Available online at www.sciencedirect.com
ScienceDirect
Journal of Controlled Release 127 (2008) 137–145
journal of controlled release
www.elsevier.com/locate/jconrel

Evaluation of *in vivo–in vitro* release of dexamethasone from PLGA microspheres

Banu S. Zolnik [1], Diane J. Burgess *

Department of Pharmaceutical Sciences, University of Connecticut, Storrs, CT 06269, United States

Received 11 October 2007; accepted 8 January 2008
Available online 24 January 2008

Abstract

Two poly(lactic-*co*-glycolic acid) (PLGA) microsphere formulations, with different polymer molecular weights were investigated to determine whether an *in vitro* and *in vivo* relationship could be established for dexamethasone release. A USP apparatus 4 was used for *in vitro* testing. The *in vivo* release kinetics and pharmacodynamic effects of dexamethasone were evaluated using a Sprague Dawley rat model. The *in vitro* release from both formulations followed the typical triphasic profile of PLGA microspheres (initial burst release, followed by a lag phase and a secondary zero-order phase). The *in vivo* release profiles differed in that the lag phase was not observed and drug release rates were faster compared to the *in vitro* studies. It is speculated that the lack of lag phase *in vivo* may be a result of different PLGA degradation mechanisms *in vivo* as a consequence of the presence of enzymes as well as other *in vivo* factors such as interstitial fluid volume, and local pH. This may result in degradation of the PLGA microspheres proceeding from the surface inward *in vivo*. Whereas, *in vitro* an "inside out" degradation is thought to occur in some PLGA microsphere systems as a result of the autocatalytic degradation process where build up of acidic oligomeric units can occur within the microspheres. A linear *in vitro–in vivo* relationship was established after normalization of the time required to reach plateau for the *in vitro* and *in vivo* data and the *in vitro* release data were predictive of the *in vivo* release. Inflammation was significantly reduced in the tissue surrounding the dexamethasone microspheres compared to the positive control (empty microspheres) and the number of inflammatory cells was similar to that of normal tissue within one to three days.

Keywords: Drug delivery; *In vivo–in vitro* relationship; PLGA microspheres; Dexamethasone; USP apparatus

1. Introduction

Microsphere systems have been used for localized drug delivery to reduce side effects as well as to improve the therapeutic response at the local site [1,2]. Dexamethasone, a synthetic steroidal anti-inflammatory drug, has been incorporated into microspheres and liposomes to control the inflammatory reaction at the site of implantable devices such as biosensors and metallic stents, respectively [3–5]. If unchecked such devices can result in severe tissue response including acute (one to five days) and chronic inflammation (two to four weeks), as well as fibrosis [6]. In another application, microspheres containing both dexamethasone and bupivacaine have been prepared where dexamethasone acts as a vasoconstrictor to extend the pharmacological activity of bupivacaine [7,8].

In order to evaluate the performance of such microsphere systems, *in vitro* release methods with *in vivo* relevancy must be developed [9]. *In vitro* release data can be used for *in vivo* and *in vitro* correlation (IVIVC) to aid in setting dissolution specifications and to act as a surrogate for bioequivalence studies (for example, in the case of formulation, equipment, process and manufacturing site changes) [10]. A variety of *in vitro* release testing methods (dialysis sac diffusion, reversed dialysis sac, sample-and-separate, and continuous flow) have been used for injectable dispersed systems [11–13]. The dialysis sac method involves suspending microspheres or other dispersed systems in a dialysis sac with a semi-permeable membrane that allows diffusion of the drug, and then drug concentration is

* Corresponding author. Tel.: +1 860 486 3760; fax: +1 860 486 0538.
E-mail address: d.burgess@uconn.edu (D.J. Burgess).
[1] Current address: Nanotechnology Characterization Laboratory, SAIC-Frederick, Inc., National Cancer Institute, Frederick, MD 21702, United States.

0168-3659/$ - see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jconrel.2008.01.004

monitored in the receiver chamber. Disadvantages of this method include: i) potential for microsphere aggregation due to the lack of agitation, and ii) violation of sink conditions may result when drug release from the microspheres is faster than drug diffusion through the membrane [11]. The reversed dialysis sac method, where the dispersed system is placed in the chamber and the dialysis sacs contain only media, has been utilized to overcome the problem of violation of sink conditions that can occur with the traditional dialysis sac method [11]. The sample-and-separate method utilizes USP apparatus 2, and determination of release requires sampling and separation of the microspheres from the media. Disadvantages of this method include aggregation of the microspheres and the potential for loss of microspheres during the separation step leading to erroneous release profiles [13]. The continuous flow method utilizes flow-through cells, where microspheres are contained, and the media is continuously pumped from a reservoir through the microsphere bed [14]. This method has the advantage that sampling and separation are not required, and media volume is easily adjusted. In addition, a modification of this method has been reported where the microspheres are interspersed with glass beads in the flow-through cell to avoid aggregation [13].

Ideally, *in vitro* methods should be selected with the view to attaining a correlation between *in vivo* and *in vitro* release data [9]. To achieve this, researchers have investigated miniaturized methods considering that the volume at the subcutaneous site is low [15,16]. However, such methods can suffer from: violation of sink conditions, due to the small volumes used; and microsphere aggregation, as a result of limited agitation [17]. To overcome these problems the flow-through method has been used. This method avoids aggregation as discussed above. USP apparatus 4 also allows the use of small volumes since media replenishment can be achieved easily and without microsphere loss thus facilitating the maintenance of sink conditions [13]. In addition to the choice of testing method, media conditions may be varied to aid in the development of a relationship between *in vivo* and *in vitro* release data. To this end, the use of co-solvents, the addition of surfactants and enzymes, as well as variation in pH, ionic strength and temperature have been investigated [18–25]. For example, acidic media has been used to mimic drug release from PLGA microspheres *in vivo* [25,26]. The *in vitro* and *in vivo* performance of drug loaded PLGA microspheres has been evaluated [25,27–37]. However, there are a few examples in the literature where a correlation between *in vitro* and *in vivo* release data of single controlled release parenteral formulations has been reported [25,36,37]. Moreover, in order to establish a relationship between *in vitro* and *in vivo* release, more than one formulation must be investigated [38]. In addition, the *in vitro* method should be able to discriminate between formulations with different *in vivo* release characteristics.

