel composites has been shown to suppress the inflammatory tissue reaction in response to subcutaneously implanted foreign material for a period of one month. The scope of the present work is to investigate whether suppressing the initial acute inflammatory phase with fast releasing dexamethasone-PLGA microsphere/PVA composites (that release the drug over a period of one week) would prevent the development of a foreign body reaction in response to implantation in the subcutaneous tissue using a rat model.

Methods:
Dexamethasone loaded PLGA microspheres were prepared using the solvent evaporation method. In vitro release from microspheres was analyzed using USP apparatus 4 in phosphate buffered saline (PBS) at 37°C. Composites were fabricated in 18G needles by freeze-thaw cycling the PVA/microsphere dispersion. The composites were implanted in the subcutaneous tissue of anesthetized rats. The pharmacodynamic effect was evaluated by histological examination of the tissue surrounding the composites at pre-determined time points.

Results:
In vitro release studies showed that most of the drug entrapped in the microspheres was released within one week. At days 3 and 8, these fast releasing dexamethasone containing composites suppressed the acute phase of inflammation but did not prevent the development of an inflammatory reaction after dexamethasone was completely released from the composites. By day 30, chronic inflammation and fibrosis were observed in the tissue surrounding the drug-containing composites. On days 3 and 8, the number of inflammatory cells in the vicinity of the dexamethasone containing composites was similar to that in normal tissue. However, the number of inflammatory cells was higher in drug-containing composites as compared to drug-free composites by day 30. This was due to the inflammation being in a more advanced stage in drug-free composites where a granulomatous reaction had already developed.

Conclusion:
Fast release of dexamethasone from PLGA/PVA composites did not provide long-term protection against the foreign body reaction in response to implantation. It would appear that a sustained delivery of anti-inflammatory agents such as dexamethasone is necessary to suppress inflammation throughout the implant life-time.


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**Introduction**

Real-time monitoring of various metabolic analytes that control the function and physiology of the human body is important for the treatment of diseases such as diabetes. An intensive insulin regimen requires multiple blood glucose measurements every day and for optimal insulin administration continuous glucose monitoring is desirable. Continuous monitoring provides not only the glucose level at a specific time point, but also the rate and direction of change in glucose levels and is important for early detection of hypoglycemic/hyperglycemic states. Development of glucose biosensors is an area of intense research and the recent advances in microfabrication techniques have opened up the possibility of totally implantable glucose biosensors. For such sensors the subcutaneous tissue is regarded as an appropriate site for implantation since it is easily accessible for implantation and removal. In addition, it has been reported that the glucose concentration in the subcutaneous fluid is directly correlated to blood glucose concentration. Bolinder et al reported that in patients with diabetes, the absolute glucose concentration in s.c. tissue was the same or almost the same as the blood glucose concentration (87% to 101% of the blood glucose value). In rats, the s.c. glucose content has been shown to be closely related to blood glucose content.

A major obstacle to the development of implantable biosensors is the gradual loss of function that occurs following implantation. The biological factors responsible for the instability of biosensors are tissue trauma and biofouling. Tissue trauma results from injury during implantation and the long-term presence of the implant. This triggers a cascade of inflammatory and wound healing responses typical of a foreign body reaction. The initial acute phase, characterized by fluid and protein exudation and neutrophilic reaction, lasts up to a few days. The chronic phase consists of the influx of macrophages, the formation of multinucleate giant cells, neo-vascularization, and the deposition of collagen with subsequent formation of a fibrotic capsule around the implant (i.e. scarring) and lasts from days to years. Biofouling is the adhesion of proteins and cells onto foreign materials and can occur immediately after implantation. Inflammatory cells release various reactive oxygen species and secrete proteolytic enzymes resulting in decreased tissue pH. These changes may damage sensor components and impair sensor function. In addition, fibrosis and biofouling can decrease the transport of analyte to the sensor thus compromising device sensitivity. Wisniewski et al have reported that resistance to glucose mass transport due to biofouling and tissue reaction increased between days 0 and 8 after implantation. It was established that the tissue effects had a 3 to 5 times greater impact on glucose transport compared to the impact of biofouling. In addition, it is considered that biofouling may be a result of secretions from inflammatory cells. Therefore, controlling the inflammation and fibrotic encapsulation at the implant site would appear to be critical to achieve a functional biosensor with extended life time.

