SUSTAINED RELEASE OF A SOFT GLUCOCORTICOID LOTEPREDNOL ETABONATE USING PLA MICROSPHERES FOR THE PREVENTION OF ISLET TRANSPLANTATION REJECTION

By

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LIST OF ABBREVIATIONS

AE	Δ^1-cortienic acid etabonate
ANOVA	Analysis of variance
BAD	Proapoptotic Bcl-2-associated death promoter protein
CA	Δ^1-cortienic acid
Ca^{2+}	Calcium ion
cmc	Critical micelle concentration
CO_2	Carbon dioxide
DCM	Dicholoromethane
DDS	Drug delivery system
DDW	Double distilled water
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dQ/dt	Rate of drug released
D_T	Total amount of drug added to the formulation
D_W	Amount of drug found in the wash solution
EDTA	Ethylenediaminetetraacetic acid
EE	Encapsulation efficiency
FBS	Fetal bovine serum
G	Glucocorticoid
GA	Glycolic acid
GR	Glucocorticoid receptor
H_2O	Water
HCl	Hydrochloric acid

HFIP	Hexafluoroisopropanol
HPLC	High performance liquid chromatography with UV-VIS (Ultraviolet – visible) detection
HSP90	Heat-shock protein 90
LA	Lactic acid monomer
LdE	Loading efficiency
LE	Loteprednol etabonate
LE-PLA	Poly(D,L-lactic) acid microspheres doped with loteprednol etabonate
LE-PLGA	Poly(D,L-lactic-co-glycolic) acid microspheres doped with loteprednol etabonate
IC_{50}	50% inhibitory concentration
ID	Inner diameter of infusion tube
IEQ	Islet equivalents
IL	Interleukin
IR	Infusion rate of organic phase into aqueous phase
IS	Immunosuppressant
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MW	Molecular weight
PBS	Phosphate buffer saline
PLA	Poly(D,L-lactic acid)
PLGA	Poly(D,L-lactic-co-glycolic) acid
P_N	Mean particle diameter based on the number distribution
PP-2B	Calcineurin
P_R	Polydispersity index; Ratio of P_V/P_N
PTFE	Polytetrafluoroethylene
P_V	Mean particle diameter based on the volume distribution

PVA	Polyvinyl alcohol
PXRD	Powder X-ray diffraction
Q	*In vitro* drug release amount
Q_∞	Total amount of drug in the microspheres (mg)
Q_t	Amount of drug released as a function of time (mg)
R^2	Correlation coefficient
SDS	Sodium dodecyl sulfate
SE	Standard error
SEM	Scanning electron microscope
$t_{1/2}$	Biological half-life
US	United States
x_{exp}	Amount of drug quantified for a given amount of the formulation
x_{LE}	Total amount of drug added in preparing the formulation
x_m	Amount of formulation analyzed
x_P	Total amount of polymer added in preparing the formulation

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Type I Diabetes Mellitus is an autoimmune disease in which the insulin producing β cells are permanently destroyed leaving the patient dependant on exogenous insulin for the rest of his/her life. Islet transplantation can offer a possible treatment for the most severe forms of the disease. The islet allograft replaces the destroyed β cells with viable ones. Dr. Buchwald's group, at the University of Miami's Diabetes Research Institute, has developed a novel method of islet transplantation. The donor islets are infused into a subcutaneous, neovascularized biohybrid implant device. The neovascularized device localizes the islets allowing for ease of implantation and retrieval of donor islet cells. This procedure still has complications that need to be refined – one being a need for a suitable, long-term immunosuppressant regimen to prevent the alloimmune rejection of the islets with low side effects to the patient.

The soft glucocorticoid loteprednol etabonate (LE) was chosen as the immunosuppressant since it breaks down into inactive metabolites reducing systemic accumulation of the drug and adverse side effects. LE and the islets are placed into the neovascularized device allowing for localized delivery which also minimizes systemic side effects as opposed to other routes of

administration (i.e. oral). It is desired to have a therapeutic drug concentration within the device that can last over a range of three months to one year.

The overall objective was to develop a sustained release formulation of LE suitable for use in the biohybrid transplant device for islet transplantation. By incorporating the drug into a biodegradable polymer matrix, we can have controlled release of the drug to the localized islet cells. Poly(D,L-lactic) acid (PLA) and poly(D,L-lactic-co-glycolic) acid (PLGA) microspheres prepared by solvent evaporation were used for sustained delivery of LE. The formulation process parameters were optimized using factorial design analysis to produce 5 to 50 micron-sized, monodispersed, smooth microspheres having sustained drug release ranging from three months to a few years.

The LE-PLA microspheres were analyzed for its *in vitro* cell toxicity and *in vivo* efficacy. The drug LE had a threshold concentration of 10 μM and IC_{50} of 20 μM on the MIN-6 insuloma cell line. However, the LE-PLA microspheres had no cytotoxicity. The LE-PLA microspheres were tested to find the immunosuppressive activity in chemically diabetic rats receiving islet transplantation using the novel implant device. The survival duration of the rats with the LE-PLA microspheres was double that of the control (no drug). These results show a great promise in the sustained release LE-PLA microspheres improving the success of islet transplantation by making the way for a more patient compliant and effective immunosuppressant treatment.

CHAPTER 1
INTRODUCTION

Type 1 Diabetes

In 2002, diabetes was listed as the sixth leading cause of death on United States (U.S.) death certificates [1]. About 5 to 10 % of all diagnosed cases had type 1 diabetes—estimating that about one in every 400 to 600 children and adolescents in the U.S. suffered from the disease [1]. In type 1 diabetes, the body's immune system destroys the insulin-producing pancreatic beta cells. Risk factors may be autoimmune, genetic [2], viral infections [3, 4], or environmental factors [5]. Patients suffer from a lower quality of life as a result of the long-term hyperglycemic complications—diabetic retinopathy, nephropathy, neuropathy, and vasculopathy. More serious complications can include hypoglycemic episodes which can lead to a coma, seizure, or even death. Daily administration of exogenous insulin is needed to maintain glycemic control.

Islet transplantation is recommended treatment for those suffering from the more severe forms of type 1 diabetes. Currently, islet transplantation is given to patients suffering from brittle or unstable diabetes or to those undergoing other transplantations (i.e. kidney or liver) requiring immunosuppressive therapy [6]. Patients who have received islet transplants have improved glycemic control and do not suffer from severe hypoglycemia [7, 8]. In addition, they have improved cardiovascular, renal, neural, and retinal functions [9-11]. Islet transplantation has been used over the past three decades and could be a very promising treatment or cure for type 1 diabetes.

Islet Transplantation

History

In 1967, Lacy et al. came across a collangenase-based method for isolating islets [12] and was successful, in 1972, in transplanting the cells into rodent models. In 1974, the first clinical

allogeneic islet transplantation was performed at the University of Minnesota [13]. However, no patients achieved insulin independence. Over the years, experts all over from Miami, Milan, Edmonton, Minnesota, and Pittsburg attempted to improve the islet purification methods, immunosuppressant protocols, and ultimately the clinical success of islet transplantation [14-16].

A major success occurred in 2000 when Shapiro et al. developed the Edmonton protocol which was 100% successful in seven recipients achieving insulin independence following islet transplantation using a strong immunosuppressant protocol. [17]. Follow-up studies revealed that 80% of the recipients remained insulin independent after one year [18, 19] and 10% after 5 years [20]. The median duration of insulin independence was 15 months. While there is still a need for improvement, the Edmonton protocol brought back interest in islet transplantation.

Current Methods of Islet Transplantation

For a successful transplant, about one million islets are needed and isolated from two or three donor pancreases. Ricordi et al. devised a suitable method for the large-scale isolation of human islets [21]. A collagenase solution is inserted into the main pancreatic duct and the gland is placed in a warm digestion chamber where the collagenase digests the exocrine tissue. The digested tissue is centrifuged and the islets (characterized by a lower density) migrate to the upper layer. The purified islets are infused into the portal vein of the liver using a closed gravity fed bag system [22] as shown in Figure 1-1. The liver (as opposed to the pancreas) makes a better residence site for the transplanted islets due decreased organ morbidity, damage during surgery, and a greater physiological advantage (since the liver is a major site of insulin function) [23].

While intrahepatic islet infusion through the portal vein is the recommended route of transplantation, the procedure is still associated with problems. Bleeding is the most common followed by portal vein thrombosis and elevated pressure [16, 24]. In addition, the lack of

vacularization and oxygen increase islet loss and impairment [25]. Alternative sites of transplantation could improve islet engraftment and long-term function. Various sites tested include the spleen [26], omental pouch [27], intramuscular tissue [28], and subcutaneous tissue [29].

Novel Bioartificial Pancreas

At the University of Miami's Diabetes Research Institute, Pileggi et al. [30] decided to focus on subcutaneous islet transplantation since it is less invasive as compared to the other sites. However, subcutaneous tissue still has the problem of vascularization serving as an ill environment for the transplanted islets in providing insufficient nutrients from the blood stream and waste removal [31, 32]. Neovascularized biohybrid devices for subcutaneous islet transplantation have been successful in providing a suitable environment for islets [31, 33]. Hence, Pileggi et al. developed their own neovascualarized biohybrid implant device.

The biohybrid implant device (Figure 1-2) consisted of a stainless-steel mesh cylinder (2 x 0.6 cm) with two polytetrafluoroethylene (PTFE) stoppers and a plunger [30]. The device (with the plunger insert) is implanted into the subcutaneous tissue in the dorsal region of the rat forty days before islet transplantation. This period is sufficient for the connective tissue to surround the device and for vascularization to occur. The plunger is removed prior to transplantation to provide a bed/void for inserting the islets. The device is then plugged with the stopper.

Pileggi et al. [30] was able to maintain normal non-fasting blood glucose levels (< 200 mg/dL) in chemically diabetic rat recipients with the biohybrid device. In addition, the recipients had long-term survival duration (> 5 months) and islet function. Hence the reversal of diabetes was successful with the novel biohybrid device. However, an immunosuppressant is still needed to reduce alloimmune rejection of the device and islets.

Immunosuppressant Therapy

As with all transplantations, immunosuppressants are necessary to prevent alloimmune rejection of transplants. Initial islet transplants utilized the immunosuppressants cyclosporine A, prednisone, azathioprine, and 15-deoxyspergualin [34, 35]. However, these immunosuppressant regimens were not successful considering that fewer than 10% of patients achieved insulin independence [36]. The first successful regimen for islet transplantation came with the Edmonton protocol. The Edmonton protocol consisted of sirolimus (rapamycin or Rapamune), FK506 (tacrolimus or Prograf), and humanized anti-IL-2 receptor α mAb antibody (daclizumab or Zenapax) [17]. More recent successful studies have included everolimus, basiliximab, FTY720, and lisofylline [37-39] in immunosuppressant regimens.

Problems with Current Immunosuppressive Agents

The risk-to-benefit ratio has to be taken into consideration when selecting effective immunosuppressive agents. Even with the success of the Edmonton protocol, the immunosuppressant regimen contained potent drugs that are associated with serious side effects [40, 41]. The calcineurin inhibitor tacrolimus has been associated with neurotoxicity, nephrotoxicity, and impaired islet graft function [42-45]. Sirolimus is known to cause oral ulcers, dyslipidemia, myelotoxicity, and impaired islet graft function [46, 47].

Other commonly used immunosuppressants are also associated with side effects. Cyclosporine causes nephrotoxicity and impairs islet graft function [45, 48]. Azathioprine can cause nephrotoxicity and pulmonary toxicity [49, 50]. 15-deoxyspergualin can cause anorexia, diarrhea, leucopenia, and sepsis [51]. Daclizumab is associated with significant morbidity and mortality since it can increase the risks of infections [52]. While glucocorticoids are also

affiliated with serious side effects, a more localized, low-dose delivery (within the biohybrid implant) with the newer glucocorticoids may be therapeutically beneficial [53-57].

Glucocorticoids as Immunosuppressants

Glucocorticoids elicit their immunosuppressive effects by attacking the cells that control the body's natural immune response. Dexamethasone induces apoptosis in lymphocytes (T cells and B cells) and thymocytes (T cell precursors) [58-60]. Glucocorticoids cause cell shrinkage, chromatin condensation, and DNA cleavage by caspase-3 activation [61]. They induce their immunosuppressive effects through the expression of cytoplasmic glucocorticoid receptor to repress genes involved in eliciting an immune response. For example, glucocorticoids inhibit NF-κB, antiapoptotic transcription factor, by increasing production of NF-κB inhibitor, IκB [62-64]. In addition, glucocorticoids down-regulate proinflammatory mediators TNF-α, IL-1β, and IL-6 that are secreted by monocytes and macrophages during an immune response [65-68]. The immunosuppressive effects of glucocorticoids make them suitable for use in preventing allograft rejection.

Loteprednol Etabonate

Loteprednol etabonate (LE) is a soft glucocorticoid that breaks down into inactive metabolites Δ^1-cortienic acid etabonate (AE) and then into Δ^1-cortienic acid (CA) *in-vivo* [69]. The clearance mechanism of LE reduces accumulation of the drug and systemic side effects. LE is 1.5, 8, and 15 times more potent than dexamethasone, prednisolone, and hydrocortisone [70, 71]. LE has been used for treatment of ophthalmic [72-76], pulmonary [77, 78], dermatological [79], arthritic [80], and gastrointestinal diseases [81].

A current immunosuppressant protocol looks at reducing the rejection of transplanted islets by infusing the islets and the anti-inflammatory drug LE into a neovascularized biohybrid

implant device [53]. It was found that the dose (45 μg for 3 months to 180 μg for one year) of LE necessary to maintain therapeutic levels (~0.5 μg/day) within the device over a three-month to one-year period is fatal to the beta islet cells. In addition, due to the soft properties of the drug, the stability of LE ($t_{1/2}$ ~ 18 hrs at 37^{o}C in dog blood and plasma) [82] is also a problem. This specific project aims to develop a sustained release formulation of LE.

Localized Sustained Release Technologies

There are various commercially available drug delivery systems (DDS) that focus on localized sustained delivery. They range from infusion pumps [83] to monolithic devices [84, 85] to biodegradable microspheres [86], etc. This project focuses on using biodegradable microspheres for localized drug delivery due to their versatility of different administration routes (oral, intramuscular, subcutaneous, etc…), excellent storage stability, suitability for industrial production, and safe and ease of elimination of the DDS by biodegradation [87, 88]. Biodegradable microspheres have been developed for the following types of polymers: gelatin, albumin, polyorthoesters, polyanhydrides, and polyesters. Most commonly used are polyesters specifically poly(D,L-lactic) acid (PLA) and poly(D,L-lactic-co-glycolic)acid (PLGA).

PLGA Microspheres

PLA and PLGA microspheres have been used as DDS for years [86]. They are able to encapsulate and provide sustained release of both hydrophilic and lipophilic drugs [89]. PLA and PLGA microspheres can decrease unwanted side effects while maintaining therapeutic effects since they can be utilized for targeted delivery [90]. The polymers degrade *in vivo* by hydrolysis of their ester linkages to CO_2 and H_2O making the polymer biodegradable and biocompatible [91]. The biodegradation rate of PLGA can vary from less than one month to a period of a few years depending on the copolymer composition [92].

Outline of This Dissertation

The purpose of this study was to develop a sustained release formulation of LE suitable for use in a biohybrid implant device for islet transplantation. We proposed that by incorporating the drug into a biodegradable polymer matrix, we can have a controlled release of the drug to the localized islet cells. The sustained release formulation was developed by solvent evaporation using the biodegradable PLGA microspheres as the drug delivery vehicle. In developing the formulation, the process parameters were optimized to produce 5 to 50 micron-sized, monodispersed, smooth microspheres having sustained release ranging from three months to a few years.