In vivo release from controlled release parenterals such as microspheres may be affected by the environment at the site of administration. *In vivo* factors that affect drug release can be classified as, delivery system independent and delivery system dependent. Delivery system independent factors include: barriers to drug diffusion (*e.g.* fluid viscosity, and connective tissue); drug partitioning at the site (for example, uptake into fatty tissue); the fluid volume available at the site; and in the case of intramuscular injection muscle movement may also be an important factor. Delivery system dependent factors are those specific to a particular delivery system and include: enzymatic degradation of susceptible polymers; protein adsorption; phagocytosis as well as any possible inflammatory reaction to the delivery system. For example, the acute phase of inflammation results in edema and an influx of phagocytic cells at the local site. Whereas, the chronic phase of inflammation can lead to fibrosis which in turn results in isolation of the delivery system and a consequent reduction in the fluid volume.

In vitro–in vivo correlation (IVIVC) can be categorized as Levels A, B, C, D and multiple Level C. Level A is a point-to-point correlation over the entire release profile. For Level B, the mean *in vitro* dissolution time is compared to either the mean residence time or the mean *in vivo* dissolution time. Level C is a single point correlation between a dissolution parameter (for example, the amount dissolved at a particular time or the time required for *in vitro* dissolution of a fixed % of the dose) and an *in vivo* parameter (for example, C_{max} or AUC). A multiple Level C correlation is also possible and this involves a correlation at several time points in the release profile. Level D is a rank order correlation (qualitative) [10]. IVIVC can be achieved either by mathematical modeling (*e.g.* time variant non-linear modeling) of the *in vitro* and *in vivo* data [38] or by alteration of the *in vitro* release method to obtain *in vitro* data that mimics the *in vivo* data [17,24]. In order for the *in vitro* method to be used as surrogate for bioequivalence studies, a Level A IVIVC is required.

In the present study, two PLGA formulations with different *in vivo* release characteristics were chosen in order to investigate the relationship between *in vivo* and *in vitro* release. To develop an *in vitro* release method that is able to discriminate between formulations which have different *in vivo* release characteristics, the USP apparatus 4 method was selected since it offers advantages, as discussed above, and this method has been recommended for controlled release microspheres [9]. The *in vivo* pharmacodynamic affect of dexamethasone (i.e. control of inflammation at the site) was also investigated in this study as this may affect the *in vivo* release kinetics.

2. Materials and methods

Poly(D,L, lactic-*co*-glycolic acid) (PLGA) polymers, PLGA Medisorb 50:50 DL 2A and 2.5A (Mw: 13,000 and 28,000, respectively), were gifts from Astra Zeneca. Methylene chloride and tetrahydrofuran (Optima grade) were obtained from Fisher Scientific (Pittsburgh, PA). Dexamethasone, poly(vinyl alcohol) (PVA), carboxymethylcellulose sodium salt and sodium chloride were obtained from Sigma (St. Louis, MO).

2.1. Preparation of microspheres

An oil-in-water emulsion solvent evaporation technique was used for dexamethasone microsphere formulation. 2 g of PLGA was dissolved in 8 mL of methylene chloride, and 200 mg of dexamethasone was dispersed in this solution using a homogenizer at 10,000 rpm for 1 min. This organic phase was added

slowly to 40 mL of a 1% (w/v) aqueous PVA (Av. Mw 30,000–70,000) solution and homogenized at 10,000 rpm for 3 min. This emulsion was added to 500 mL of a 0.1% (w/v) aqueous PVA solution and stirred at 250 rpm under reduced pressure for 6 h at 25 °C. The resulting microspheres were filtered (Durapore Membrane Filter, 0.45 μm, Fisher Scientific, Pittsburgh, PA), washed three times with double distilled deionized sterile water and vacuum dried for 24 h.

2.2. Characterization of microspheres

2.2.1. Particle sizing

An Acusizer (optical particle sizer model 770,Santa Barbara, CA, USA) was used to determine the mean particle diameter and distribution. Microspheres were suspended in 0.1% (w/v) PVA solution in water. A 500-μL portion of this suspension was diluted with 25-mL deionized water prior to detection.

2.2.2. High performance liquid chromatography (HPLC)

The concentration of dexamethasone was determined using HPLC. The HPLC system consisted of a Constametric 4100 pump (Thermoseparation), an automatic sample injector (Bio-Rad) and a UV absorbance detector (Bio-Rad) set at 242 nm. The mobile phase consisted of acetonitrile:water:phosphoric acid (30:70:0.5 (v/v/v)). The analytical column was a Nova-Pak® C_{18} (9 mm × 150 mm, pore size 4 μm) (Millipore Corp, Waters, Milford, MA). The flow rate was set at 1 mL/min. The retention time of dexamethasone was 5 min, and total run time of HPLC analysis was 15 min. The chromatograph was analyzed by PeakSimple Chromatography System (Model 203, software 3.29, SRI instruments, Torrance, CA). This HPLC method is adapted from that of Spangler and Mularz [39].

2.2.3. Drug loading

10 mg of microspheres were dissolved in 10 mL of THF, filtered (Millex-HV, 0.45 μm, Fisher Scientific, Pittsburgh, PA) and analyzed using the HPLC method described above for dexamethasone content. All measurements were conducted in triplicate and the mean values and standard deviations are reported.

2.2.4. Differential scanning calorimeter (DSC)

Samples were analyzed using a TA Instruments 2920 DSC. Samples were heated to 150 °C and cooled to −30 °C at a rate of 5 °C/min. The second cycle was used to determine the glass transition temperature (Tg) for characterization of the microspheres as prepared. Samples were analyzed in aluminum pans with pinhole lids.

2.2.5. Gel permeation chromatography (GPC)

The Mw of the PLGA microspheres was determined using GPC (Waters) with an evaporative light scattering detector (ELSD, Polymer Laboratories PL-ELS 1000). Four Jordi Flash (high-speed) columns were connected in series. The mobile phase was THF with a flow rate of 3 mL/min. The columns were held isothermally at 40 °C. Microspheres (10 mg) were dissolved in 10 mL in THF and filtered (Whatman Puradisc PVDF(Polyvinylidene Fluroide) Syringe Filters with 0.22 μm pore size). All glass equipment (vials and syringes) was used to minimize possible contamination from plastic materials. The polymer solution injection volume was 100 μL. The data collection and analysis were performed using Waters Millennium software. Weight average molecular masses were calculated based on polystyrene standards (1,250,000; 400,000; 200,000; 43,000; 17,600; 6930; 2610; 982; and 472 Da).

2.2.6. In vitro release studies

In vitro release studies were conducted using a modified USP apparatus 4 (Sotax *CE7 smart*, or *CY 7 piston pump*, Sotax, Horsham, PA) with flow-through cells (12 mm diameter) packed with glass beads (1 mm) (to prevent microsphere agglomeration and to achieve laminar flow [13]) in a closed system mode at 37 °C. 45 mg of microspheres were dispersed in the flow-through cells and 250 mL of 0.1 M phosphate buffered saline (PBS) pH 7.4 (prepared according to the USP monograph) with 0.1% (w/v) sodium azide was circulated through a fiberglass filter (0.45 μm). A flow rate of 20 mL/min was used. This flow rate was chosen since this is the standard flow rate for USP apparatus 4 and we have previously reported [1] that the flow rate does not affect the release rate for PLGA microspheres of high molecular weight (such as the formulations reported here) where release is erosion/diffusion controlled.