Inflammation and fibrosis at the implant site may be controlled with the use of steroidal and non-steroidal anti-inflammatory drugs. Since long-term systemic use of these drugs leads to unwanted side effects, localized and sustained delivery of anti-inflammatory drugs has been investigated. Microsphere and nanoparticle based drug delivery systems, hydrogels, microparticles embedded in hydrogel matrices and implants containing drug-filled reservoirs have been investigated as means to deliver anti-inflammatory drugs to the implant site. Earlier, we reported the use of a dexamethasone/PLGA microsphere system to suppress the tissue response at the implant site for at least one month. A mixture of pre-degraded and newly formulated microspheres was used in this study to provide sustained release of dexamethasone over a one month period. The microsphere system containing dexamethasone adequately controlled inflammation and fibrosis in the vicinity of a subcutaneously implanted suture model. Recently, we reported the development and evaluation of a composite coating for implantable devices, based on physically cross-linked poly(vinyl alcohol) (PVA) hydrogels containing dexamethasone-loaded poly(lactic-co-glycolic acid) (PLGA) microspheres, that controlled both the acute and chronic phases of inflammation over a one month period. However, it remains to be investigated whether long-term release of dexamethasone is imperative or a short-term delivery would provide adequate protection against the foreign body reaction.

In the present study, composites containing fast releasing (within one week) dexamethasone/PLGA microspheres were investigated to determine whether these can provide long-term protection against inflammation and fibrotic encapsulation. This will elucidate whether control of the acute phase of inflammation will prevent progression to the chronic stage and eliminate the need for long-term release of dexamethasone at the implant site. Histopathological examination of the subcutaneous tissue in the vicinity of the implant was performed over a time period of one month to evaluate the pharmacodynamic effects of dexamethasone released from these composite coatings.
Experimental Methods

Materials

PVA (average mol wt 30,000-70,000 Da), humic acid and dexamethasone were purchased from Sigma (St. Louis MO). PVA (99% hydrolyzed; mol mass 133KDa; lot number 440424) was purchased from Polysciences, Inc., (Warrington PA). PLGA 50:50 (mol wt 5KDa; lot #1285-535) was purchased from Alkermes, Inc., (Wilmington OH). Methylene chloride was purchased from Fisher Scientific (Pittsburgh PA).

Preparation of PLGA microspheres

Dexamethasone-loaded/blank microspheres were prepared using a solvent evaporation method as described previously.13 Briefly, 2 g PLGA was dissolved in 8 ml methylene chloride. For dexamethasone-loaded micro-spheres, 200 mg of dexamethasone was dispersed in this solution. This organic phase was emulsified in 40 ml of a 1% (w/w) PVA (average mol wt 30,000-70,000 Da) solution and homogenized at 10,000 rpm for 1.5 min using a PowerGen 700D Homogenizer (Fisher Scientific, Pittsburg, Pennsylvania). The resultant emulsion was poured into 500 ml of a 0.1% (w/w) PVA (average mol wt 30,000-70,000 Da) solution and stirred under vacuum to achieve rapid evaporation of methylene chloride. The hardened microspheres were washed 3 times with deionized water and collected by filtration (0.45 µm). The prepared microspheres were kept under vacuum overnight and later stored at 4°C until further use.

In vitro release from microspheres

In vitro release studies were conducted as described previously.14 Briefly, in vitro release was analyzed at 37°C using a modified USP apparatus 4 (Sotax CY 7 piston pump, Sotax, Horsham, Pennsylvania) with flow-through cells (12 mm diameter) packed with glass beads (1 mm), to prevent microsphere agglomeration and to achieve laminar flow in a closed system mode. Approximately 40 mg of microspheres were dispersed in the flow-through cells and 250 ml of 0.1 M phosphate-buffered saline (PBS) was circulated through the implantation with 2% isoflurane in oxygen. The back of each animal was shaved and washed with betadine solution. All animal studies were conducted at the University of Connecticut in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines using an approved protocol (#A05-015). All procedures were performed as discussed previously.10 Composites were implanted into the dorsolateral s.c. tissue of male Sprague–Dawley rats using an 18G needle/plunger system. The same needles that were used to fabricate the composites were used for implantation. One drug free and one drug containing composite was implanted into each rat. For this purpose, rats were initially anesthetized using isoflurane (Forane; Baxter; Deerfield, Illinois). The anesthesia was maintained during the implantation with 2% isoflurane in oxygen. The back of each animal was shaved and washed with betadine solution. All of the procedures were conducted under aseptic conditions. Tissue inflammation response was determined through serial sacrifice to investigate both the acute (day 3) and the chronic (8 and 30 day) stage anti-inflammatory effect (n=6 at each time point). A histologic evaluation of excised tissue samples from the site of implantation was performed after staining with hematoxylin and eosin (H&E) stain and Massons’ trichome stain for inflammation and collagen, respectively. Tissue samples were fixed in 10% neutral buffered formalin processed through serial alcohol and toluene and embedded in paraffin. Tissue sections of 5µm thickness were cut and stained with H&E or trichome. Tissue samples were observed and digitally stored using an Olympus microscope (model A051, Olympus America, Melville, NY) and Bioquant software (BQ-TCWV3.00.6). The pictures were taken at different magnifications. The tissue images taken at magnification of 200X were used