Chapter 2 of this dissertation investigated the effect of process parameters on the development of the LE-PLGA microspheres. The microspheres were characterized for their encapsulation efficiency, drug loading efficiency, initial burst of the microspheres in the dissolution media, particle size and morphology, and *in vitro* drug release profile. Factorial design analysis was performed to optimize the process using the infusion method during the emulsification stage. In addition, varying the percentage of emulsifier polyvinyl alcohol (PVA) in the aqueous phase and the monomer composition of the copolymer PLGA was investigated to determine a formulation with an optimal drug release profile.

Chapter 3 investigated the cytotoxicity of the LE-PLA microspheres on the MIN-6 insuloma beta cells. A dose of 45 μg for a 3 month period of LE is necessary to maintain therapeutic levels for a one-time administration of the immunosuppressant into the implant. However, exposing the beta cells to such a high dose is toxic. It is hypothesized that by incorporating the drug into the biodegradable polymer matrix will reduce the dose exposure to

the islets hence protecting them from the toxic effects. In addition, the polymer microspheres should not cause any additional cytotoxicity.

Chapter 4 investigated the *in vivo* efficacy of the LE-PLA microspheres. The three month release of LE-PLA microspheres with a mean particle diameter of 5 microns were inserted into the novel biohybrid implant device. The device was implanted into chemically diabetic rats receiving islet transplantation. The mean survival duration of the rats was used as an indicator of the transplant rejection time point. It is hypothesized that the mean survival duration of the recipients receiving the LE-PLA microspheres will be significantly greater than the control. In addition, the mean survival time of the recipients should last for three months.

The final chapter, Chapter 5, will summarize the findings and address future works for the project.

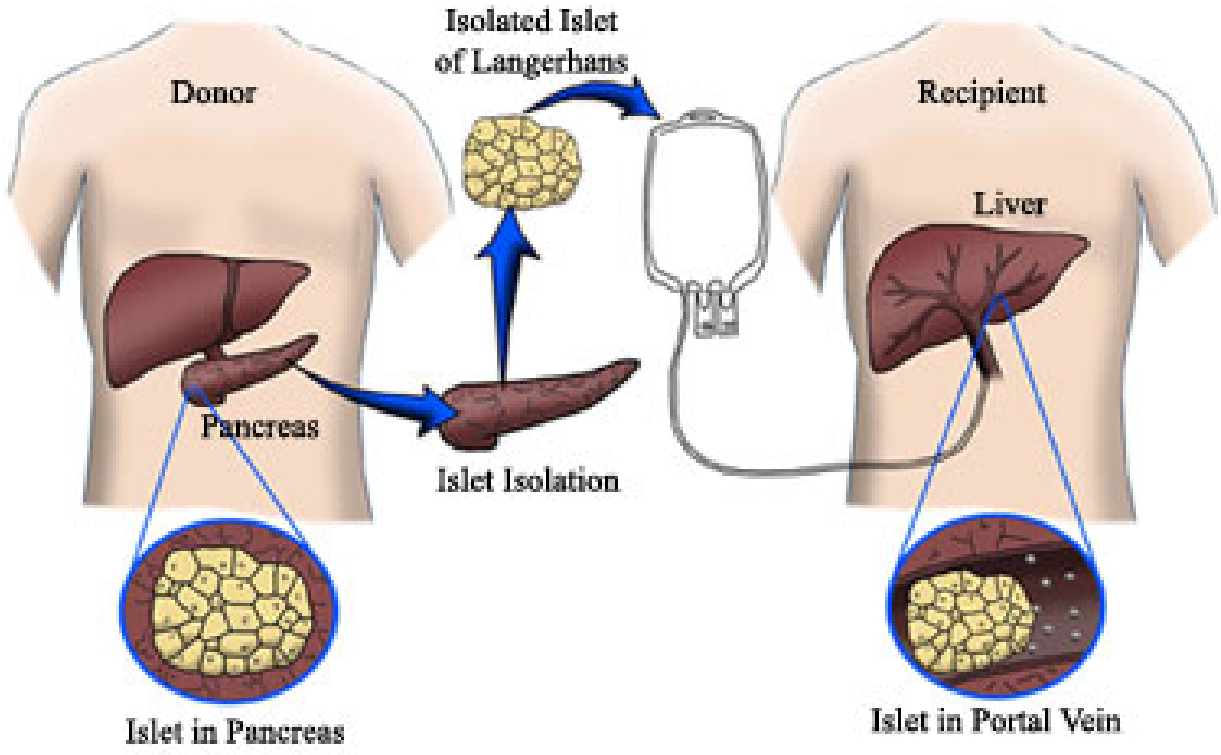


Figure 1-1. Current method of islet transplantation
(http://www.cumc.columbia.edu/news/in-vivo/Vol1_Iss4_feb25_02/pictures/islet-isolation.jpg)

Figure 1-2. Neovascularized biohybrid implant device to be used in the animal study (http://www.diabetesresearch.org/Newsroom/NewsReleases/DRI/NewDeviceforIsletTransplantation.htm)

CHAPTER 2
PREPARATION AND CHARACTERIZATION OF THE SUSTAINED RELEASE LE-PLGA MICROSPHERES

Introduction

Poly(DL-lactic) acid (PLA) and poly(DL-lactic-co-glycolic) acid (PLGA) are commonly used biodegradable synthetic polymers for sustained release drug delivery. The polymers have excellent tissue biocompatibility, biodegradation property, and safety profile [93-95]. Solvent evaporation is a commonly used method for preparing PLGA microspheres. Sustained release PLGA microspheres have been previously made using this method having drug release over a month [94, 96-99] and will be used in this project for developing the LE-PLGA microspheres.

The goal of this study was to develop LE-PLGA microspheres having sustained release ranging from three months to one year. The reason for using microspheres, as opposed to nanospheres, is that nanospheres may migrate out through the pores of the biohybrid implant device. In addition, nanospheres exhibit much faster drug release over a shorter period of time as opposed to microspheres [100, 101]. However, microspheres that are very large have a disadvantage of getting stuck in the syringe needle. To avoid the problems of using nanospheres and very large microspheres, the LE-PLGA microspheres will be made in the size range of 5 to 50 microns.

In developing the LE-PLGA microspheres, various process parameters were varied to determine the effect on formulation properties: encapsulation efficiency, loading efficiency, particle diameter, particle morphology, and *in vitro* drug release. The process parameters varied are:

a) The type of emulsification method used
b) The theoretical drug loading
c) Use of surfactant in wash solution
d) Inner diameter of infusion tube (ID)
e) Infusion rate of oil phase into aqueous phase (IR)

f) The percentage of emulsifier polyvinyl alcohol (PVA) in the aqueous phase
g) The LA:GA monomer composition of the copolymer (% LA)

Various emulsification methods can be employed in solvent evaporation to produce PLGA microspheres. Bath sonication and the infusion method were chosen in this study. Bath sonication produces low levels of sonic waves that provide enough energy to the system to form microspheres and not nanospheres [102]. The infusion method utilizes a needle based system that infuses droplets of the oil phase into an aqueous phase forming an o/w emulsion [103]. Based on the size of the emulsion droplets, which can be controlled by the stirrer speed, microspheres can be formed.

The ideal LE-PLGA microspheres would have a very high drug content or drug loading. One way to control this factor would be to start off with a high theoretical/initial drug loading. However formulations with high drug loading have a problem – a high initial burst [104]. An initial burst is undesirable since it can result in acute toxicity. So in developing the LE-PLGA microspheres, the formulation with the highest drug loading have to be determined without having a high initial burst.

Aside from modifying the theoretical drug loading, there are other ways of reducing the initial burst. Akbugcara was able to reduce the initial burst by modifying the ratio of various polymers Eudragit L, Eudragit RS, and Eudragit S in the furosemide loaded microspheres [105]. Xu et al. produced hydroxyapatite-coated PLGA microspheres loaded with amoxicillin [106]. The coating produced microspheres with low initial burst. In addition, Xu et al. used an anionic surfactant sodium dodecyl sulfate (SDS) which was also able to produce microspheres with low initial burst. In this project, we investigated whether adding SDS to the wash media will reduce the initial burst.

Due to the promising results with the infusion method, factorial design analysis was used to obtain a mathematical model for predicting the optimal formulation. We investigated the effect on the LE-PLGA microspheres' physicochemical properties by varying the ID versus IR, ID versus % PVA, and % PVA versus % LA. Chacon et al. reported that using slower infusion rates and larger diameter needles produced larger microspheres [107]. Chu et al. noticed that increasing the percentage of PVA in the aqueous phase produced smaller microspheres with lower encapsulation efficiency [108]. Increasing the lactic monomer composition in the PLGA copolymer produced microspheres that had more sustained release [109, 110]. By varying the four parameters, we should be able to find LE-PLGA microspheres in the ideal size range and having the ideal release profile.

Hypothesis

We hypothesized that we can produce monodisperse LE-PLGA microspheres having sustained release ranging from three months to one year utilizing solvent evaporation and by varying the above mentioned process parameters.

Materials and Methods

Chemicals

Loteprednol etabonate (LE) was generously donated by Dr. Nicholas Bodor. Poly(D,L-lactic) acid (PLA) (0.68 dL/g inherent viscosity in chloroform @ 30^{o}C), poly(D,L-lactic-co-glycolic) acid 75:25 (PLGA 75:25) (0.68 dL/g inherent viscosity in chloroform), and poly(D,L-lactic-co-glycolic) acid 50:50 (PLGA 50:50) (0.55 to 0.75 dL/g inherent viscosity in HFIP) were purchased from DURECT Corporation, Pelham, Alabama. The polyvinyl alcohol (MW_{avg} 30,000 – 70,000, 87 to 90% hydrolyzed) was purchased from Sigma Chemical Co. (St. Louis, MO). The remaining chemicals were of analytical grade and were purchased from Fisher Scientific Inc. (Suwanee, GA).

Solvent Evaporation

The drug LE and polymer PLA/PLGA were added to the organic solvent dichloromethane (DCM) and sonicated until both were solubilized. The organic solution was added to the aqueous solution containing a certain percentage of the emulsifier polyvinyl alcohol (PVA) and emulsified either by (a) sonication in a bath sonicator for 5 minutes or (b) infusion of the organic phase into the aqueous phase stirred magnetically at room temperature. Figure 2-1 shows the setup of the two emulsification methods. The emulsion was constantly stirred overnight using the Bellco Multistir 9 (Catalog #7760-00303) to allow the DCM to evaporate off and form a suspension. The suspension was centrifuged at 3000 rpm for 15 minutes using the 50 mL polypropylene tubes, Beckman centrifuge (Model # J2-21), and Beckman rotor (Model # JA-20). The supernatant was collected separately and the residue resuspended in the wash solution. The collected microspheres were washed three more times. The residue was resuspended in the minimal volume of double distilled water (DDW) and frozen at –80^{o}C for at least two hours. The samples were then lyophilized using Labconco Freeze Dry System 4.5 (Kansas City, MO) for three days and stored at 4^{o}C in a desiccator until use.

Encapsulation Efficiency

The wash suspension solution was collected in a beaker. One mL of the wash solution was dried off by vacuum centrifugation in triplicate. The dried residue was redissolved in 1 mL mobile phase and 100 μL of 10X dilution was injected into a reverse-phase Waters C_{18} 150 x 4.6 mm 5 micron HPLC column. The mobile phase consisted of acetonitrile: DDW: glacial acetic acid (60:40:0.4). The flowrate used was 0.8 mL/min and wavelength 254 nm. From a 100 μg/mL stock in acetonitrile, calibration samples of 30, 20, 10, 5, 1, 0.5, and 0.1 μg/mL were made with the mobile phase. The encapsulation efficiency (EE) was calculated as follows:

$$EE = \frac{D_T - D_W}{D_T} * 100\%$$

where D_T is the total (or initial) amount (mg) of drug added to the formulation

D_W is the amount (mg) of drug found in the wash solution

Loading Efficiency

A known amount of the LE-PLGA microspheres was dissolved in 2 mL of DCM by bath sonication for 15 minutes. The DCM was removed by vacuum centrifugation. The dried residue was redissolved in 2 ml mobile phase and 100 µL of (10X or 100X) dilution was injected into a reverse-phase Waters C_{18} 150 x 4.6 mm 5 micron HPLC column. The mobile phase consisted of acetonitrile: DDW: glacial acetic acid (60:40:0.4). The flowrate used was 0.8 mL/min and wavelength 254 nm. From a 100 µg/mL stock in acetonitrile, calibration samples of 30, 20, 10, 5, 1, 0.5, and 0.1 µg/mL were made with the mobile phase. The loading efficiency (LdE) was calculated as follows:

$$LdE = \frac{Actual\ Drug\ Loading}{Theoretical\ Drug\ Loading} \cdot 100\% = \frac{x_{exp} / x_m}{x_{LE} / (x_{LE} + x_P)} \cdot 100\%$$

where x_{exp} is the amount (mg) of drug quantified by HPLC analysis for a given amount of the formulation

x_m is the amount (mg) of formulation analyzed

x_{LE} is the total amount (mg) of drug added in preparing the formulation

x_P is the total amount (mg) of polymer added in preparing the formulation

Particle Size Analysis

A known amount of the LE-PLGA microspheres was dispersed in deionized water and sonicated for 60 seconds to break apart the aggregates. The suspension was then subjected to

analysis using the Coulter LS13320 (Beckman Coulter, Fullerton, CA). The Coulter LS13320 can analyze particles in the size range of 400 nm to 2 mm utilizing laser diffraction. The refractive index was set at 1.46 (0.01*i*) and run time at 60 seconds. The runs were done in triplicate per sample.

Scanning Electron Microscope (SEM)

The LE-PLGA microspheres were analyzed using a JEOL (Model 6335F) scanning electron microscope (SEM) to obtain the size, shape, and surface morphology. The microspheres were mounted on aluminum SEM stubs with double stick carbon tape. A thin layer of carbon, approximately 10 to 15 nm thick, was evaporated onto the surface of the particles prior to SEM analysis. Characterization was performed at 2-5 kEV under vacuum.

Powder X-Ray Diffraction (PXRD)

A known amount of LE-PLGA microspheres were stuck onto a microscope slide using double sided tape. The Philips APD X-ray diffractometer was used. The taped samples were exposed to Cu radiation (40 kV, 20 mA) and scanned from 5^{o} to 40^{o}, 2θ at a step size of 0.02^{o} and step time of 1 second.

***In-Vitro* Drug Release**

The *in vitro* drug release studies were performed at 37^{o}C using 200 mL dissolution media in a capped 250 mL erlenmeyer flask and continuously stirred. The dissolution media consisted of 1X phosphate buffer saline (pH 7.4), 0.025% sodium azide, and 1% sodium dodecyl sulfate (SDS). Sodium azide was used as a preservative. SDS was used to enhance the stability of the drug and to keep the *in vitro* drug release assay under sink conditions. For accessing the drug released within the implant device, the "sample and separate" technique was used with a stir rate of 30 rpm. However, considering that the sustained release formulations will take 6 months to

one year for the drug to release, an accelerated *in vitro* release assay was used (i.e. the stir rate used was around 550 rpm) to make the study more feasible and correlated to the assay performed under real time conditions (data not shown).