1.3-mL samples were withdrawn and replenished with 1.3 mL of fresh media. The samples were analyzed by HPLC (as explained above) and any drug degradation was accounted for in the cumulative release data reported. When the drug concentration reached 5% (w/v) of the solubility of dexamethasone, half of the total media volume was replenished. This media replenishment was taken into account in the calculation of the cumulative percent release. All measurements were conducted in triplicate and the mean values and standard deviations are reported.

2.2.7. In vivo release studies

Dexamethasone PLGA microspheres were prepared under aseptic conditions. Microspheres were suspended in a viscosity enhanced diluent (30 mg/mL carboxymethylcellulose sodium salt, 9 mg/mL NaCl and sterile water) [40] in order to prevent any sticking of the microspheres in syringes/needles during injection. Rats were anesthetized in an induction chamber filled with a 4.5% (v/v) mixture of isofluorane in oxygen. The back of each animal, where microspheres were injected, was shaved prior to injection. PLGA Microspheres with Mw 13,000 and 28,000 formulations known herein as formulations 13K and 28K, respectively were injected subcutaneously using 18 gauge needles. Microspheres were administered at 4 mg dexamethasone encapsulated dose per animal for both the 13K and 28K formulations. Five rats were sacrificed at each of the following time intervals: 1, 3, 5, 8, 11, 12, 14 days for 13K formulations and 1, 3, 5, 8, 11, 12, 14, 17, 22, 26, 30 days for 28K formulations and 1, 3, 5, 8, 14, 17, 22, and 26 days for control. Subcutaneous tissues including the microspheres were removed from the injection site. The microspheres were separated from

the tissue samples. The drug content of the degraded microspheres *in vivo* was determined according to the HPLC method described above. The released drug amount was calculated by subtraction of the drug content in the degraded microspheres from the total drug loaded into the microspheres. All animal studies were conducted at the University of Connecticut in accordance with Institutional Animal Care and Use Committee guidelines using an approved protocol (number E2901201).

2.2.8. Pharmacodynamic study of dexamethasone microspheres

Tissue samples from the various rat studies described above were fixed in 10% formalin and sections were immersed in paraffin and cut using a microtome. The pharmacodynamic effect of the dexamethasone microspheres was determined via histological examination using hematoxylin and eosin (H&E) staining. Empty microspheres (28K) were used as a positive control and untreated subcutaneous tissue samples were used as a negative control. Photomicrographs of the histology slides were taken and digitally stored using an Olympus microscope (model Ax70, Olympus America, Melville, NY) at a 10× magnification. Visual counting of the purple stained nuclei of the inflammatory cells was performed to reduce error that might occur as a result of the software counting two or more cells in close proximity as one. The magnified images of the photomicrographs were quantified visually using Microsoft® Power Point® 2002 program which allowed placement of 42 uniformly sized boxes for ease of counting. This method of evaluating the number of inflammatory cells has been previously reported [41,42].

3. Results

3.1. Polymer selection and microsphere characterization

In order to investigate whether an *in vitro–in vivo* relationship could be established, two microsphere formulations were prepared and characterized for molecular weight, glass transition temperature (Tg), and drug loading (Table 1). Although the molecular weights were different (13,000 and 28,000) the Tg values were similar (44 °C and 45.6 °C) for the formulations known herein as formulations 13K and 28K, respectively. The particle size was similar for both formulations (number-weight average particle size 20 μm). The drug loading for the 13K and 28K formulations were 7.64±0.28 and 8.25±0.25%, respectively and therefore, should not affect the release characteristics. These formulations were selected since they had similar physical characteristics, but were expected to have different release rates since they were prepared using different molecular weight PLGA and therefore could be used to test the discriminatory ability of the *in vitro* release method.

Table 1
PLGA microsphere formulation and physico-chemical characterization

	Ratio of LA:GA	Mw (kDa)	Tg (°C)	Drug loading (%)
13K	50:50	13	44	7.64±0.28
28K	50:50	28	45.6	8.25±0.25

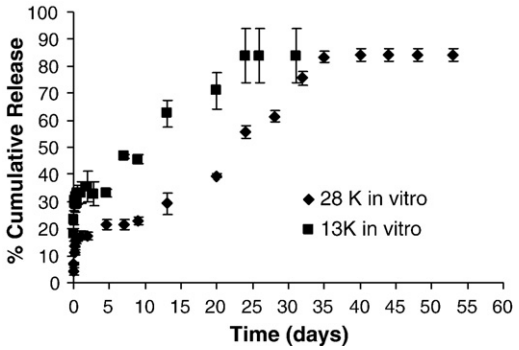

Fig. 1. Dexamethasone release from PLGA microspheres at 37 °C in PBS buffer (pH 7.4) using USP apparatus 4: (◆) 28K formulation, and (■) 13K formulation (mean±SD; $n=3$).

3.2. In vitro studies

Dexamethasone release from both formulations 13K and 28K showed a triphasic release profile, with an initial burst release, followed by a lag phase and then a secondary zero-order release phase. Such triphasic release profiles are typical of PLGA microspheres [43]. The 13K and 28K formulations exhibited an initial burst phase of approximately 30% and 20% with a lag phase until days 5 and 9, respectively. The observed decrease in the initial burst with increase in polymer Mw may be a result of decreased diffusion pathway in the higher Mw microspheres (28K). We have previously reported similar trends for other PLGA microsphere formulations of differing MW (5 kDa, 25 kDa, 70 kDa) [18]. The longer lag phase with the 28K formulation compared to the 13K formulation is considered to be due to the higher Mw which requires more time for degradation to take place (Fig. 1).

3.3. In vivo studies

The *in vivo* release profiles showed an initial burst release of approximately 48 and 15% for formulations 13K and 28K, respectively followed by an apparent zero-order release phase. The time to reach plateau was 8 and 12 days for formulations 13K and 28K, respectively (Fig. 2).