Preparation of composites

Microsphere/PVA composites were prepared as described previously.10 Briefly, PVA (99% hydrolyzed; mol wt 133 KDa) was dissolved in Millipore quality deionized water at 80°C to obtain a 10% (w/w) solution and sterilized by autoclaving. A 4 % (w/w) humic acids (HAs) solution was prepared in deionized water and sterilized by filtration. Equal volumes of PVA and HAs solutions were mixed to obtain 5% (w/w) PVA solution containing 2% (w/w) HAs. One hundred milligrams of microspheres were dispersed per ml of this PVA/HAs solution. This dispersion was then filled into 18G needles (1.5 inches long X 0.42 mm in diameter) and subjected to 3 freeze-thaw cycles (-20°/24°C) to fabricate the composites. Each freeze-thaw cycle comprised 2 h of freezing at -20°C followed by 1 h thawing at 24°C. The prepared composites were stored at 4°C until further use. All the procedures were conducted under aseptic conditions. The rationale for the use of HAs in these composites has been discussed previously.10,13

In vivo pharmacodynamic study

All animal studies were conducted at the University of Connecticut in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines using an approved protocol (#A05-015). All procedures were performed as discussed previously.10 Composites were implanted into the dorsolateral s.c. tissue of male Sprague–Dawley rats using an 18G needle/plunger system. The same needles that were used to fabricate the composites were used for implantation. One drug free and one drug containing composite was implanted into each rat. For this purpose, rats were initially anesthetized using isoflurane (Forane; Baxter; Deerfield, Illinois). The anesthesia was maintained during the implantation with 2% isoflurane in oxygen. The back of each animal was shaved and washed with betadine solution. All of the procedures were conducted under aseptic conditions. Tissue inflammation response was determined through serial sacrifice to investigate both the acute (day 3) and the chronic (8 and 30 day) stage anti-inflammatory effect (n=6 at each time point). A histologic evaluation of excised tissue samples from the site of implantation was performed after staining with hematoxylin and eosin (H&E) stain and Massons’ trichome stain for inflammation and collagen, respectively. Tissue samples were fixed in 10% neutral buffered formalin processed through serial alcohol and toluene and embedded in paraffin. Tissue sections of 5µm thickness were cut and stained with H&E or trichome. Tissue samples were observed and digitally stored using an Olympus microscope (model A051, Olympus America, Melville, NY) and Bioquant software (BQ-TCWV3.00.6). The pictures were taken at different magnifications. The tissue images taken at magnification of 200X were used.
to count the inflammation mediating cells. For this purpose 10 square boxes of 75 μm X 75 μm were made randomly per sample and the number of cells was counted manually.

**Results and Discussion**

Figure 1 shows the *in vitro* release profile of the microspheres in 0.1M PBS at 37°C. It is observed that approximately 90% of the drug is released within 4 days *in vitro*. The burst release accounts for about 65% of the drug released and is complete within 12 hours. The microspheres were therefore classified as fast-releasing.