A predetermined amount of the LE-PLGA microspheres were added to the bulk media. The amount of sample used was adjusted to keep the total released drug concentration in the dissolution media below 15% of the drug's saturation solubility (126 μg/mL). At distinct time intervals, 0.5 mL of the bulk media was taken out and analyzed using the HPLC. The samples (0.5 mL) were centrifuged at 13,200 rpm for 10 minutes. The supernatant (100 μL) was injected into a reverse-phase Waters C_{18} 150 x 4.6 mm 5 micron HPLC column. The mobile phase consisted of acetonitrile: DDW: glacial acetic acid (60:40:0.4). The flowrate used was 0.8 mL/min and wavelength 254 nm. Previous stability studies (data not shown) of LE showed that the degradation of the drug in aqueous media to AE only and not to CA over time indicating no need for detecting CA. Calibration samples of 20, 10, 5, 1, 0.5, 0.1, and 0.05 μg/mL were prepared by diluting a 100 μg/mL LE and AE stock (in 100% acetonitrile) in dissolution media. Quality control samples consisted of 10, 1, and 0.1 μg/mL (LE and AE). The calibration curve was plotted in Excel using peak heights of the absorbance of the standard solutions. The trendline was used to calculate the sample concentrations.

At the end of the study, the solids were collected by centrifugation to access the amount of drug remaining. DCM was added to the solids to dissolve the PLA and release the remaining drug. The suspension was vortexed and dried by vacuum centrifugation. The dried residue was reconstituted with 0.5 mL mobile phase and 100 μL was injected into the HPLC using the conditions mentioned above. Calibration standards consisted of 30, 20, 10, 5, 1, 0.5, 0.1, and

0.05 μg/mL of LE and AE. Quality control standards consisted of 10, 1, and 0.1 μg/mL of LE and AE.

Statistical Analysis

Design-Expert® Version 7.1.5 (Stat-Ease, Inc., Minneapolis, MN) and SigmaPlot for Windows Version 10.0 (Systat Software, Inc., San Jose, CA) were used for curve fitting the experimental data. The goodness of fit was determined by the correlation coefficient (R^2). The analysis of variance (ANOVA) was determined using Design-Expert®

Results and Discussion

Emulsification Method

The type of emulsification method used made a difference in the morphology of the microspheres produced. Emulsification using sonication (Figure 2-2A) produced a polydispersed formulation of deformed/deflated microspheres due to the energy generated by sonication and premature precipitation of the emulsion droplets into solid microspheres. Emulsification by infusion of the organic phase into the aqueous phase stirred at a set speed (Figure 2-2B) formed monodispersed, smoother microspheres due the slower precipitation of the emulsion droplets allowing more time for more uniform droplets to form. As shown in Figure 2-3, the more monodispersed microspheres (F2) had a more sustained zero-order release as opposed to the polydispersed microspheres (F1) prepared by sonication. Formulation F1 had faster release since it contained clusters of nanospheres while F2 did not. Nanospheres have much larger specific surface area than microspheres so drugs can diffuse out of nanospheres at a faster rate [100].

Theoretical Drug Loading

The theoretical drug loading of the LE-PLA microspheres were analyzed at 5, 10, 20, and 30% LE. Increasing the theoretical drug loading from 5% to 10% had a decrease in the

encapsulation efficiency (Figure 2-4) and loading efficiency (Figure 2-5). However from 10% to 30% drug loading, there was an increase in the encapsulation and loading efficiency. The initial decrease in the encapsulation and loading efficiency was a result of the poorer incorporation of the drug into the polymer matrix due to a low affinity of the drug to the polymer [111]. Further increasing the drug loading provided a more saturated environment in the organic phase which forced more drug to incorporate into the polymer matrix. Hence the results were microspheres with higher encapsulation and loading efficiency.

There was a dramatic difference in the initial burst (Figure 2-6) for the four formulations. The 5% LE loaded microspheres had a very low initial burst of 0.2% (± 0.4%) and was significantly different from the 10% – 30% LE loaded microspheres which had an initial burst of 78.5% to 94.4%. The PXRD plots gave a clearer explanation for the initial bursts observed. The pure drug LE powder (Figure 2-7A) had distinct sharp peaks at 16, 17, and 19 2θ angles. The blank PLA microspheres (Figure 2-7B) did not have any distinct peaks. When looking at the physical mixtures containing 5, 10, 20, and 30% drug and blank PLA microspheres (Figure 2-7C, E, G, and I), the three 2θ angle peaks had increased intensity as the % drug in the mixture increased. The 5% LE loaded PLA microspheres (Figure 2-7D) showed no peaks at the three 2θ angles. However the 10, 20, and 30% LE loaded PLA microspheres did. The absence of peaks for the 5% LE loaded PLA microspheres indicated that there was very little or no drug on the surface of the microspheres. Since the 10, 20, and 30% drug loaded PLA microspheres (Figure 2-7F, H, and J) contained peaks, this indicates that there was still unencapsulated drug in the form of drug crystals on the surface of the microspheres [112]. The initial burst observed with the 10, 20, and 30% LE loaded PLA microspheres was due to the immediate dissolution of the unencapsulated drug.

Surfactant in Wash Media

Using the surfactant sodium dodecyl sulfate (SDS) in the wash media significantly reduced the initial burst (Figure 2-8) as compared to the formulation washed with double distilled water (DDW). At 1% SDS or 1 g/100 mL, the concentration of SDS in water was above it's critical micelle concentration (cmc) which is 0.24 g/100 mL [113]. So the wash solution contained SDS micelles which have a highly hydrophobic core. The hydrophobic core attracts and solubilizes the unencapsulated drug by making it easier to remove the drug not imbedded in the PLA microspheres. Micelles are commonly utilized to extract unwanted hydrophobic chemicals from aqueous solutions [114]. So utilizing this technology was an effective and practical mean of producing formulations with a low initial burst.

Factorial Design Analysis: Inner Diameter of Infusion Tube vs. Infusion Rate of Oil Phase

A factorial design analysis study was performed to optimize the properties of the LE-PLGA microspheres using the infusion emulsification method (Figure 2-1). The inner diameter of the infusion tube (ID) and the infusion rate (IR) of the organic phase into the aqueous phase were varied according to the conditions listed in Table 2-1. The % PVA used was 0.2%. And, the microspheres were made with PLA. Table 2-2 lists the formulations' encapsulation efficiency (EE), loading efficiency (LdE), mean particle diameter based on the volume distribution (P_V), mean particle diameter based on the number distribution (P_N), and the polydispersity (P_R), the ratio of the means of the two distributions, which is calculated as follows:

$$P_R = \frac{P_V}{P_N}$$

The number distribution is a statistical representation of the actual mean diameter of the sample. Visually it would depict the particle size distribution observed if the microspheres' diameter were individually measured under SEM. The volume distribution is commonly used when

modeling or understanding systems that are concentration dependent (in our case the *in vitro* drug release studies). The volume distribution is weighed to emphasize the larger microspheres. When looking at concentration dependent systems, the larger particles contain a significantly greater amount of drug. However, a considerably smaller particle contains a minute or negligible amount of drug in comparison. Since the large particles contain more drug, they tend to dictate the concentration dependent characteristics of a formulation making P_V an important parameter to investigate. The ratio P_R represents the polydispersity of a sample with 1 being monodisperse and greater than 2.0 being polydispersed.

The contour plot in Figure 2-9 is a graphical representation of the encapsulation efficiency results listed in Table 2-2. It can be observed that the highest encapsulation efficiency occurred when the ID was 0.020 inches and the IR was either 1.0 or 0.8 mL/min. The similar trend was also observed with the loading efficiency (Figure 2-10). The contour plot, Figure 2-11, showed a more parabolic relationship where the ID of 0.015 and 0.055 inches produced microspheres with a larger mean diameter (based on the volume distribution P_V) and where the IR of 0.8 mL/min produced the smaller microspheres. The P_N (Figure 2-12) also showed the same trend with the ID significantly increasing as P_N increased ($P<0.01$). No noticeable trend was observed with the IR. With the P_R (Figure 2-13), using larger IDs produced more polydispersed microspheres ($P<0.05$) whereas the IR had no significant effect.

As previously reported by Chacon et al., larger microspheres were formed with an increase in ID [107]. However, so did the polydispersity (P_R) making the larger IDs not suitable in optimizing the formulation. The IR had no effect on the P_V, P_N, and P_R. However, an IR of 1.0 or 0.8 mL/min gave the highest encapsulation and loading efficiency. In the future studies, an IR of 0.8 mL/min was chosen in optimizing the formulations.

Factorial Design Analysis: Percent Polyvinyl Alcohol vs. Inner Diameter of Infusion Tube

Further studies were performed using factorial design analysis looking at the effect of the percent of polyvinyl alcohol (PVA) and the inner diameter of the infusion tube (ID) on the physicochemical properties of the LE-PLA microspheres. The process parameters were varied according to the conditions listed in Table 2-3. The infusion rate (IR) used was 0.8 mL/min. And, the microspheres were made with PLA. Table 2-4 lists the formulations' encapsulation efficiency (EE), loading efficiency (LdE), mean particle diameter based on the volume distribution (P_V), mean particle diameter based on the number distribution (P_N), and the polydispersity (P_R).

The encapsulation efficiency (Figure 2-14) decreased as the % PVA decreased. With the loading efficiency (Figure 2-15), the same trend was observed. The formulations made with 0.2% PVA had a much lower loading efficiency as opposed to those made with the 0.5% and 1.0% PVA. The P_V (Figure 2-16) significantly decreased as the % PVA decreased ($P<0.005$) with IDs above 0.020 inches. The ID relationship with P_V was a parabolic one ($P<0.05$) with an ID of 0.030 inches producing the smallest microspheres and the 0.055 and 0.015 inches producing the larger microspheres. The similar trend was observed with P_N (Figure 2-17). The P_N generally decreased with decreasing % PVA ($P<0.05$). A parabolic relationship was again observed with varying IDs with 0.030 inches producing the smallest microspheres. The P_R (Figure 2-18) was smallest when using higher % of PVA ($P<0.05$) and lower ID.

As reported by Chu et al., increasing the % PVA in the aqueous solution did produce smaller microspheres [108]. PVA reduced the surface tension between the aqueous and organic phase by forming emulsions where the hydrophobic end of PVA was absorbed in the oil phase and hydrophilic end was absorbed in the aqueous phase. The more PVA added, the more stable and smaller the emulsion droplets [115]. The formation of the smaller emulsion droplets was due

to the PVA increasing the viscosity of the aqueous solution. The increase in the viscosity of the aqueous solution reduced the frequency of the emulsion droplets collisions which prevented the coalescence of the emulsion droplets into larger ones. As DCM evaporated, the polymer in the emulsion droplets precipitated into the small microspheres.

Factorial Design Analysis: Percent Lactic Acid vs. Percent Polyvinyl Alcohol

Further studies were performed using factorial design analysis looking at the effect of the percent of monomer lactic acid (LA) and polyvinyl alcohol (PVA) on the physicochemical properties of the LE-PLGA microspheres. The process parameters were varied according to the conditions listed in Table 2-5. The infusion rate (IR) used was 0.8 mL/min. Since the 0.015 inches inner diameter of the infusion tube (ID) produced larger, more monodispersed microspheres, this tubing was used for the study. The LE-PLGA microspheres were made with PLA, PLGA 75:25, and PLGA 50:50. Table 2-6 lists the formulations' encapsulation efficiency (EE), loading efficiency (LdE), mean particle diameter based on the volume distribution (P_V), mean particle diameter based on the number distribution (P_N), and the polydispersity (P_R).

The encapsulation efficiency (Figure 2-19) and loading efficiency (Figure 2-20) initially decreased as the % PVA decreased to 2.0%, increased to 1.0%, and decreased to 0.2%. The % PVA had a significant effect on the encapsulation efficiency ($P<0.0005$) and loading efficiency ($P<0.05$). Varying the % LA had no significant effect. With the P_V (Figure 2-21), smaller microspheres were formed with increasing % PVA ($P<0.005$). No noticeable effect was observed with varying % LA. With the P_N (Figure 2-22), smaller microspheres were again formed with increasing % PVA ($P<0.05$). However with 0.2% to 0.5% PVA for PLGA 50:50 and PLGA 75:25, the microspheres' diameter decreased. With decreasing % PVA ($P<0.05$), the P_R (Figure 2-23) increased. No significant effect was observed with varying % LA.

Figure 2-24 shows the *in vitro* drug release profiles of the LE-PLGA 50:50 microspheres. Even though the microsphere diameter (listed in Table 2-7) increased when the % PVA increased from 0.2% to 0.3%, the LE release rate increased and the duration of the release shortened. This indicates that the release profile was not just based on the particle size of the microspheres but also on another phenomena. Enhanced internal porosity (or decreased density) of the LE-PLGA microspheres can have a higher drug release rate as opposed to microspheres that are completely dense [112]. There was a decrease in the LE release rate when the % PVA increased from 0.3% to 2.0%. This indicated that the LE-PLGA microspheres formed at 2.0% PVA were denser than the ones formed at 0.5% and at 0.3% PVA. When the % PVA increased from 2.0% to 5.0%, the microsphere diameter decreased and the LE release rate increased. This resembles expected release rates based on the diameter of the microspheres indicating that the density of the microspheres play little or no role on the release profiles.

With the PLGA 50:50 microsphere LE release profiles, the smallest microspheres (5% PVA) had a concave downward profile. This was typical of diffusion-controlled release. In other words, the drug diffusion rate was much faster than the polymer degradation rate. The fast diffusion rate was probably due to smaller microspheres having a larger specific surface area [100] and increased water uptake resulting in swelling of the microspheres [101]. The larger microspheres (0.2% to 2.0% PVA) had more of an S-shaped release profile. The reason for the S-shaped release profile was due to two different phases [100]. First, there was an initial diffusion of the drug from the immediate surface layer of the microsphere. Since large microspheres have low specific surface area, this diffusion rate was slow. The drug that was not in immediate exposure to the aqueous bulk environment had to travel through the dense polymer matrix to reach the bulk media. Second, the microspheres swelled due to water penetration.

During this phase, more drug was exposed to the aqueous bulk environment so the diffusion and deadsorption of the drug from the microspheres occurred very rapidly. Polymer erosion also played a role since it can increase drug diffusivity through the enlargement of aqueous pores in the polymer matrix [116].

A similar trend was observed with the LE-PLGA 75:25 microspheres, the *in vitro* drug release profiles (Figure 2-25) were more linear. When the % PVA increased from 0.3% to 0.5%, the microsphere diameter (listed in Table 2-8) increased and the LE release rate decreased. Again there was a decrease in the LE release rate when the % PVA increased from 0.5% to 1.0% possibly due to a change in the microsphere density. When the % PVA increased from 1.0% to 5.0%, the microsphere diameter decreased and the LE release rate increased.

The similar trend continued with the LE-PLA microspheres. The *in vitro* drug release profiles (Figure 2-26) were more linear. Even though the microspheres diameter (listed in Table 2-9) increased when the % PVA increased from 0.2% to 0.3%, the LE release rate increased due to a change in the density of the microspheres. When the % PVA increased from 0.3% to 0.5%, the microsphere diameter decreased and so did the LE release rate. Finally, when the % PVA increased from 0.5% to 5.0%, the microsphere diameter decreased and the LE release rate increased.

As expected, the LE release rates of the LE-PLGA microspheres (Figure 2-27) decreased as the percentage of lactic acid (LA) increased as a result of an increase in the polymer decomposition and erosion [109, 110]. As shown in Table 2-10, the microspheres chosen had similar mean volume (P_V) and number (P_N) diameters and polydispersity (P_R). SEM pictures were taken of the PLGA 50:50 (Figure 2-28), PLGA 75:25 (Figure 2-29), and PLA (Figure 2-30) microspheres at 0, 4, 8, and 12 months during the *in vitro* drug release study. For all three types

of microspheres, the surface was initially very smooth. With the LE-PLGA 50:50 microspheres, rippling on the surface was observed at month 4 and the microsphere's structure collapsed and eroded at month 8 and 12. After the microspheres collapsed, they seemed to be sticky and agglomerated with other microspheres to form larger particles which could be seen by the eye (picture not shown). The LE-PLGA 75:25 microspheres maintained their structure but some bubbling on the surface was seen at 12 months. The LE-PLA microspheres were smooth up to 4 months but afterwards the microspheres ruptured possibly due to internal pressure buildup within the microspheres over time as a result of osmotic forces [117].