3.4. Evaluation of relationship between in vitro and in vivo release

It has been recommended that IVIVC should be conducted on formulations with the same *in vitro* mechanism of release [44]. When more than one mechanism of release occurs for a single formulation, each release phase should be correlated separately [38,44]. It has also been recommended that burst release from systems such as microspheres should be investigated separately [9,45]. The secondary zero-order phase of both formulations was used to establish a relationship between the *in vitro* and *in vivo* release data (IVIVR). As shown in Figs. 1 and 2, the time to reach a plateau was different for the *in vitro* and *in vivo* data. Therefore, the time required to reach plateau was normalized for the *in vitro* and *in vivo* data for both formulations. Normalization was achieved by plotting *in vitro* time on the *x*-axis and *in vivo* time on

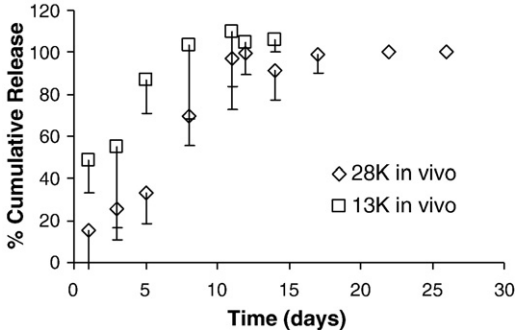

Fig. 2. Dexamethasone release obtained from PLGA microspheres *in vivo*. Sprague Dawley male rat model, $n=5$ for each data point): (□) 13K formulation, and (◊) 28K formulation.

the *y*-axis for both formulations. A linear relationship between the *in vitro* time and *in vivo* time was obtained where the time scaling parameters (slope) were 0.4984 and 0.5008 and the time shifting parameters (intercept) were 1.4276 and 3.8255 for formulations 13K and 28K, respectively. Time shifting/scaling allowed the *in vitro* data to be on the same time scale as the *in vivo* data. This, in turn made it possible to establish a relationship between *in vitro* and *in vivo* release data by plotting the *in vitro* release data on the *x*-axis and the *in vivo* release data on the *y*-axis, while time points (*z*-axis, not shown) were on the same scale. Consequently, a linear relationship between the *in vitro* and *in vivo* data was established for both the 13K and 28K formulations (Fig. 3).

3.5. Prediction of in vivo release based on in vitro and in vivo relationship

The *in vitro* and *in vivo* relationship established for the 28K formulation was utilized to predict *in vivo* release of dexamethasone from the 13K formulation. The relationship between *in vitro* and *in vivo* release of the 28K formulation was obtained via linear regression (circles in Fig. 3) as follows:

$$\%\ \text{cumulative } \textit{in vivo} \text{ release} = 1.6381(\%\ \text{cumulative } \textit{in vitro} \text{ release}) - 24.457$$

with a correlation coefficient of 0.9862. This relationship was used to predict % cumulative *in vivo* release of the 13K formulation using the experimental *in vitro* data obtained for the 13K formulation. Time scaling was obtained from the time scaling/shifting parameter of the 28K formulation since time scale differences between *in vivo* and *in vitro* release were observed. Experimental and predicted percent cumulative release versus time *in vivo* data were plotted (Fig. 4). A model independent approach using a difference factor (f_1) and a similarity factor (f_2) was utilized to compare experimental and predicted percent cumulative *in vivo* data. The difference factor was defined in the *FDA Guidance for Industry: Dissolution Testing of Immediate Release Solid Oral Dosage Forms* [46]) as "calculation of the percent difference between the two curves at each time point and is a measurement of the relative error between the two curves":

$$f_1 = \left\{ \frac{\sum_{t=1}^{n} |R_t - T_t|}{\sum_{t=1}^{n} R_t} \right\} * 100.$$

The similarity factor is a measurement of the similarity in the percent release between the two curves and is defined as the "logarithmic reciprocal square root transformation of the sum of the squared error" in the FDA guidance document [46].

$$f_2 = 50 * \log\left\{ \left[1 + (1/n) \sum_{t=1}^{n} (R_t - T_t)^2 \right]^{-0.5} * 100 \right\}$$

where n is the number of time points, R_t is the experimental data at time t, and T_t is the predicted *in vivo* percent cumulative release at time t.

The experimental and predicted *in vivo* cumulative release data from days 1, 3 and 8 were used since a model independent approach requires the use of the same time points. The difference factor (f_1) and similarity factor (f_2) were calculated as 16 and 58, respectively. It is noted in the FDA guidance document that generally, f_1 values of 15 and f_2 values greater than 50 suggest equivalence of the two profiles.

3.6. Histology studies

Figs. 5 and 6 are representative micrographs of histology sections taken at different times representative of acute and chronic inflammation for the 13K and 28K formulations together with a positive control (empty PLGA microspheres,

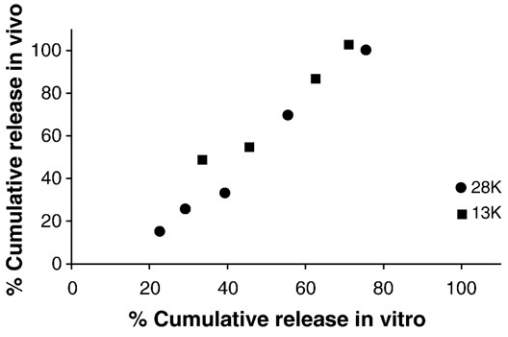

Fig. 3. An *in vitro–in vivo* relationship for dexamethasone release from PLGA microspheres: % released *in vivo* was plotted against % released *in vitro* for formulations: (●) 28K, and (■) 13K.

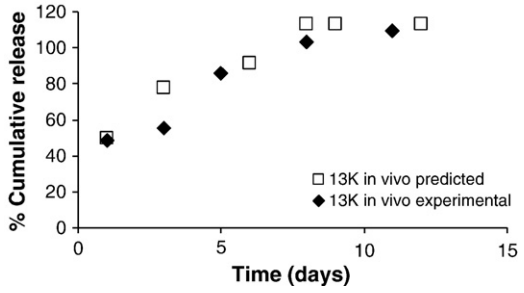

Fig. 4. Experimental (*in vivo*) and predicted % cumulative release data of 13K formulation. (■) *in vivo* experimental, (□) predicted.