![Figure 1: In vitro release profile of dexamethasone from PLGA (50:50; mol wt 5KDa) microspheres in phosphate buffered saline (PBS), 0.1M, pH 7.4 at 37°C. Each value represents average of three replicates (± SD).](image)

The pharmacodynamic effects of composites containing dexamethasone were evaluated by comparison with drug-free composites. Figure 2 shows tissue sections taken from the vicinity of the implants on day 3 after implantation (acute phase), where Figures 2A, 2B, and 2C are representative of drug free composites; Figures 2D, 2E, and 2F are representative of drug containing composites and Figure 2G is representative of s.c. tissue without an implant. The inflammatory cells stain basophilic (purple) and the lacy s.c. connective tissue surrounding the implant stain eosinophilic (pink) with H&E staining. The photomicrographs at lower magnification (85X) provide information about the intensity of the inflammatory reaction around the entire implant. The photomicrographs at the magnification of 200X were used to count the number of inflammatory cells in the vicinity of the implant, while the highest magnification photomicrographs 400X clearly differentiate the different types of inflammatory cells involved (i.e., neutrophils, macrophages and giant cells). On day 3, composites without dexamethasone produced an intense inflammatory response characterized by a large number of polymorphonuclear leukocytes (PMNs or neutrophils) surrounding the implant and in some sections extending into the skeletal muscle and the adjoining connective tissue (Figures 2A, 2B, and 2C). An edematous reaction in the connective tissue was also observed around these implants, which is one of the first responses of the body to injury and dissipates with time.

On the other hand, dexamethasone loaded composites suppressed the acute inflammatory reaction (Figures 2D, 2E, and 2F) and these tissue sections were comparable to that of normal tissue (Figure 2G). Only a few lymphocytes and plasma cells were present in these tissue sections. Figure 3 shows fibrosis and deposition of collagen in tissue surrounding the implant at each time point (Figures 3A, 3B, and 3C are representative of drug free composites; Figures 3D, 3E, and 3F are representative of drug containing composites and Figure 3G is representative of s.c. tissue without implant). Trichome, which stains collagen blue, was used to evaluate fibrosis. Collagen is the main fibrous structural protein of subcutaneous connective tissue (Figure 3G). Neither fibroblasts nor collagen deposition were observed at day 3 in either the group that received the drug containing implants or the group that received the drug free implants (Figures 3A, 3D). This is as expected, as such deposition is associated with chronic foreign body reaction and not the early acute phase. The chronic response is marked by a dense collagen deposition of active fibroblasts surrounding the implant and leads to the development of a fibrous capsule. Such collagen deposition is not evident at day 3 (acute phase; Figures 3A and 3D) in either the drug free or drug containing composites, but is apparent at the later time points (chronic phase; Figures 3B, 3C, 3E and 3F). The blue staining in figure 3D is due to the collagen normally present in s.c. connective tissue similar to Figure 3G, which is representative of normal tissue. This is in contrast to the deposition of collagen by active fibroblast during the chronic phase of inflammation (refer to Figures 3B, 3C, 3E, 3F for collagen and Figures 4 and 5 for fibrous connective tissue).
Figure 2: Pharmacodynamic changes in representative tissue sections on day 3 taken from the subcutaneous tissue of rats implanted with fast releasing PLGA microspheres/PVA hydrogel composites: (A, B, and C) without dexamethasone; (D, E, F) with dexamethasone; and (G) normal subcutaneous tissue. Hematoxylin & Eosin (H&E) stains inflammation mediating cells basophilic (purple) and s.c. connective tissue eosinophilic (pink). Magnification: A, D, G - 85X; B, E - 200X; C, F - 400X. The asterisk indicates the implant; the light green arrow indicates aggregates of neutrophils; and the black arrow indicates lymphocytes. The bar in each figure measures 100 µm.
Figure 3: Pharmacodynamic changes in representative tissue sections from the subcutaneous tissue of rats implanted with fast releasing PLGA microspheres/PVA hydrogel composites: (A, B, C) without dexamethasone; (D, E, F) with dexamethasone; (G) normal subcutaneous tissue. Magnification: 400X. A, D - day 3; B, E - day 8 and C, F - day 30. Trichome stains collagenous fibrous connective tissue blue. The asterisk indicates the implant. The bar in each figure measures 100 µm.
Figure 4 shows tissue sections taken from the vicinity of the implants on day 8 after implantation (Figures 4A, 4B, and 4C are representative of drug free implants and Figures 4D, 4E, and 4F are representative of drug containing composites). By day 8, a chronic inflammatory tissue reaction was observed in the vicinity of the composites without dexamethasone, marked by the presence of dense fibrotic connective tissue as well as a large number of lymphocytes, neutrophils and macrophages (Figures 4A, 4B, 4C). Neo-vascularization was also observed by this time point in the drug free composites, which is evident by the presence of thin-walled blood vessels in the surrounding fibrous tissue (Figures 4B and 4C). On the other hand, neovascularization was not observed in the tissue surrounding the drug containing composites at day 8 (Figures 4E and 4F). Figure 3B shows that the fibroblasts have started to deposit collagen in the connective tissue surrounding the drug-free composites signifying the start of a granulomatous reaction. However, no such response was visible in the tissue surrounding the dexamethasone containing composites (Figure 3E) at day 8. The absence of fibrosis in these tissue sections is in agreement with previous reports that dexamethasone impairs wound healing and cutaneous hypersensitivity reactions. The absence of neo-vascularization around the dexamethasone containing composites indicates suppression of the immunogenic reaction. This indicates that the body fails to recognize the composites as a foreign object. The suppression of acute inflammation by dexamethasone containing composites is consistent with previous investigations on dexamethasone loaded microspheres and composites.4,9,10