The Effect of Polydispersity (P_R) on the *In Vitro* Drug Release Profile

An interesting observation was made in relation to the polydispersity (P_R) and the *in vitro* drug release profiles of the LE-PLA microspheres. Figure 2-31 shows the release of LE from PLA-microspheres having varying P_V and P_N and constant P_R (Table 2-11). When the P_V diameter ranged from 39 to 56 μm with constant P_R, the LE release rate did not vary at all. Figure 2-32 shows the release of LE from PLA-microspheres having similar P_V and varying P_R (Table 2-12). With increasing P_R, the LE release rate increased. Figure 2-33 shows the release of LE from PLA-microspheres having similar P_N and varying P_R (Table 2-13). Increasing the P_R, decreased the LE release rate. But this decrease seems to be saturable when the P_V is above 24 μm.

Polydisperse PLGA microspheres usually have two distinct phases [112] as observed in Figure 2-33. The first phase consisted of a faster drug release rate followed by a slower rate. During the first phase, the smaller microspheres released the drug more rapidly since smaller microspheres have much larger specific surface area for diffusing out the drug [100]. Larger

microspheres released the drug at a much slower rate because of the lower specific surface area and the slow diffusion of the drug through a greater distance (the radius of the microsphere).

Conclusion

- Emulsification using the infusion method produced more monodispersed, spherical microspheres with a more sustained and zero-order release.
- 5% drug loading produced LE-PLA microspheres with the lowest initial burst due to low or no drug adsorbed onto the surface of the microsphere.
- Using 1% SDS in the wash solution did a great job of removing the drug adsorbed on the microsphere surface. The resultant microspheres had a very low initial burst.
- The optimal IR was 0.8 mL/min. Increasing the ID produced larger LE-PLA microspheres but also increased the polydispersity.
- Increasing the % of PVA in the aqueous solution, produced smaller microspheres with higher polydispersity. The encapsulation and loading efficiency was also increased with increasing % PVA. In looking at the *in vitro* drug release of the LE-PLGA microspheres, another parameter beside the size (possibly the density/porosity) of the microspheres influenced the drug release.
- Varying the composition of the lactic and glycolic acid monomer in the PLGA copolymer had no significant effect on the encapsulation and loading efficiency, the particle size, and polydispersity of the microspheres. However, in decreasing the % LA, the drug release rates also was increased. SEM pictures show that the LE-PLGA 50:50 microspheres internal structure collapsed and eroded over time. LE-PLA microspheres ruptured after 4 months of *in vitro* drug release.
- Monodisperse LE-PLA microspheres had very little difference on the *in vitro* LE release rates when varying the size of the microspheres. However, varying the mean volume diameter, mean number diameter, and the polydispersity made a big difference and should to be taken in consideration when predicting the *in vitro* drug release profiles.
- Formulation M7 was chosen for the *in vitro* cell toxicity studies (Chapter 3) and the *in vivo* feasibility study (Chapter 4) since it had a desired duration of release of 3 months.

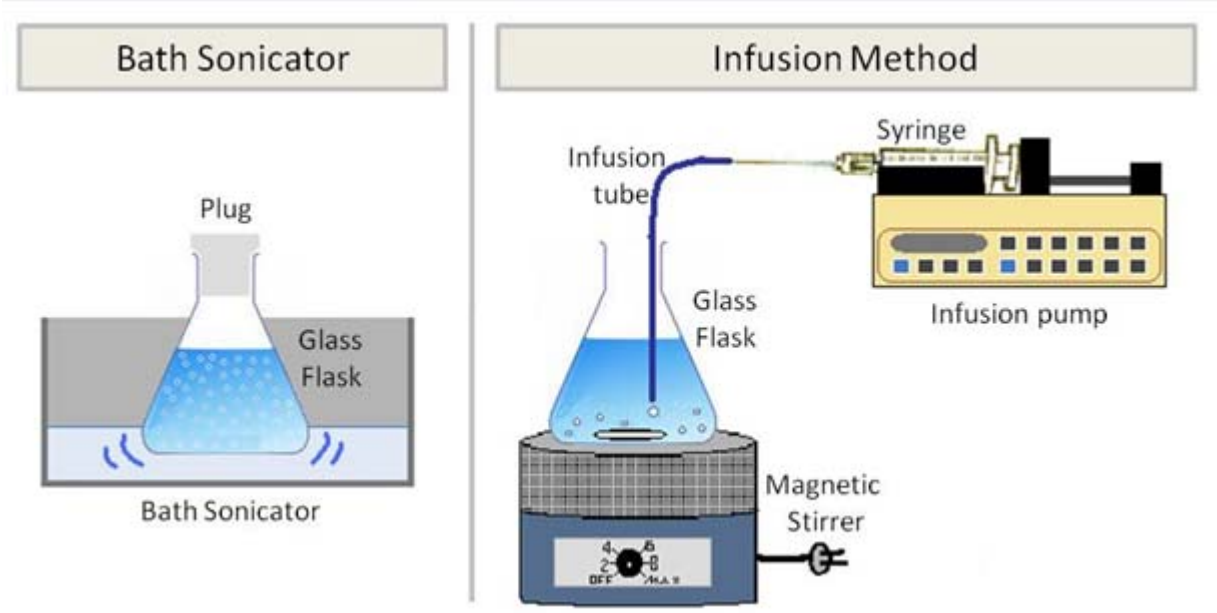


Figure 2-1. Different emulsification methods used: (A) sonication and (B) infusion method

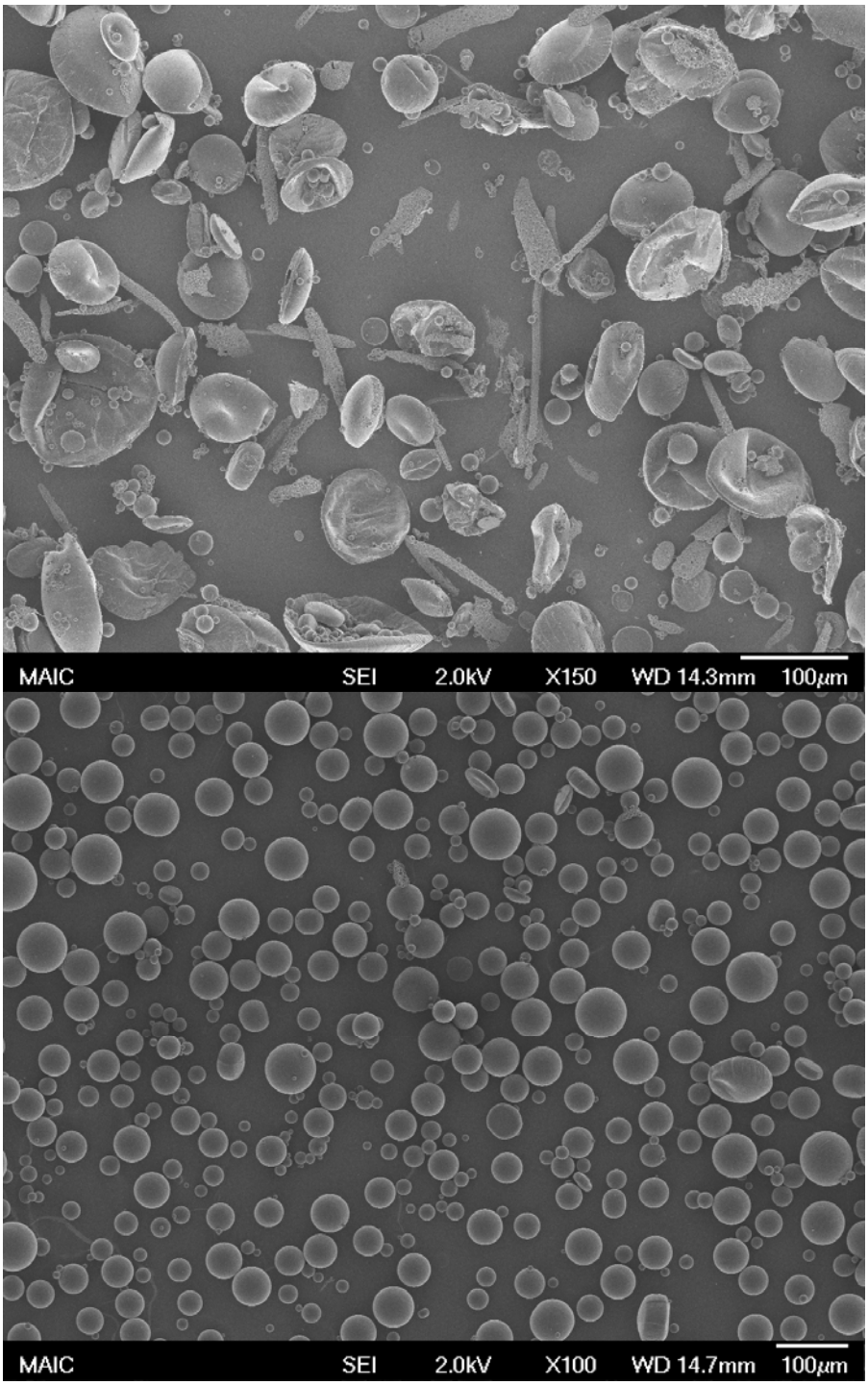


Figure 2-2. SEM pictures showing the surface morphology of LE-PLA microspheres prepared by (A) sonication and (B) infusion method

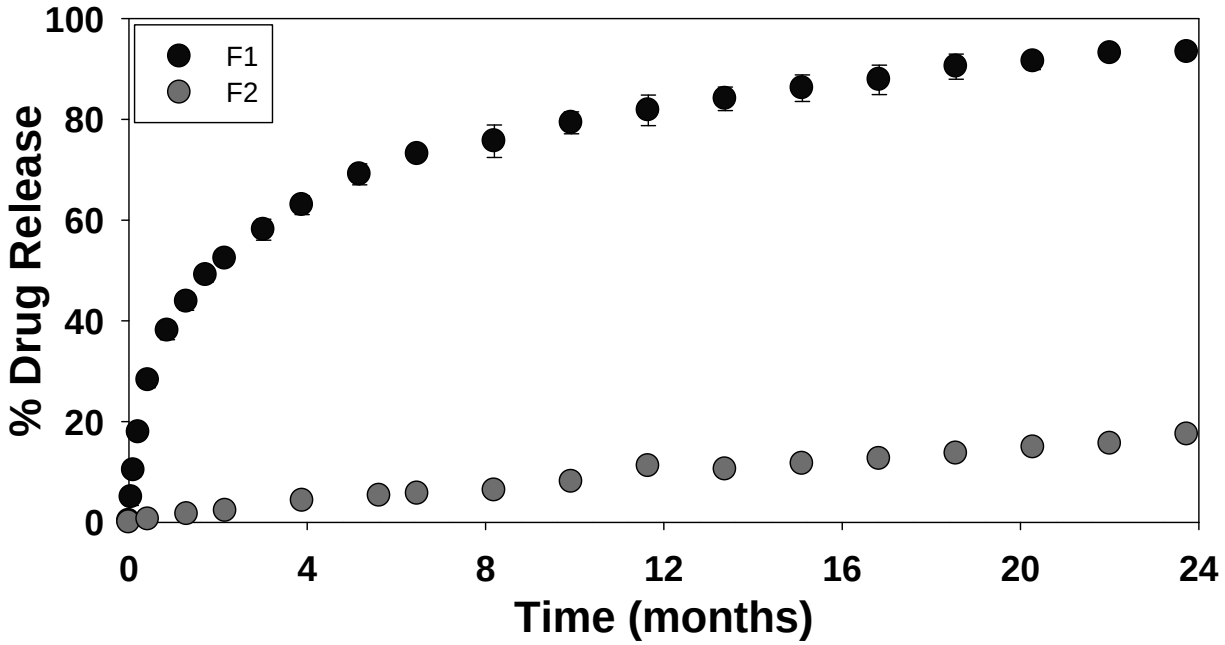


Figure 2-3. *In vitro* drug release of LE-PLA microspheres prepared by sonication (F1) and infusion method (F2) (n=3). Studies were preformed under accelerated conditions and correlated to expected real-time conditions.

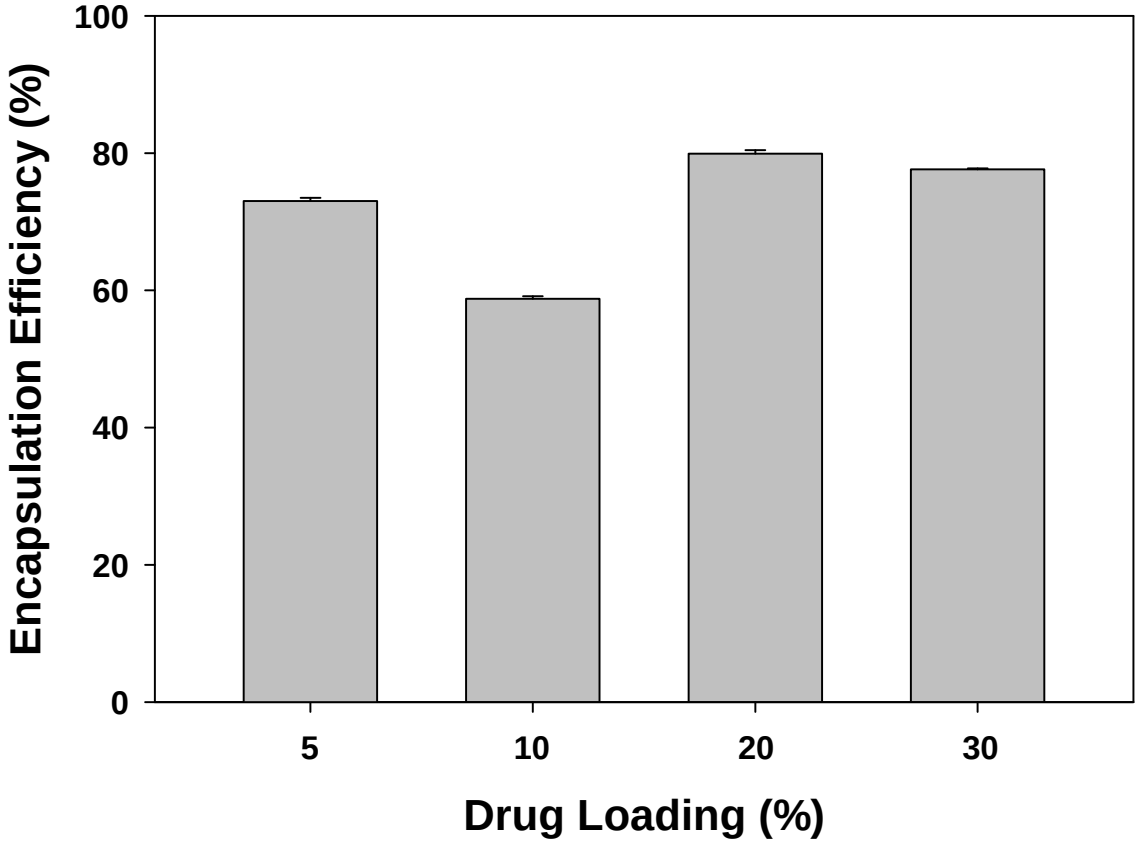


Figure 2-4. Encapsulation efficiency of LE-PLA microspheres prepared with 5, 10, 20, and 30% loteprednol etabonate in formulation (n=3) Significant difference determined by Student t-test ($P<0.05$).