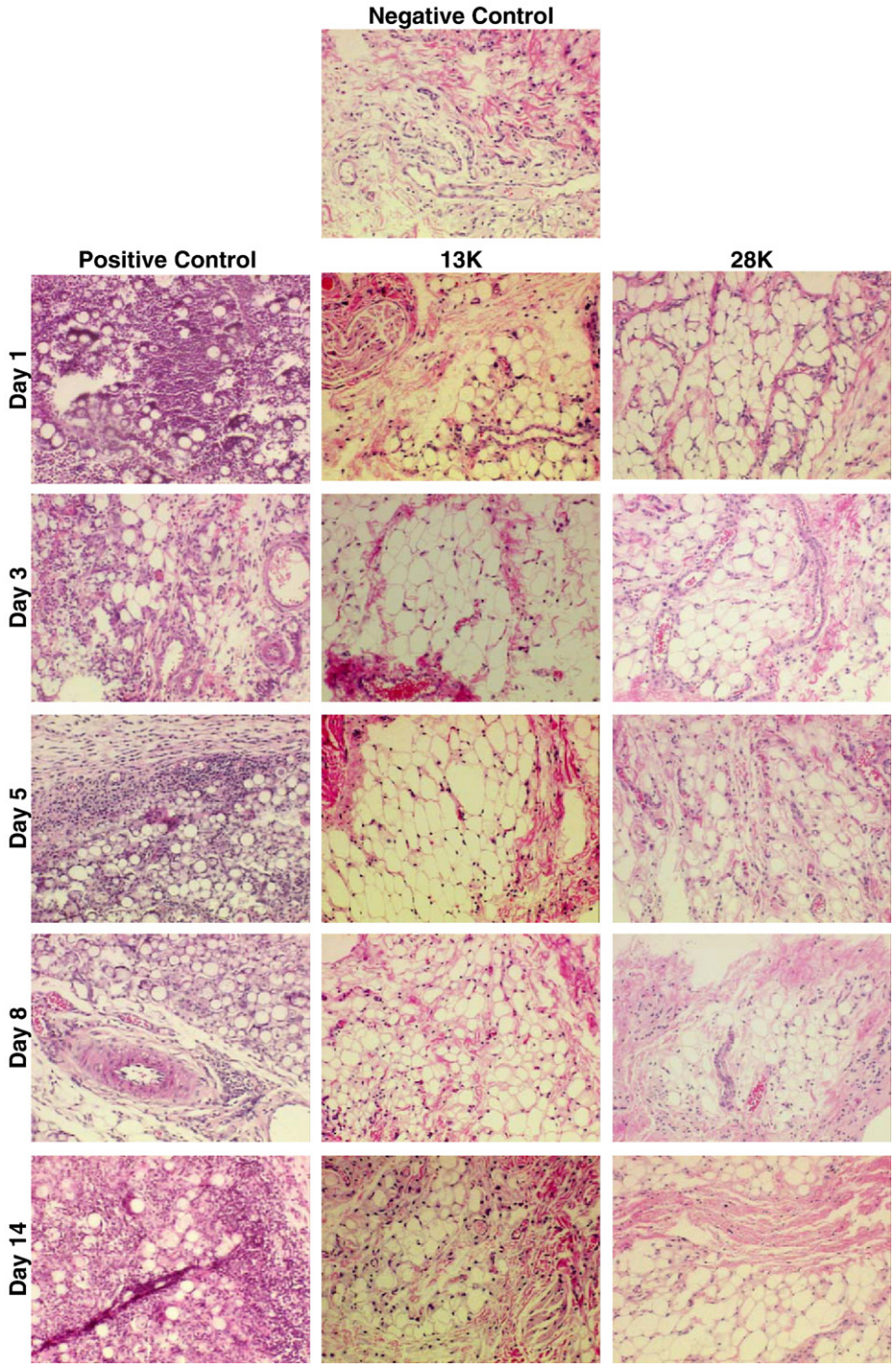

Fig. 5. Pharmacodynamic changes in representative tissue sections on days 1, 3, 5, 8 and 14. Sprague Dawley rats were subcutaneously injected with 13K and 28K formulations. The rats were sacrificed and the implant site was dissected, processed and stained using hematoxylin and eosin (H&E). Inflammation is evident in the positive control (blank PLGA microspheres — left column) while normal tissue can be seen in untreated tissue sections (negative control — top panel). Minimal inflammation is seen in those animals treated with 13K formulation (middle column) and 28K formulation (right column).

28K formulation) and a negative control (untreated tissue section). Hematoxylin–eosin (H&E) stains the nuclear material of the inflammatory cells purple, while the normal tissue remains pink. The inflammatory cells were predominately monocytes and with a small percentage of polymorphonuclear leukocytes (PMNs). The change in the number of inflammatory cells with time is shown numerically in Table 2. The positive control shows a severe inflammatory response due to the presence of the PLGA microspheres and the tissue trauma resulting from the needle injection. The inflammatory response to the PLGA microspheres may be partially due to PLGA degradation resulting in release of acidic oligomeric units in the immediate vicinity of the microspheres. The typical inflammatory response of acute followed by chronic inflammation is clearly seen in the positive

control with the numbers of the inflammatory cells peaking at day 1 (approximately 2300) and gradually decreasing to approximately 950 by day 28 (Table 2). The inflammatory response to the formulation 13K is much less severe than that to the positive control, with an initial number of inflammatory cells (560) which is only slightly greater than the negative control (456). In addition, the 13K formulation is able to control inflammation to values below that of the negative control for the remainder of 14 day study period. The 28K formulation also showed a less severe inflammatory response compared to the positive control. The number of inflammatory cells at day 1 was higher (753) than the negative control (456) but reduced over the study period and by day 5 the number of inflammatory cells was comparable to the negative control.

Table 2
Effect of dexamethasone on the inflammation mediation cell population at the vicinity of the PLGA microspheres

Formulation	Control	13K	28K
1	2300**	560	753
3	1297	208	620
5	1150	282	455
8	701	287	448
14	1766**	397	377
17	794	NA	153
22	754	NA	426
26	950**	NA	469

Positive control, blank microspheres (no dexamethasone); 13K formulation; and 28K formulation. The numbers are counted from 42 boxes across the entire tissue section. **The numbers are estimated from the representative boxes across the tissue section since some parts of the section could not be counted due to the density of inflammatory cells causing overlap and therefore, the number maybe slightly underestimated. NA: not applicable.

4. Discussion

The major differences between the *in vivo* and *in vitro* release from the 13K and 28K dexamethasone microsphere formulations are the lack of lag phase and the faster release kinetics for the apparent zero-order release phase of the *in vivo* data. In addition, the *in vitro* and *in vivo* burst phases appeared not to correlate, however the number of *in vivo* data points may well be sub-optimal to fully characterize the burst release. The faster release kinetics *in vivo* may be a result of enhanced polymer degradation due to the presence of enzymes as well as other *in vivo* factors such as interstitial fluid volume, and local pH. Accelerated enzymatic PLGA degradation *in vivo* has also been reported by other researchers [17,47]. For example, Clark et al. have reported faster *in vivo* compared to *in vitro* release kinetics for PLGA microspheres [17]. However, there is some controversy in the literature with respect to role of enzymes in PLGA degradation [48].