Figure 4: Pharmacodynamic changes in representative tissue sections on day 8, taken from the subcutaneous tissue of rats implanted with fast releasing PLGA microspheres/PVA hydrogel composites: (A, B, C) without dexamethasone; and (D, E, F) with dexamethasone. Hematoxylin & Eosin (H&E) stains inflammation mediating cells basophilic (purple) and s.c. connective tissue eosinophilic (pink). Magnification: A, D - 85X; B, E - 200X; C, F - 400X. The asterisk indicates the implant; the dark green arrow – fibrous connective tissue; light blue arrow - aggregates of mixed inflammatory cells; light blue circle – blood vessels; and black arrow indicates lymphocytes. The bar in each figure measures 100 µm.
Figure 5 shows tissue sections taken from the vicinity of the implants on day 30 after implantation (Figures 5A, 5B, and 5C are representative of drug free implants and Figures 5D, 5E, and 5F are representative of drug containing composites). A granulomatous inflammatory reaction was observed in the vicinity of the implants without dexamethasone at day 30, characterized by the presence of macrophages, fibroblasts and multinucleated giant cells, 30-50 µm in diameter (Figures 5A, 5B, and 5C). In comparison, those implants containing dexamethasone show a chronic inflammatory reaction by day 30 as characterized by a dense network of fibrous tissue together with lymphocytes and macrophages (Figures 5D and 5E). A band of fibrous connective tissue with accompanying deposition of collagen has encapsulated these composites, which is the usual reaction of the body to the continuous presence of any non-absorbable foreign material.

![Image of tissue sections](image-url)

The microspheres investigated here, formulated with low molecular weight (5KDa) PLGA 50:50, release dexamethasone within one week in vitro (Figure 1). The in vivo anti-inflammatory effect observed is in agreement with the in vitro release profile. The fast release of dexamethasone from these composites effectively prevented the acute inflammatory response up to day 8. The amount of dexamethasone used to prepare the fast releasing microspheres was the same as that used in the previous study but the release rates were different. The release was over a one month period in the previous study.
The fast release of dexamethasone reported here resulted in a greater suppression of the acute inflammatory phase than observed previously for the composites that release drug over a one month period. This was further confirmed by counting the number of inflammatory cells in the vicinity of the composites (Figure 6). On days 3 and 8 post implantation, dexamethasone containing composites have a substantially lower number of inflammatory cells (similar to that occurring in normal tissue) than those in the vicinity of composites without dexamethasone. There was no significant change in the number of inflammatory cells surrounding the dexamethasone coated implants between days 3 and 8, further confirming that the composites provide protection up to 8 days. The decrease in the number of inflammatory cells in the tissue surrounding the drug free implants is due to the progression from the acute to the chronic inflammatory phase. The number of inflammatory cells in the vicinity of the dexamethasone containing composites on day 30 is higher than that at the previous time points. This demonstrates that a delayed foreign body reaction develops in response to the implant after dexamethasone is completely released from the composites. Whereas, we have previously shown the implants that release drug over a one month period suppress inflammation and fibrous for a one month period.

**Conclusions**

These results indicate that the presence of dexamethasone adequately controlled the acute inflammatory response and once dexamethasone is completely released from the composites, the body recognizes the composites as a foreign material and an immunogenic reaction develops due to the continuous presence of the composite in the body. An initial rapid release of dexamethasone simply delays rather than suppresses inflammation. Therefore, it would appear that a continuous sustained delivery of dexamethasone is necessary throughout the lifetime of the implant and merely suppressing the acute phase of inflammation is insufficient.

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**References:**