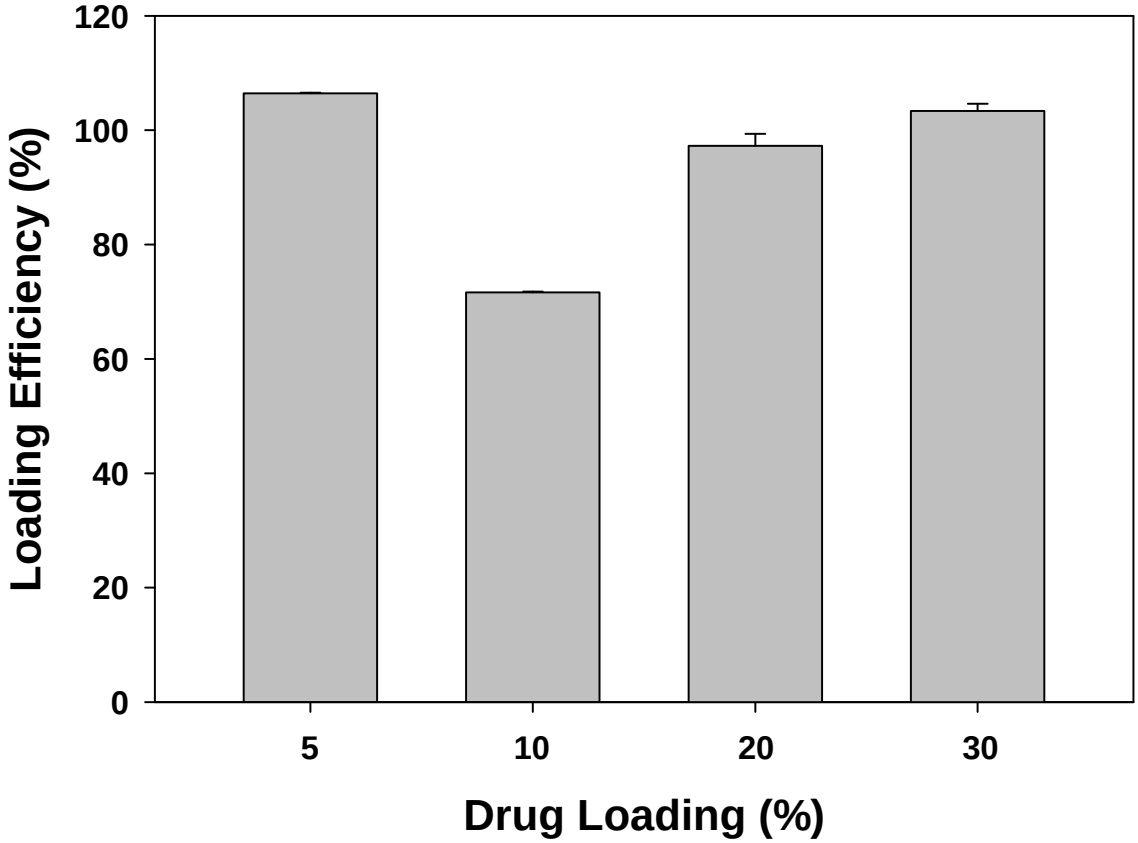


Figure 2-5. Loading efficiency of LE-PLA microspheres prepared with 5, 10, 20, and 30% loteprednol etabonate in formulation (n=3) Significant difference determined by Student t-test ($P<0.05$).

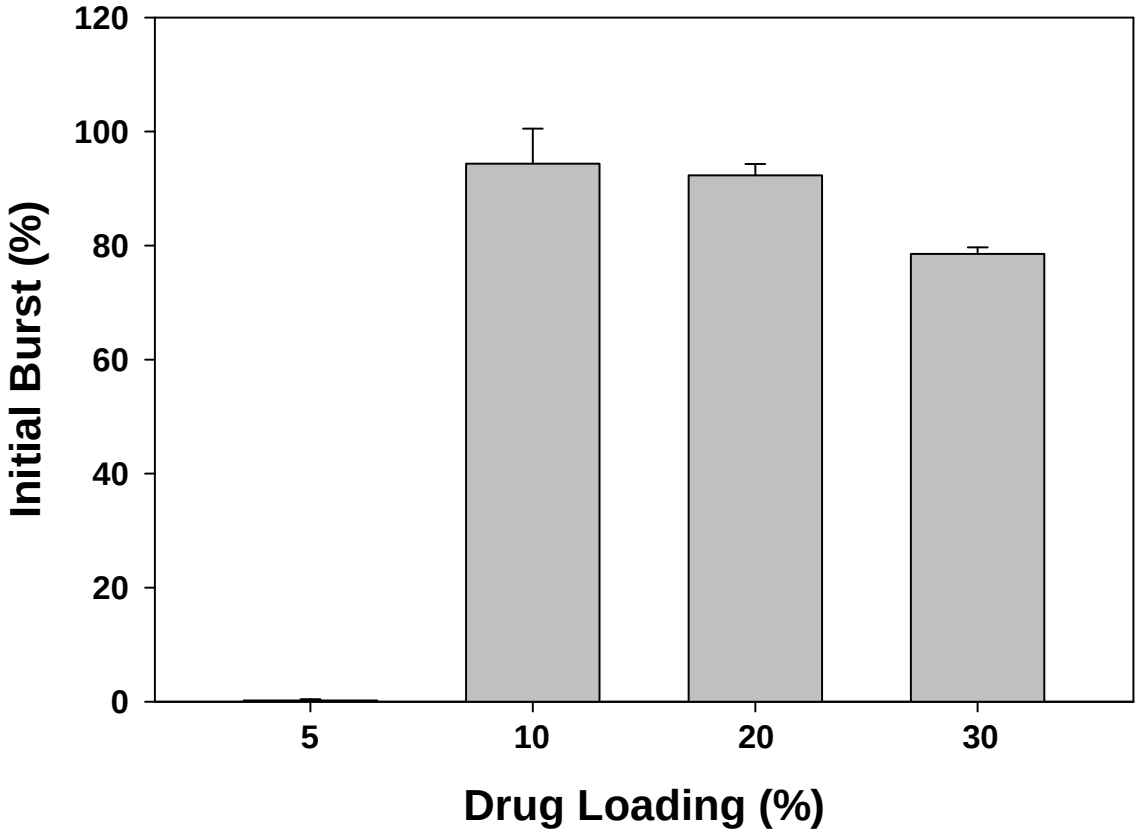


Figure 2-6. Initial burst of LE-PLA microspheres prepared with 5, 10, 20, and 30% loteprednol etabonate in formulation (n=3) Significant difference determined by Student t-test ($P<0.05$).

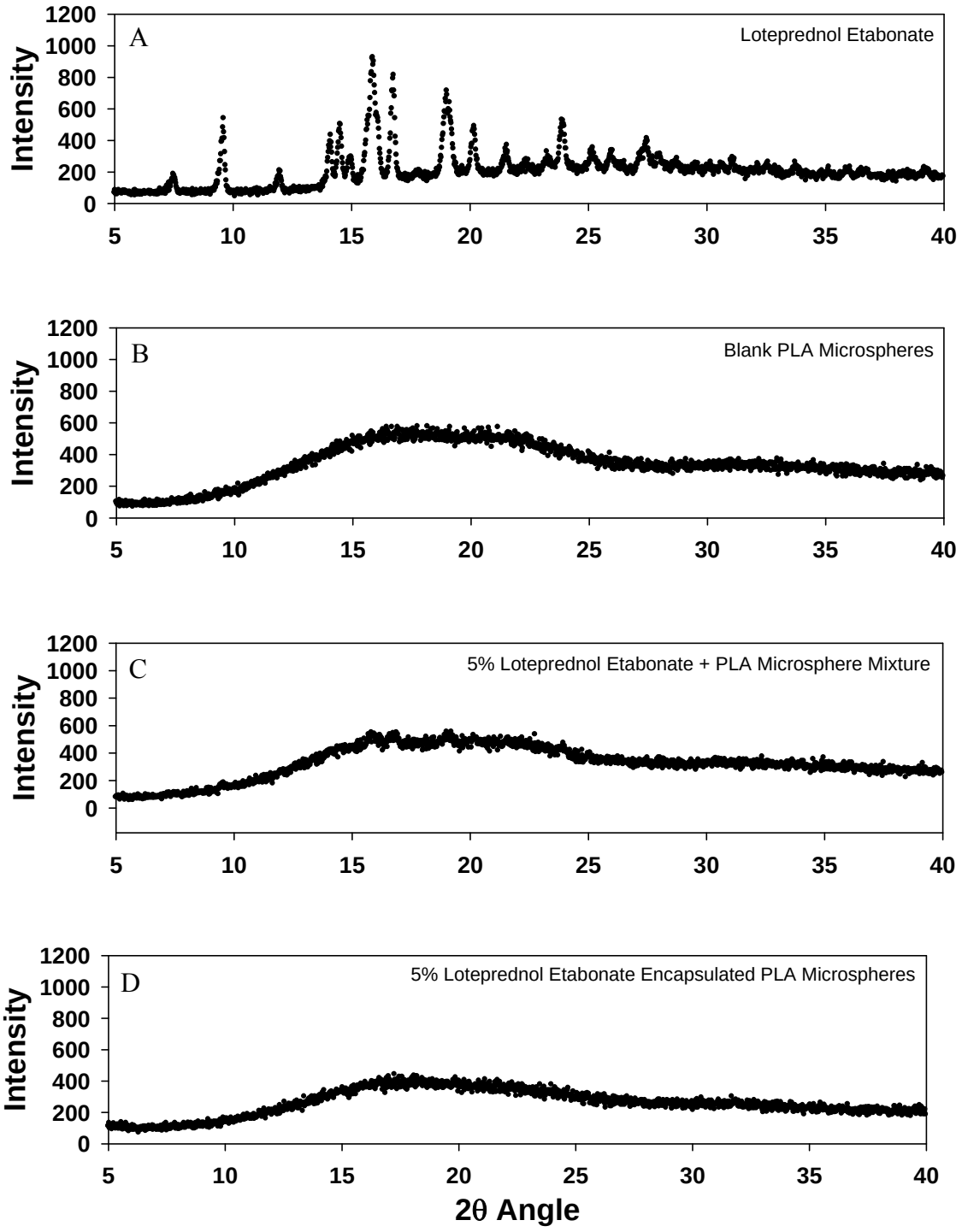


Figure 2-7. PXRD patterns of LE, physical mixture, and LE-PLA microspheres of varying drug loadings

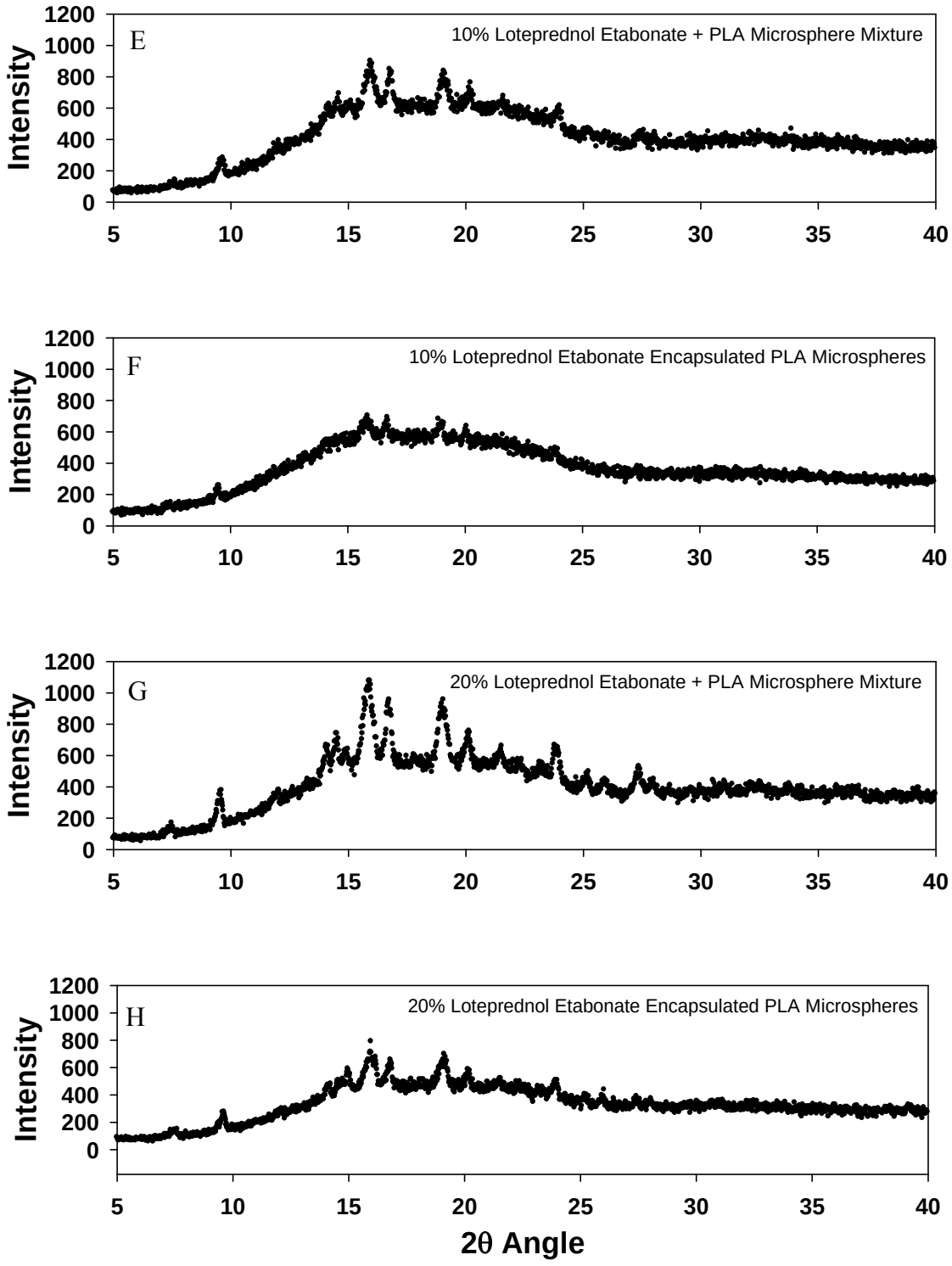


Figure 2-7 Continued.

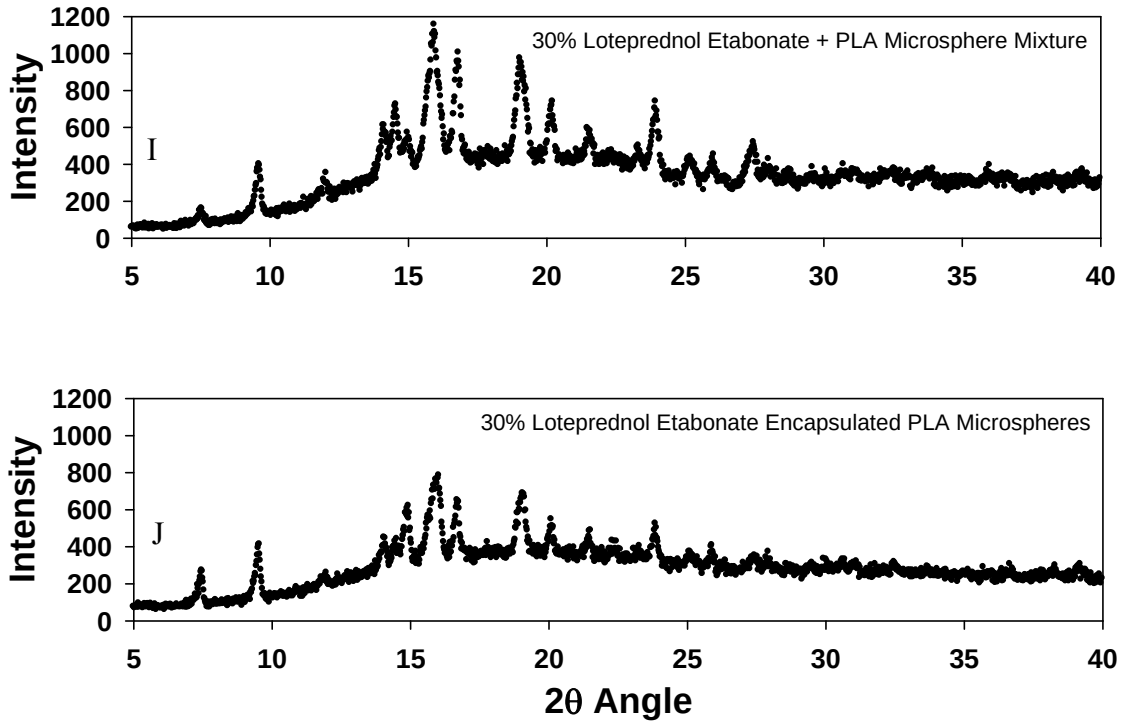


Figure 2-7 Continued.