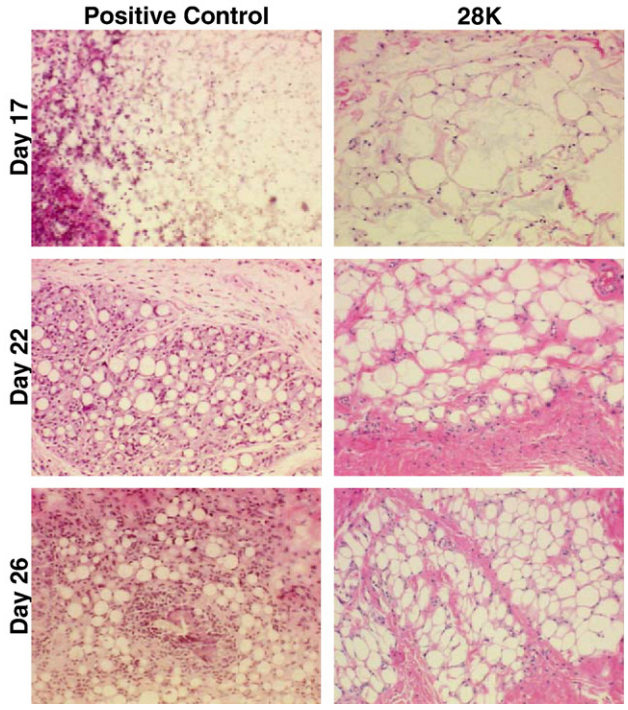

Fig. 6. Pharmacodynamic changes in representative tissue sections on days 17, 22 and 26. Sprague Dawley rats were subcutaneously injected with 28K formulations. The rats were sacrificed and the implant site was dissected, processed and stained using hematoxylin and eosin (H&E). Inflammation is evident in the positive control (blank PLGA microspheres — left column) while normal tissue can be seen in untreated tissue sections (negative control — Fig. 5 top panel). Minimal inflammation is seen in those animals treated with 28K formulation.

The release kinetics from the 13K formulation were faster, both *in vivo* and *in vitro*, compared to the 28K formulation due to the rapid degradation of the low Mw PLGA. The lack of lag phase *in vivo* for both the 13K and 28K formulations may be due to a different polymer erosion mechanism compared to *in vitro*. It is considered that *in vitro* degradation of PLGA microspheres occurs as a result of autocatalysis due to the build up of acidic oligomers within the microspheres and this has been reported to result in "inside out" erosion [49,50]. Whereas, *in vivo* enzymatic degradation is more likely to occur from the surface in and therefore, a lag phase might not be expected to occur. In a previous publication from our laboratory, it has been reported that release kinetics of vascular endothelial growth factor (VEGF) from PLGA microspheres was slower *in vivo* compared to *in vitro* [51]. A possible explanation for this is the severe inflammatory reaction that occurred in the presence of these microspheres. This is considered to be a result of both tissue reaction to the PLGA microspheres as well as to the foreign protein (human VEGF was used in a rat model). In the present study, dexamethasone release from the microspheres reduced the number of inflammatory cells down to that of normal tissue (negative control) by day 3 for the 13K formulation and by day 5 for the 28K formulation. The initial level of inflammation for both formulations was significantly less than the positive control of blank microspheres. The number of inflammatory cells at day 1 was less for the 13K formulation compared to the 28K formulation and this may be attributed to higher burst release of dexamethasone from the 13K formulation (48%) compared to the 28K formulation (15%).

A linear *in vitro* and *in vivo* relationship was established for formulations 13K and 28K after normalization of the time scales of the *in vitro* and *in vivo* data. A bio-relevant *in vitro* release

method (USP 4 method) and Sprague Dawley rat model were utilized to obtain this relationship. *In vitro* and *in vivo* correlation (IVIVC) for microspheres and implants have been previously reported, however these correlations were made on single formulations or different types of correlations were established on formulations with different release characteristics (*e.g.* diffusion and erosion controlled) [36,37].

The current study is one of a few reports showing the same *in vitro* and *in vivo* relationship for more than one controlled release parenteral formulation [52,53]. The *in vivo* release profile of the 13K formulation was predicted using IVIVR and time scaling/shifting data obtained using the 28K formulation *in vitro* and *in vivo* data. The equivalence of the experimental and predicted *in vivo* data was assessed using a model independent approach. The difference and similarity factors indicated that the percent cumulative release *in vivo* prediction of the 13K formulation was comparable to that of the experimental *in vivo* data based on the IVIVR established for the 28K formulation. A possible explanation for the calculated f_1 and f_2 values, 16 and 58, respectively being close to the threshold values of 15 and 50, respectively is that only three time points were used since this model independent approach requires the use of the same time points. When the complete profiles of both curves are compared as shown in Fig. 4, it is apparent that both predicted and experimental curves are in agreement. In addition, it should be noted that the USP apparatus 4 method was able to discriminate between two PLGA microsphere formulations with different *in vivo* release characteristics.

5. Conclusions

The *in vitro* and *in vivo* release characteristics of dexamethasone from two PLGA microsphere formulations (13K and 28K) were investigated to determine whether an IVIVR could be established. *In vitro* release of dexamethasone from both formulations exhibited a typical triphasic profile with an initial burst release, a lag phase, followed by an apparent zero-order release phase. A modified USP apparatus 4 method that eliminated problems of microsphere aggregation, which typically occur with other *in vitro* release methods, was used for *in vitro* release testing. Such a method is important for the development of an *in vitro–in vivo* relationship since the microspheres are not aggregated *in vivo* as is apparent from the histology data presented. *In vivo*, these microspheres exhibited a biphasic release profile with an initial burst followed by an apparent zero-order release phase. The lack of a lag phase *in vivo* is speculated to be a result of enzymatic degradation which leads to “outside in” erosion compared to the typical “inside out” erosion that is considered to occur *in vitro*. In addition, drug release was faster *in vivo* compared to *in vitro*. It is speculated that this may be a result of enzymatic degradation as well as the lack of a severe inflammatory response to these microspheres. Histology data revealed that both formulations were able to control inflammation at the local site. The 13K formulation was superior to 28K formulation with respect to the initial inflammatory response and this is considered to be a result of the higher burst release from the 13K formulation. A linear relationship between the *in vivo* and *in vitro* data post burst phase was established after normalization of the time required to reach a plateau.

Acknowledgments

The authors are thankful for the support from the US Army Medical Research and Material Command (W81XWH-04-1-0779 and W81XWH-05-1-0539); Office of Testing and Research CDER, FDA; CPPR-NSF; and Sotax Corp. The awarding of a USP fellowship to BSZ is greatly appreciated. The authors wish to thank Dr. David Young, AGI Therapeutics, Inc. for useful discussions.

References

[1] R. Baker, Introduction, in: R. Baker (Ed.), Controlled Release of Biologically Active Agents, vol. XV, Wiley-Interscience Publication, New York, 1987, pp. 1–18.
[2] D.J. Burgess, A.J. Hickey, Microspheres: design and manufacturing, in: D.J. Burgess (Ed.), Injectable Dispersed Systems: Formulation, Processing and Performance, vol. 149, Taylor & Francis, Boca Raton, 2005, pp. 305–353.
[3] T. Hickey, D. Kreutzer, D.J. Burgess, F. Moussy, *In vivo* evaluation of a dexamethasone/PLGA microsphere system designed to suppress the inflammatory tissue response to implantable medical devices, J. Biomed. Mater. Res. 61 (2002) 180–187.
[4] P. Kallinteri, S.G. Antimisiaris, D. Karnabatidis, C. Kalogeropoulou, I. Tsota, D. Siablis, Dexamethasone incorporating liposomes: an *in vitro* study of their applicability as a slow releasing delivery system of dexamethasone from covered metallic stents, Biomaterials 23 (2002) 4819–4826.
[5] S.D. Patil, F. Papadimitrakopoulos, D.J. Burgess, Dexamethasone-loaded poly(lactic-*co*-glycolic) acid microspheres/poly(vinyl alcohol) hydrogel composite coatings for inflammation control, Diabetes Technol. Ther. 6 (2004) 887–897.
[6] G. Majno, I. Joris, Chapter 8: Introduction to Inflammation, Oxford University Press, New York, 2004.
[7] J. Castillo, J. Curley, J. Hotz, M. Uezono, J. Tigner, M. Chasin, R. Wilder, R. Langer, C. Berde, Glucocorticoids prolong rat sciatic nerve blockade *in vivo* from bupivacaine microspheres, Anesthesiology 85 (Nov 1996) 1157–1166.
[8] J. Curley, J. Castillo, J. Hotz, M. Uezono, S. Hernandez, J.O. Lim, J. Tigner, M. Chasin, R. Langer, C. Berde, Prolonged regional nerve blockade. Injectable biodegradable bupivacaine/polyester microspheres, Anesthesiology 84 (Jun 1996) 1401–1410.
[9] D.J. Burgess, D.J.A. Crommelin, A.J. Hussain, M.-L. Chen, EUFEPS workshop report, assuring quality and performance of sustained and controlled release parenterals, Eur. J. Pharm. Sci. 21 (2004) 679–690.
[10] FDA Guidance for Industry: Extended Release Oral Dosage Forms: Development, Evaluation, and Application of *In Vitro*/*In Vivo* Correlations, Septemper, 1997.
[11] N. Chidambaram, D.J. Burgess, A novel *in vitro* release method for submicron-sized dispersed systems, AAPS PharmSci 1 (1999) Article 11.
[12] J.W. Kostanski, P.P. DeLuca, A novel *in vitro* release technique for peptide containing biodegradable microspheres, AAPS PharmSciTech 1 (2000) E4.
[13] B.S. Zolnik, J.L. Raton, D.J. Burgess, Application of USP Apparatus 4 and in situ fiber optic analysis to microsphere release testing, Dissolution Technologies 12 (2005) 11–14.
[14] USP23NF18, The United States Pharmacopeia, The National Formulary, in: (Ed.), (1995).
[15] Y.-Y. Yang, H.-H. Chia, T.-S. Chung, Effect of preparation temperature on the characteristics and release profiles of PLGA microspheres containing protein fabricated by double-emulsion solvent extraction/evaporation method, J. Control. Release 69 (2000) 81–96.
[16] M.-K. Yeh, J.-L. Chen, C. C-H, *In vivo* and *in vitro* characteristics for insulin-loaded PLA microparticles prepared by w/o/w solvent evaporation