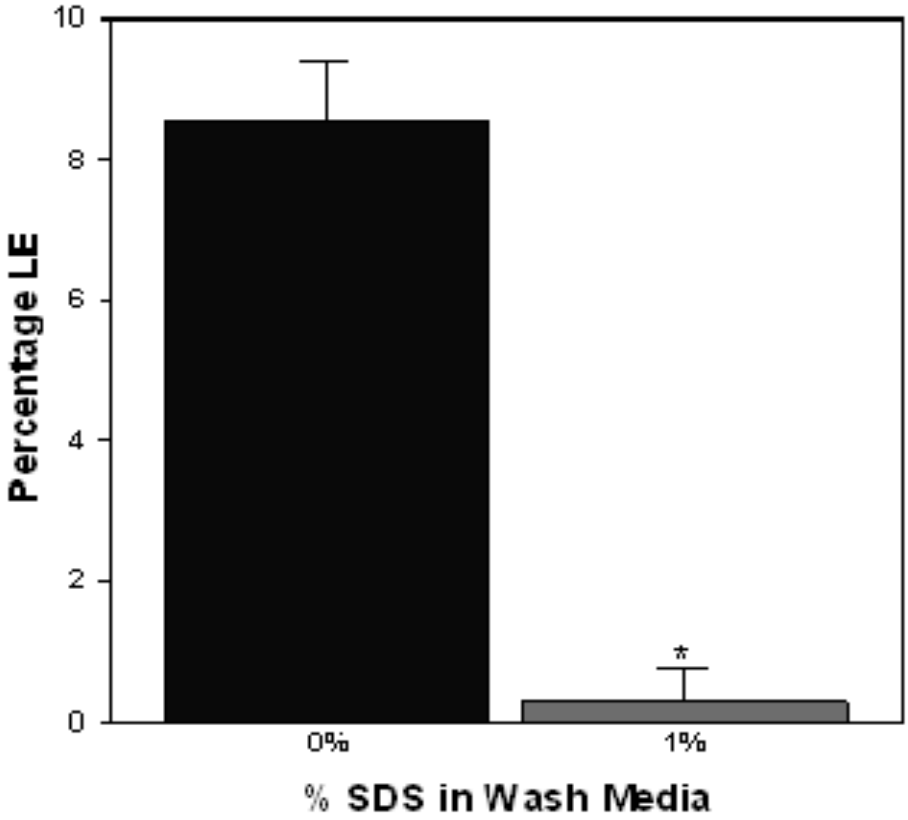


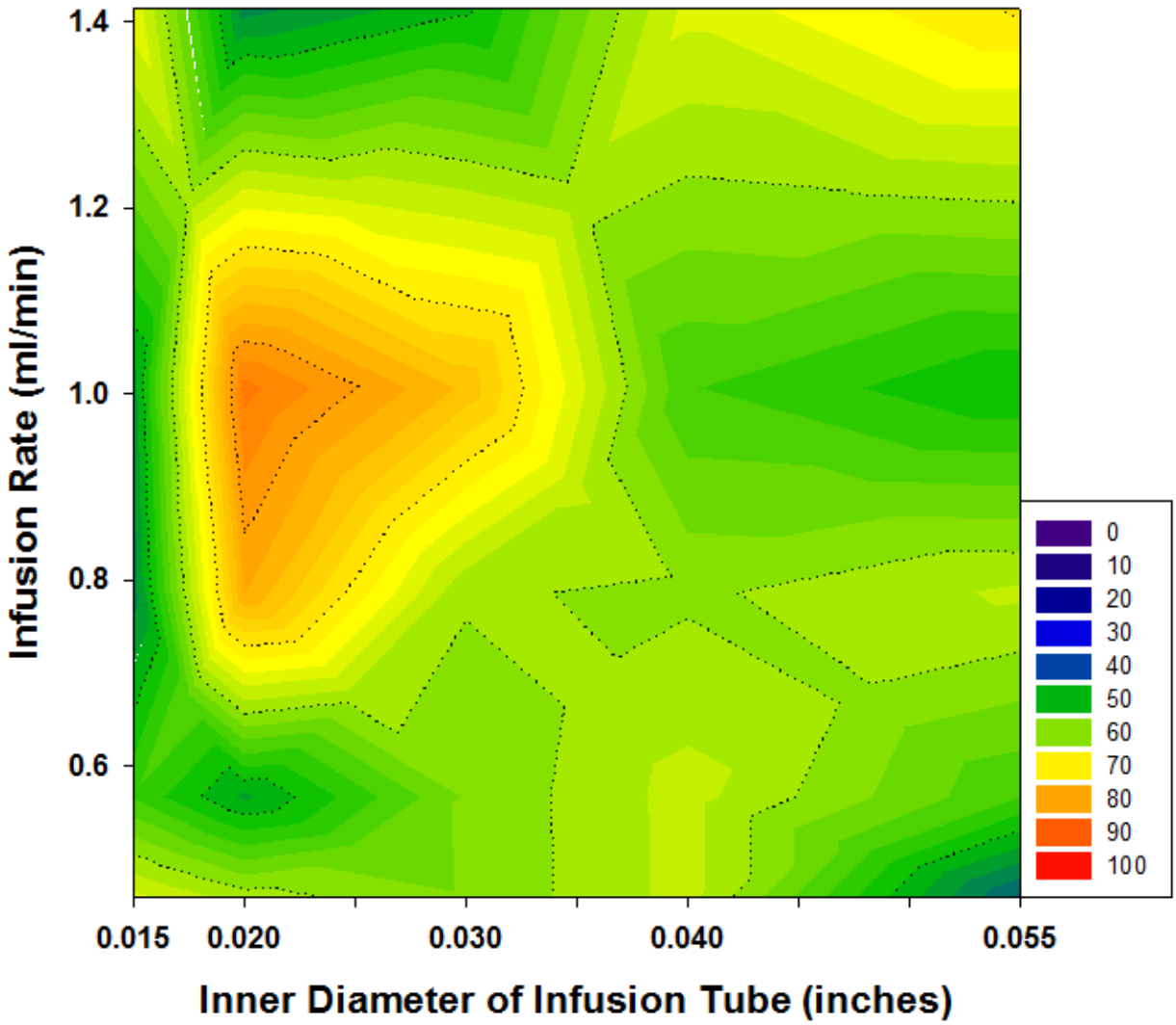
Figure 2-8. Initial burst of the LE-PLA microspheres prepared with 0% or 1% SDS in wash media (n=3). Significant difference determined by Student t-test ($P<0.05$).

Table 2-1. Experimental design (ID vs. IR)

Factor	Levels				
	1	2	3	4	5
Inner Diameter of Tube (inches)	0.015	0.020	0.030	0.040	0.055
Infusion Rate (mL/min)	0.5	0.6	0.8	1.0	1.4

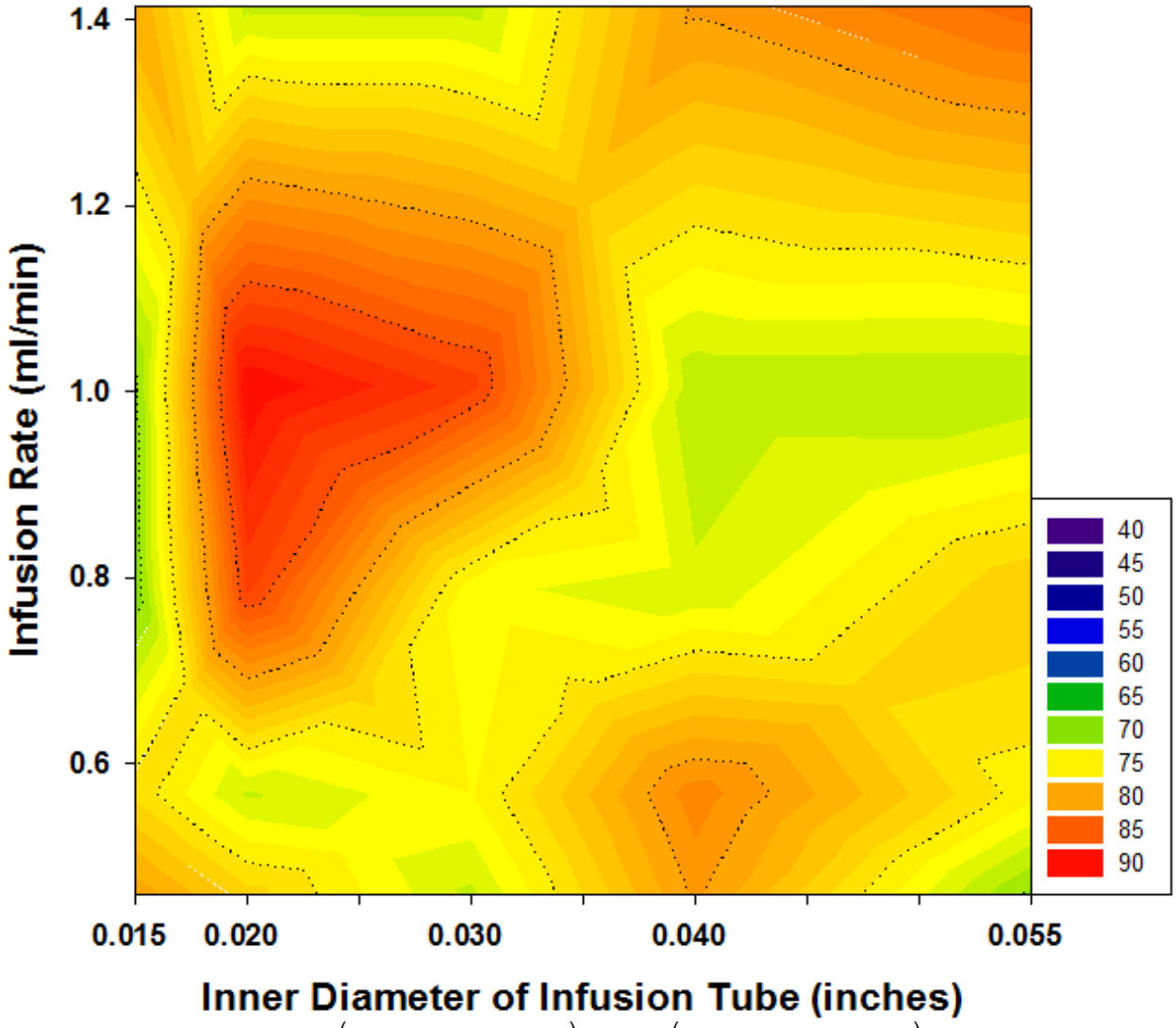
Table 2-2. Microsphere formulation variables and properties (ID vs. IR)

#	ID (inches)	IR (mL/min)	EE (%)	LE (%)	P_V (μm)	P_N (μm)	P_R
1	0.015	0.5	64.7	79.5	35.3	0.7	47.2
2	0.015	0.6	53.8	71.5	36.2	26.2	1.4
3	0.015	0.8	44.8	58.3	44.5	37.7	1.2
4	0.015	1.0	46.9	59.3	37.9	26.4	1.4
5	0.015	1.4	65.7	78.3	47.4	41.4	1.1
6	0.020	0.5	61.0	73.0	39.1	31.3	1.3
7	0.020	0.6	47.6	63.7	48.8	42.5	1.1
8	0.020	0.8	78.1	92.5	32.0	0.8	41.0
9	0.020	1.0	84.8	100.0	48.9	42.5	1.2
10	0.020	1.4	45.1	63.4	35.4	12.1	2.9
11	0.030	0.5	58.2	63.6	23.8	1.1	21.3
12	0.030	0.6	58.3	67.9	34.3	0.9	37.6
13	0.030	0.8	60.3	66.7	22.6	1.2	19.7
14	0.030	1.0	75.5	92.9	47.2	41.3	1.1
15	0.030	1.4	49.5	63.1	30.4	21.5	1.4
16	0.040	0.5	62.8	80.4	29.9	0.8	35.3
17	0.040	0.6	62.8	83.2	41.3	0.8	54.9
18	0.040	0.8	59.6	64.4	22.2	0.9	25.0
19	0.040	1.0	54.2	62.4	31.4	0.8	39.4
20	0.040	1.4	64.6	80.6	37.1	0.9	40.2
21	0.055	0.5	41.7	59.4	36.1	10.8	3.4
22	0.055	0.6	54.1	68.7	39.5	23.4	1.9
23	0.055	0.8	62.4	73.9	30.1	0.9	32.6
24	0.055	1.0	50.1	62.1	36.2	0.8	45.7
25	0.055	1.4	70.4	87.2	36.1	0.8	45.4



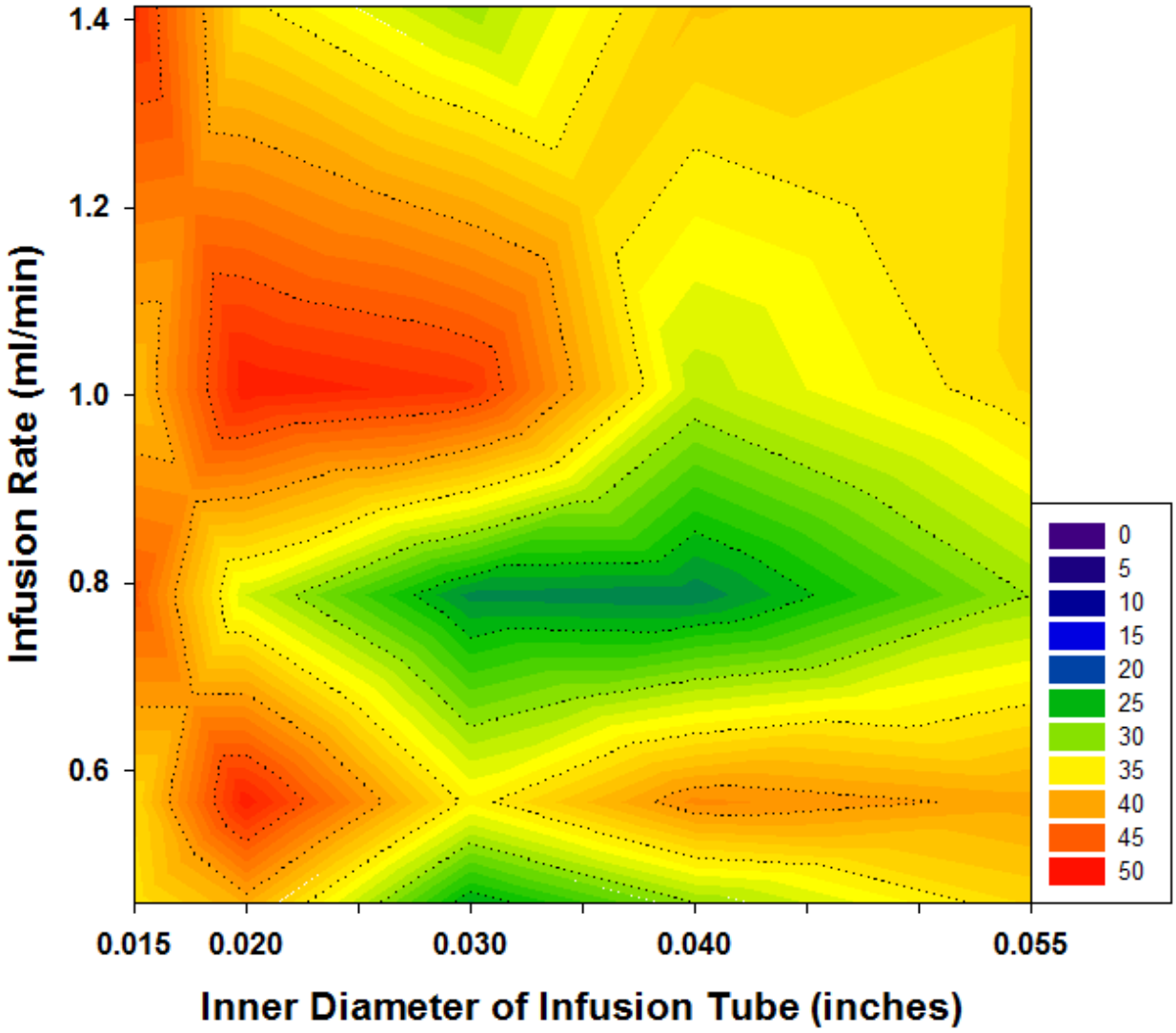
$$EE(\%) = \frac{64.8\%}{\left[1+\left(\frac{ID-0.03 inches}{0.06 inches}\right)^2\right]\cdot\left[1+\left(\frac{IR-1.03\,mL/\min}{1.74\,mL/\min}\right)^2\right]}, R^2 = 0.29$$

Figure 2-9. Encapsulation Efficiency (EE) of PLA microspheres prepared with variable inner diameter of infusion tube (ID) and infusion rate (IR)



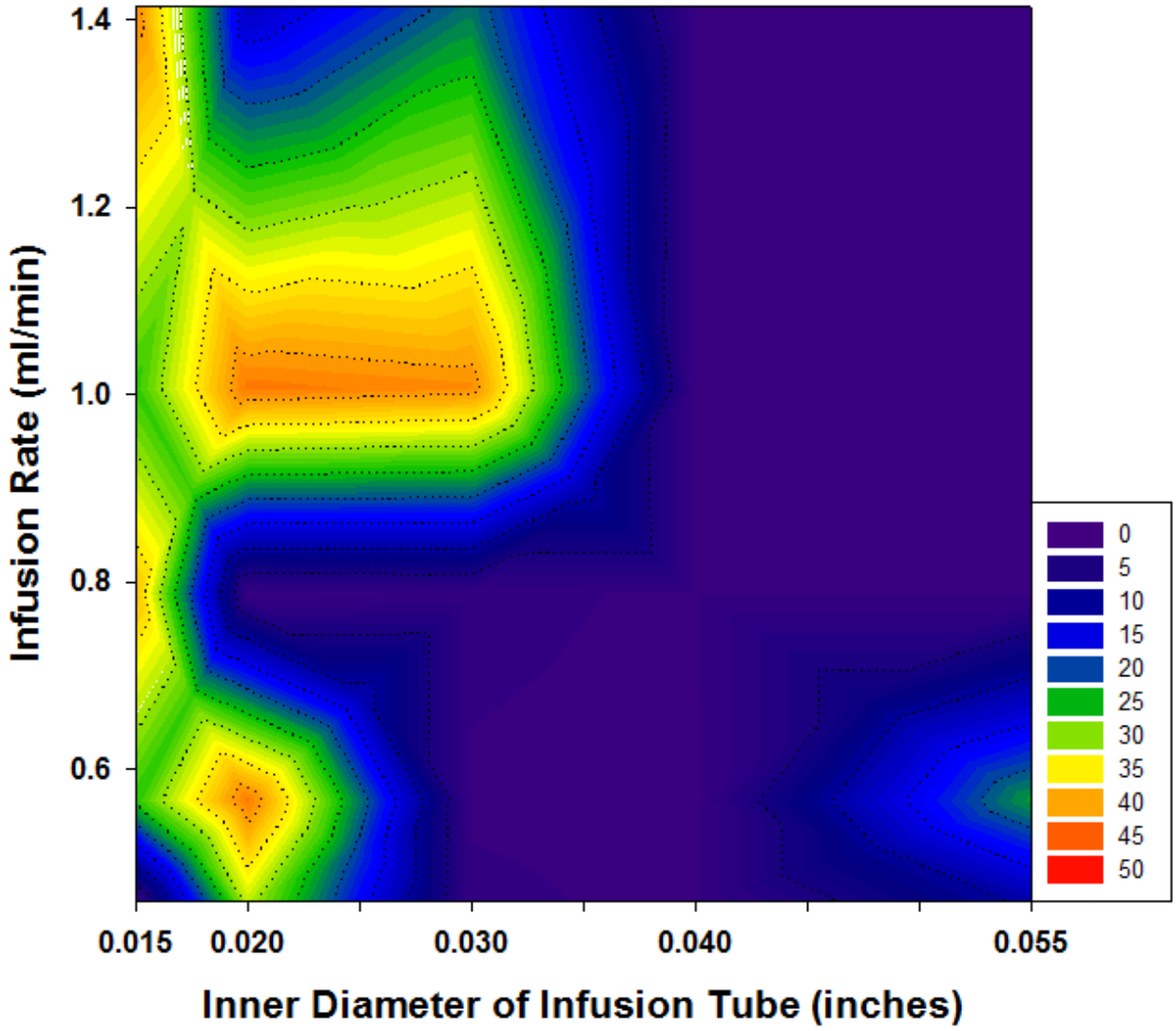
$$LdE(\%) = 61.14\% + \left(417.43\,\%/_{inches}\right)\cdot ID + \left(10.95\,\%\cdot \min/_{mL}\right)\cdot IR + \left(417.43\,\%/_{inches^2}\right)\cdot ID^2 + \left(10.95\,\%\cdot \min^2/_{mL^2}\right)\cdot IR^2 \qquad , R^2 = 0.18$$

Figure 2-10. Loading Efficiency (LdE) of PLA microspheres prepared with variable inner diameter of infusion tube (ID) and infusion rate (IR)



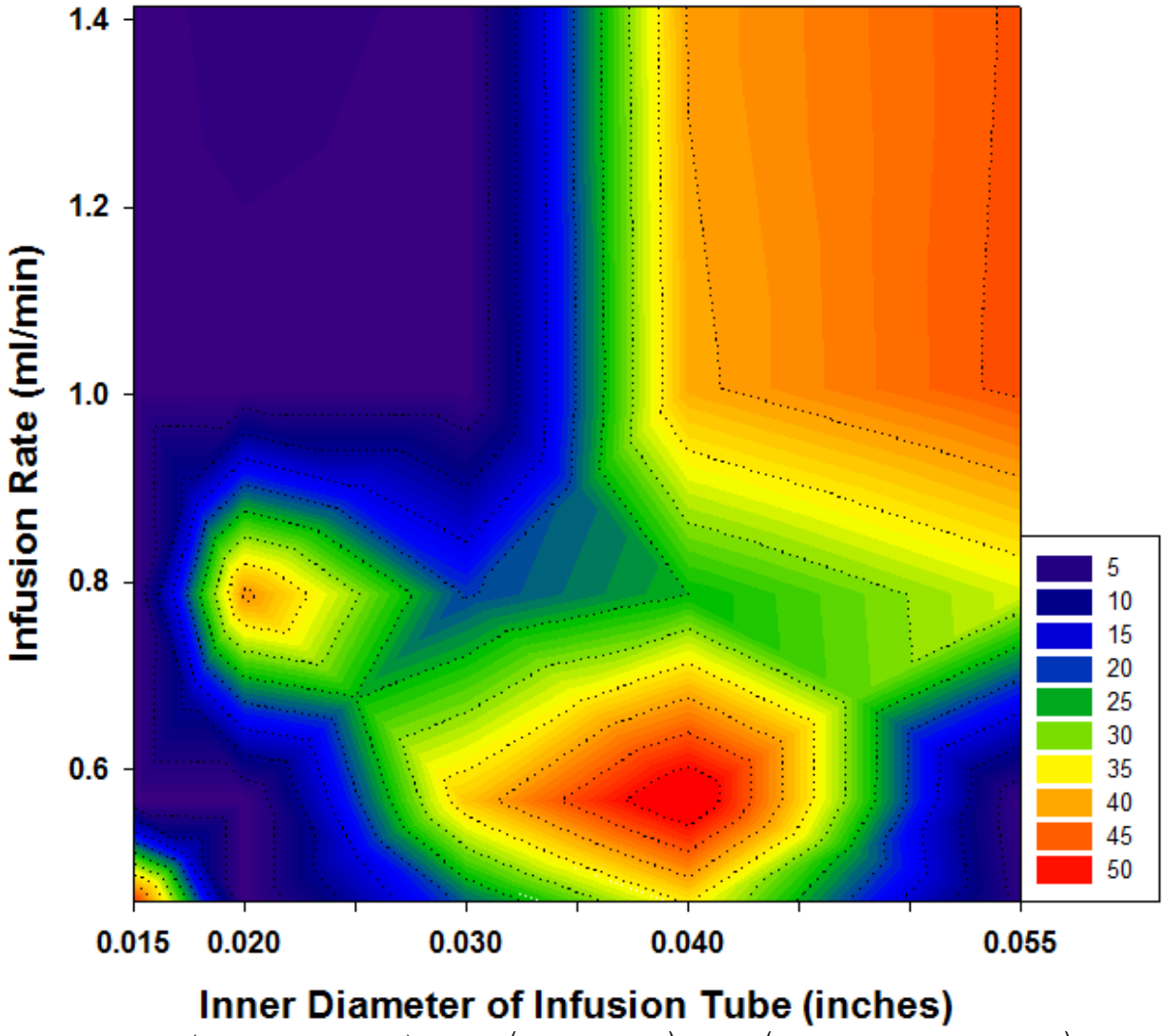
$$P_V(\mu m) = 53.03\mu m - \left(1270.87\,{}^{\mu m}/_{inches}\right)\cdot ID + \left(6.72\,{}^{\mu m\cdot \min}/_{mL}\right)\cdot IR$$
$$+\left(15972.2\,{}^{\mu m}/_{inches^2}\right)\cdot ID^2 - \left(1.86\,{}^{\mu m\cdot \min^2}/_{mL^2}\right)\cdot IR^2 \quad , R^2 = 0.49$$

Figure 2-11. Mean particle diameter based on volume distribution (P_V) of PLA microspheres prepared with variable inner diameter of infusion tube (ID) and infusion rate (IR)



$$
\begin{aligned}
{}^{1}\!/_{P_N\,(\mu m)} = {} & 3.4\,\mu m - \left(283.1\,{}^{\mu m}\!/_{inches}\right)\cdot ID - \left(0.7\,{}^{\mu m\cdot \min}\!/_{mL}\right)\cdot IR \\
& + \left(64.1\,{}^{\mu m\cdot \min}\!/_{mL\,\cdot\, inches}\right)\cdot ID\cdot IR + \left(9341\,{}^{\mu m}\!/_{inches^{\,2}}\right)\cdot ID^{2} \\
& - \left(1.7\,{}^{\mu m\cdot \min^{2}}\!/_{mL^{2}}\right)\cdot IR^{2} + \left(2499.7\,{}^{\mu m\cdot \min}\!/_{mL\,\cdot\, inches^{\,2}}\right)\cdot ID^{2}\cdot IR \\
& - \left(98.4\,{}^{\mu m\cdot \min^{2}}\!/_{mL^{2}\,\cdot\, inches}\right)\cdot ID\cdot IR^{2} - \left(118528.4\,{}^{\mu m}\!/_{inches^{\,3}}\right)\cdot ID^{3} \\
& + \left(1.6\,{}^{\mu m\cdot \min^{3}}\!/_{mL^{3}}\right)\cdot IR^{3} \qquad , R^{2} = 0.62
\end{aligned}
$$

Figure 2-12. Mean particle diameter based on number distribution (P_N) of PLA microspheres prepared with variable inner diameter of infusion tube (ID) and infusion rate (IR)



$$\sqrt{P_R} = 12.4 - \left(1143.6\, inches^{-1}\right)\cdot ID + \left(6.3\, {\min}/{mL}\right)\cdot IR + \left(195.7\, {\min}/{mL\cdot inches}\right)\cdot ID\cdot IR$$
$$+ \left(38804.5\, inches^{-2}\right)\cdot ID^2 - \left(17.2\, {\min^2}/{mL^2}\right)\cdot IR^2$$
$$+ \left(10452.6\, {\min}/{mL\cdot inches^2}\right)\cdot ID^2\cdot IR - \left(371.2\, {\min^2}/{mL^2\cdot inches}\right)\cdot ID\cdot IR^2$$
$$- \left(491990\, inches^{-3}\right)\cdot ID^3 + \left(10.1\, {\min^3}/{mL^3}\right)\cdot IR^3 \quad , R^2 = 0.61$$

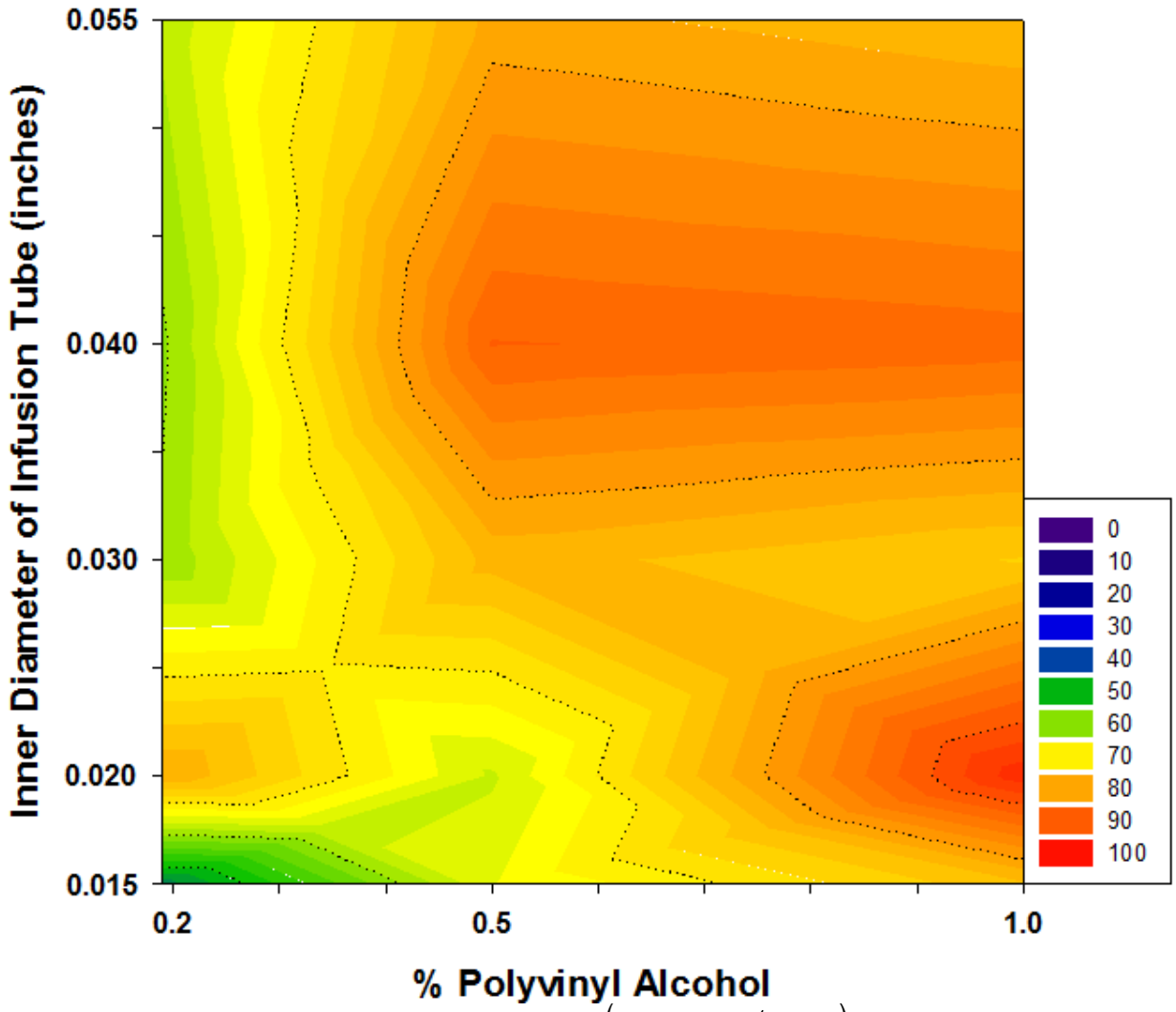
Figure 2-13. Mean particle diameter ratio (P_R) of PLA microspheres prepared with variable inner diameter of infusion tube (ID) and infusion rate (IR)