method with electrolytes in the continuous phase, J. Microencapsul 21 (2004) 719–728.
[17] B.C. Clark, P.A. Dickinson, I.T. Pyrah, Case study: *in vitro*/*in vivo* release from injectable microspheres, in: D.J. Burgess (Ed.), Injectable Dispersed Systems: Formulation, Processing and Performance, vol. 149, Taylor & Francis, Boca Raton, 2005, pp. 543–570.
[18] B.S. Zolnik, P.E. Leary, D.J. Burgess, Elevated temperature accelerated release testing of PLGA microspheres, J. Control. Release 112 (2006) 293–300.
[19] S. Li, A. Girard, H. Garreau, M. Vert, Enzymic degradation of polylactide stereocopolymers with predominant D-lactyl contents, Polym. Degrad. Stab. 71 (2000) 61–67.
[20] Y. Aso, S. Yoshioka, A. Li Wan Po, T. Terao, Effect of temperature on mechanisms of drug release and matrix degradation of poly(-lactide) microspheres, J. Control. Release 31 (1994) 33–39.
[21] K. Makino, H. Ohshima, T. Kondo, Mechanism of hydrolytic degradation of poly(lactide) microcapsules: effects of pH, ionic strength and buffer concentration, J. Microencapsul 3 (1986) 203–212.
[22] C.M. Agrawal, D. Huang, J.P. Schmitz, K.A. Athanasiou, Elevated temperature degradation of a 50:50 copolymer of PLA–PGA, Tissue Eng. 3 (1997) 345–352.
[23] M. Hakkarainen, A.-C. Albertsson, S. Karlsson, Weight losses and molecular weight changes correlated with the evolution of hydroxyacids in simulated *in vivo* degradation of homo- and copolymers of PLA and PGA, Polym. Degrad. Stab. 52 (1996) 283–291.
[24] G. Jiang, B.H. Woo, F. Kang, J. Singh, P.P. DeLuca, Assessment of protein release kinetics, stability and protein polymer interaction of lysozyme encapsulated poly(D,L-lactide-*co*-glycolide) microspheres, J. Control. Release 79 (2002) 137–145.
[25] M.J. Blanco-Prieto, K. Besseghir, P. Orsolini, F. Heimgartner, C. Deuschel, H.P. Merkle, H. Nam-Tran, B. Gander, Importance of the test medium for the release kinetics of a somatostatin analogue from poly(-lactide-*co*-glycolide) microspheres, Int. J. Pharm. 184 (1999) 243–250.
[26] T. Heya, H. Okada, Y. Ogawa, H. Toguchi, *In vitro* and *in vivo* evaluation of thyrotrophin releasing hormone release from copoly(DL-lactic/glycolic acid) microspheres, J. Pharm. Sci. 83 (1994) 636–640.
[27] T. Urata, K. Arimori, H. Nakano, Modification of release rates of cyclosporin A from polyl(L-lactic acid) microspheres by fatty acid esters and *in-vivo* evaluation of the microspheres, J. Control. Release 58 (1999) 133–141.
[28] M.J. Blanco-Prieto, K. Besseghir, O. Zerbe, D. Andris, P. Orsolini, F. Heimgartner, H.P. Merkle, B. Gander, *In vitro* and *in vivo* evaluation of a somatostatin analogue released from PLGA microspheres, J. Control. Release 67 (2000) 19–28.
[29] X.M. Lam, E.T. Duenas, A.L. Daugherty, N. Levin, J.L. Cleland, Sustained release of recombinant human insulin-like growth factor-I for treatment of diabetes, J. Control. Release 67 (2000) 281–292.
[30] H.S. Yoo, K.H. Lee, J.E. Oh, T.G. Park, *In vitro* and *in vivo* anti-tumor activities of nanoparticles based on doxorubicin-PLGA conjugates, J. Control. Release 68 (2000) 419–431.
[31] M.J. Blanco-Prieto, M.A. Campanero, K. Besseghir, F. Heimgatner, B. Gander, Importance of single or blended polymer types for controlled *in vitro* release and plasma levels of a somatostatin analogue entrapped in PLA/PLGA microspheres, J. Control. Release 96 (2004) 437–448.
[32] K.D. Hinds, K.M. Campbell, K.M. Holland, D.H. Lewis, C.A. Piche, P.G. Schmidt, PEGylated insulin in PLGA microparticles. *In vivo* and *in vitro* analysis, J. Control. Release 104 (2005) 447–460.
[33] W.H. Liu, J.L. Song, K. Liu, D.F. Chu, Y.X. Li, Preparation and *in vitro* and *in vivo* release studies of Huperzine A loaded microspheres for the treatment of Alzheimer's disease, J. Control. Release 107 (2005) 417–427.
[34] L. Feng, X.R. Qi, X.J. Zhou, Y. Maitani, S.C. Wang, Y. Jiang, T. Nagai, Pharmaceutical and immunological evaluation of a single-dose hepatitis B vaccine using PLGA microspheres, J. Control. Release 112 (2006) 35–42.
[35] R.C. Mundargi, S. Srirangarajan, S.A. Agnihotri, S.A. Patil, S. Ravindra, S.B. Setty, T.M. Aminabhavi, Development and evaluation of novel biodegradable microspheres based on poly(D,L-lactide-*co*-glycolide) and poly(epsilon-caprolactone) for controlled delivery of doxycycline in the treatment of human periodontal pocket: *in vitro* and *in vivo* studies, J. Control. Release 119 (2007) 59–68.
[36] G. Schliecker, C. Schmidt, S. Fuchs, A. Ehinger, J. Sandow, T. Kissel, *In vitro* and *in vivo* correlation of buserelin release from biodegradable implants using statistical moment analysis, J. Control. Release 94 (2004) 25–37.
[37] H. Zhong, Y. Deng, X. Wang, B. Yang, Multivesicular liposome formulation for the sustained delivery of breviscapine, Int. J. Pharm. 301 (2005) 15–24.
[38] D. Young, C. Farrell, T. Shepard, *In vitro*/*in vivo* correlation for modified release injectable drug delivery systems, in: D.J. Burgess (Ed.), Injectable Dispersed Systems: Formulation, Processing and Performance, vol. 149, Taylor & Francis, Boca Raton, 2005, pp. 159–176.
[39] M. Spangler, E. Mularz, A validated, stability-indicating method for the assay of dexamethasone in drug substance and drug product analyses, and the assay of preservatives in drug product, Chromatographia 54 (2001) 329–334.
[40] Nutropin Prescription Information http://www.gene.com/gene/products/information/opportunistic/nutropin/insert.jsp.
[41] S.D. Patil, F. Papadmitrakopoulos, D.J. Burgess, Concurrent delivery of dexamethasone and VEGF for localized inflammation control and angiogenesis, J. Control. Release 117 (2007) 68–79.
[42] U. Bhardwaj, R. Sura, F. Papadimitrakopoulos, D.J. Burgess, Fast-releasing PLGA microsphere/PVA hydrogel composites for inflammation suppression at the site of implantable devices, J. Diabetes Sci. Technol. 1 (2007) 8–17.
[43] N. Faisant, J. Siepmann, J.P. Benoit, PLGA-based microparticles: elucidation of mechanisms and a new, simple mathematical model quantifying drug release, Eur. J. Pharm. Sci. 15 (2002) 355–366.
[44] B.S. Zolnik, D.J. Burgess, *In vitro–in vivo* correlation on parenteral dosage forms, in: R. Krishna, L. Yu (Eds.), Biopharmaceutics Applications in Drug Development, Springer Science+Business Media, Inc., New York, NY., November 2007, pp. 337–358.
[45] D.J. Burgess, A.S. Hussain, T.S. Ingallinera, M.-L. Chen, Assuring quality and performance of sustained and controlled release parenterals: workshop report, AAPS PharmSci 4 (2002) Article 7.
[46] FDA Guidance for Industry: Dissolution Testing of Immediate Release Solid Oral Dosage Forms, , August, 1997.
[47] M. Sandor, J. Harris, E. Mathiowitz, A novel polyethylene depot device for the study of PLGA and P(FASA) microspheres *in vitro* and *in vivo*, Biomaterials 23 (2002) 4413–4423.
[48] F. Alexis, Factors affecting the degradation and drug-release mechanism of poly(lactic acid) and poly (lactic acid)-*co*-(glycolic acid), Polym. Int. 54 (2005) 36–46.
[49] K. Fu, D.W. Pack, A.M. Klibanov, R. Langer, Visual evidence of acidic environment within degrading poly(lactic-*co*-glycolic acid) (PLGA) microspheres, Pharm. Res. 17 (2000) 100–106.
[50] I. Galeska, T.-K. Kim, S. Patil, U. Bhardwaj, D. Chatttopadhyay, F. Papadimitrakopoulos, D.J. Burgess, Controlled release of dexamethasone from PLGA microspheres embedded within polyacid-containing PVA hydrogels, AAPS J. 7 (2005) Article 22.
[51] T.-K. Kim, D.J. Burgess, Pharmacokinetic characterization of ^{14}C-vascular endothelial growth factor controlled release microspheres using a rat model, J. Pharm. Pharmacol. 54 (2002) 897–905.
[52] C.M. Negrin, A. Delgado, M. Llabres, C. Evora, *In vivo-in vitro* study of biodegradable methadone delivery systems, Biomaterials 22 (2001) 563–570.
[53] R. van Dijkhuizen-Radersma, S.J. Wright, L.M. Taylor, B.A. John, K. de Groot, J.M. Bezemer, *In vitro*/*in vivo* correlation for ^{14}C-methylated lysozyme release from poly(ether-ester) microspheres, Pharm. Res. 21 (2004).