Table 2-3. Experimental design (PVA vs. ID)

Factor	Levels				
	1	2	3	4	5
Polyvinyl Alcohol (%)	0.2	0.5	1.0	--	--
Inner Diameter of Tube (inches)	0.015	0.020	0.030	0.040	0.055

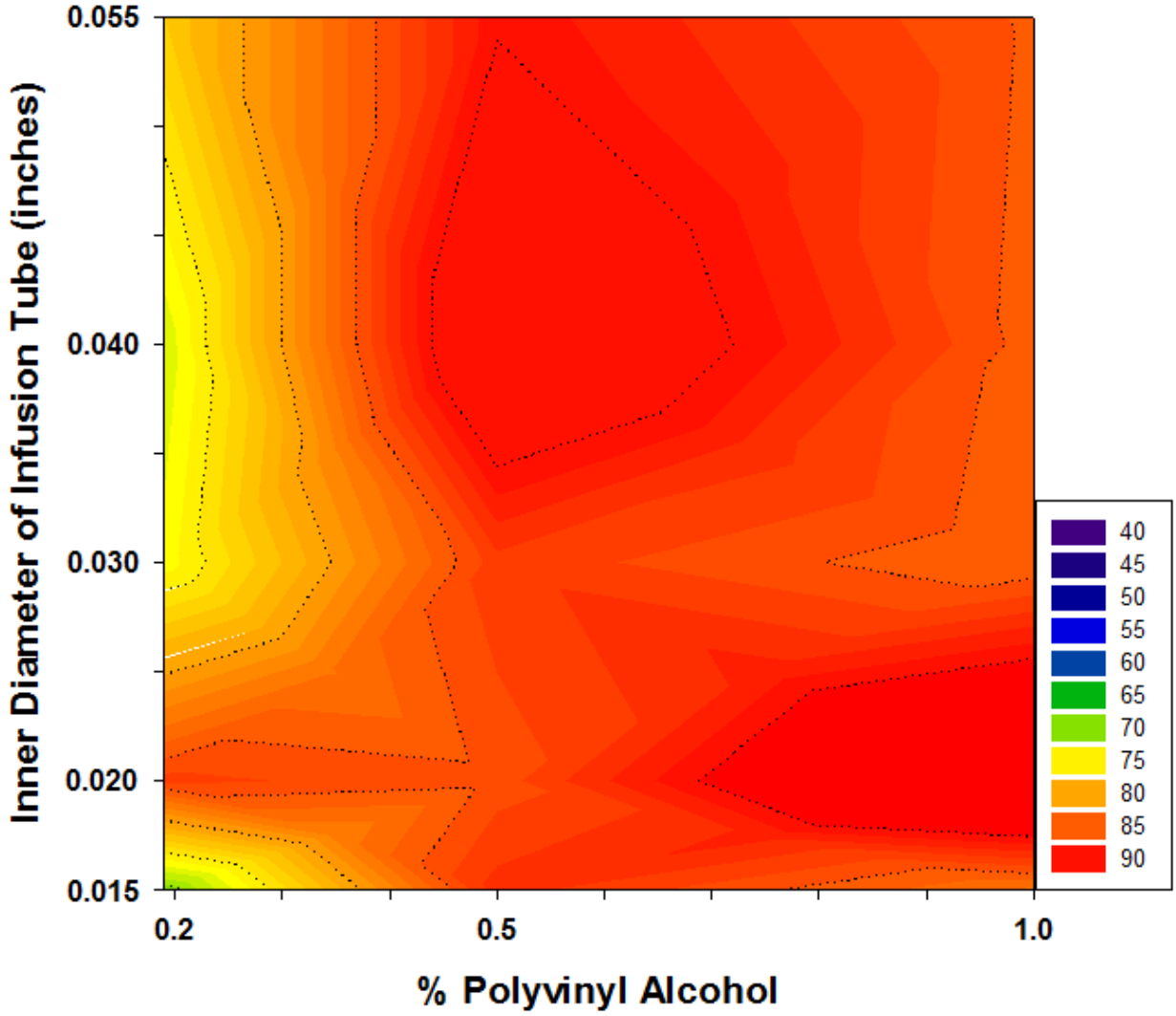
Table 2-4. Microsphere formulation variables and properties (PVA vs. ID)

#	PVA (%)	ID (inches)	EE (%)	LE (%)	P_V (μm)	P_N (μm)	P_R
1	0.2	0.015	44.8	58.3	44.5	37.7	1.2
2	0.2	0.020	78.1	92.5	32.0	0.8	41.0
3	0.2	0.030	60.3	66.7	22.6	1.2	19.7
4	0.2	0.040	59.6	64.4	22.2	0.9	25.0
5	0.2	0.055	62.4	73.9	30.1	0.9	32.6
6	0.5	0.015	65.8	94.9	48.8	44.3	1.1
7	0.5	0.020	63.7	90.9	46.7	41.0	1.1
8	0.5	0.030	76.8	93.2	27.3	0.8	34.1
9	0.5	0.040	88.2	108.6	43.1	33.3	1.3
10	0.5	0.055	78.7	99.4	40.3	26.1	1.5
11	1.0	0.015	75.6	85.7	40.7	27.6	1.5
12	1.0	0.020	95.5	115.2	41.1	26.5	1.6
13	1.0	0.030	73.9	88.0	38.6	26.7	1.4
14	1.0	0.040	87.0	89.0	43.7	35.7	1.2
15	1.0	0.055	76.4	89.5	35.8	0.5	66.2



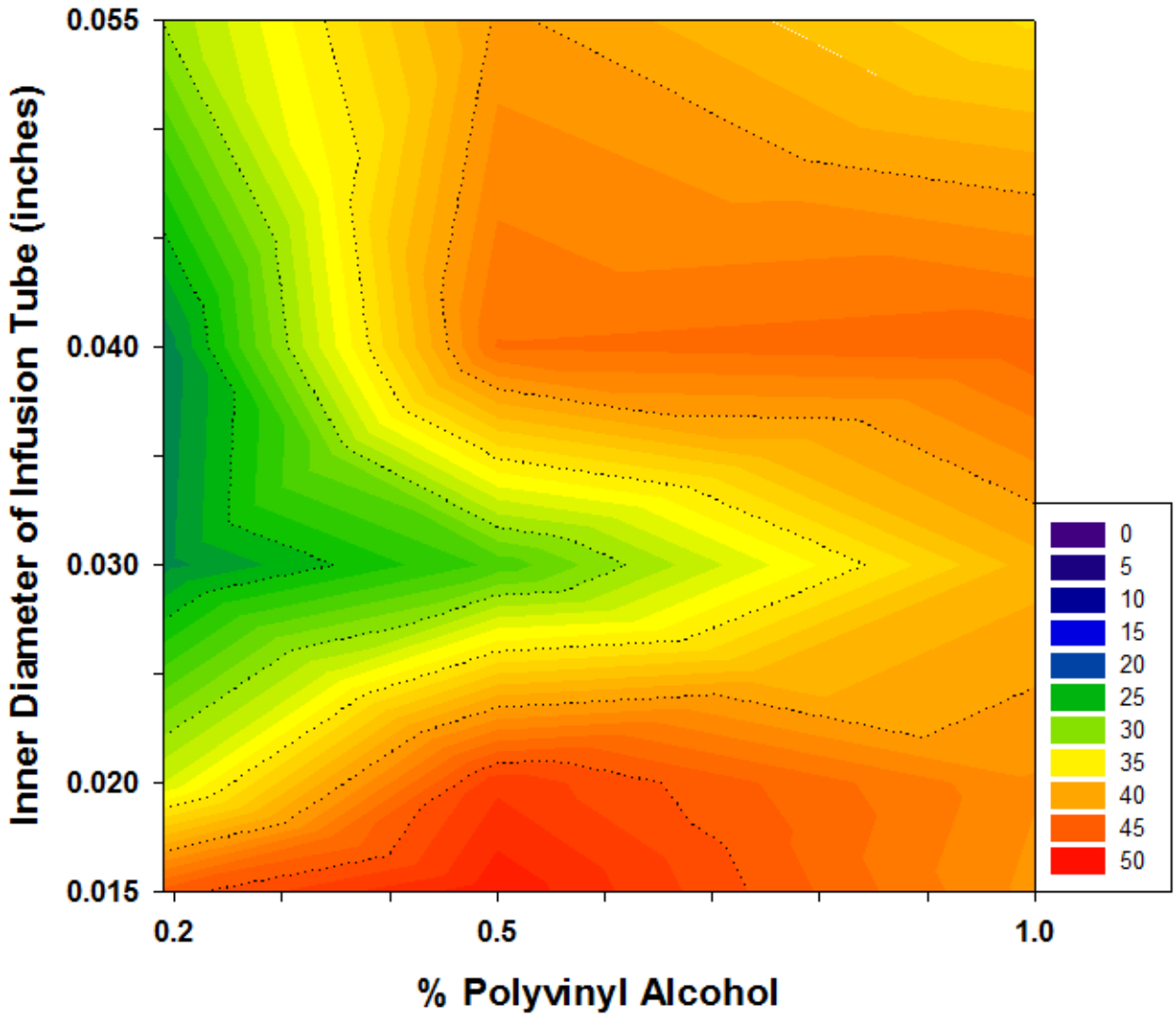
$$EE(\%) = 26.57\% + 69.25 \cdot PVA + \left(1396.2\,\%/_{inches}\right) \cdot ID - \left(36.8\,\%/_{\%}\right) \cdot PVA^2 - \left(17916.1\,\%/_{inches^2}\right) \cdot ID^2 \quad , R^2 = 0.73$$

Figure 2-14. Encapsulation efficiency (EE) of PLA microspheres prepared with variable % of polyvinyl alcohol (PVA) and inner diameter of infusion tube (ID)



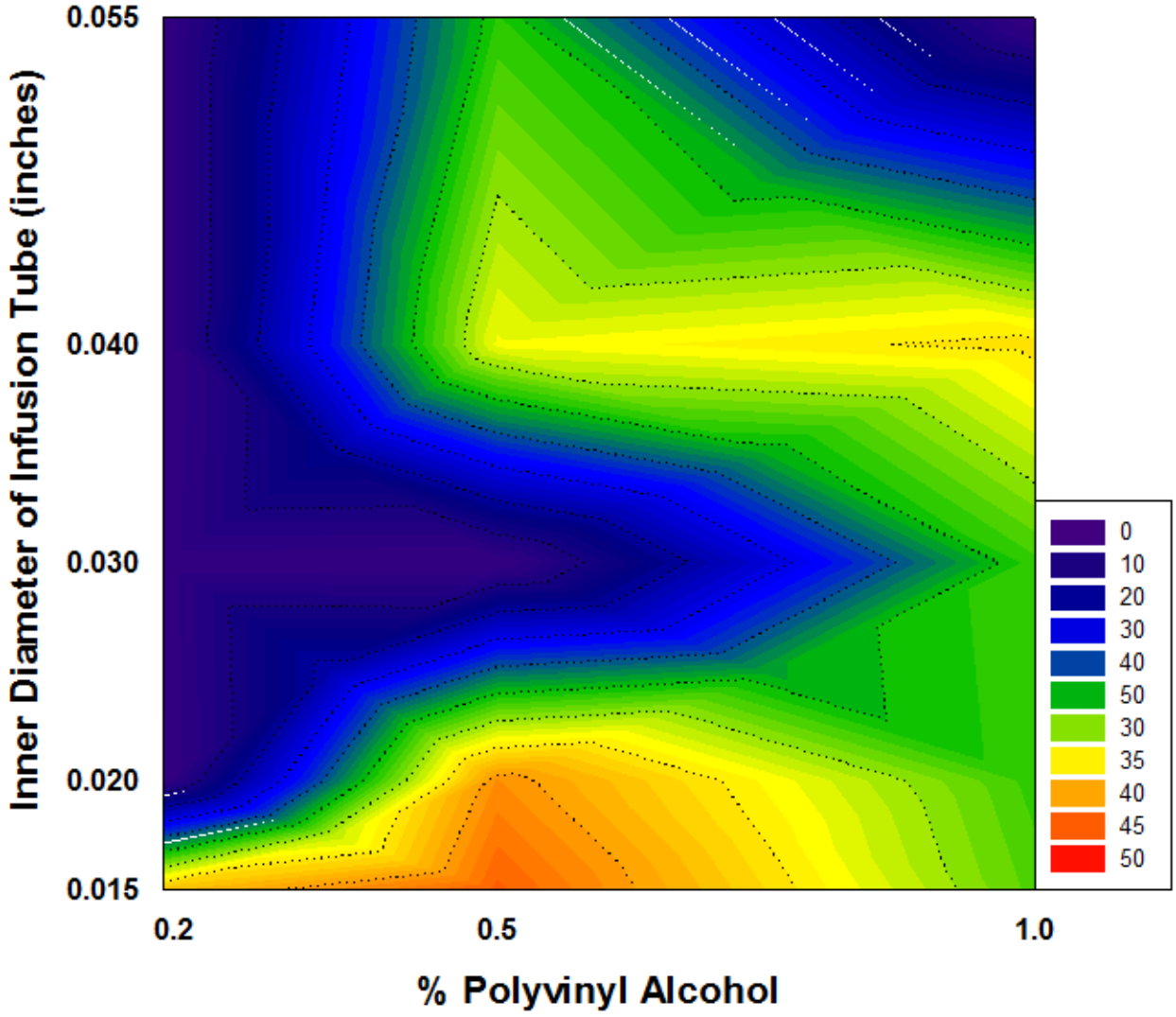
$$LdE(\%) = 41.61\% + 163.41 \cdot PVA + \left(181.64\,\%/_{inches}\right) \cdot ID - \left(114.16/_{\%}\right) \cdot PVA^2 - \left(2601.6\,\%/_{inches^2}\right) \cdot ID^2 \quad , R^2 = 0.76$$

Figure 2-15. Loading efficiency (LdE) of PLA microspheres prepared with variable % of polyvinyl alcohol (PVA) and inner diameter of infusion tube (ID)



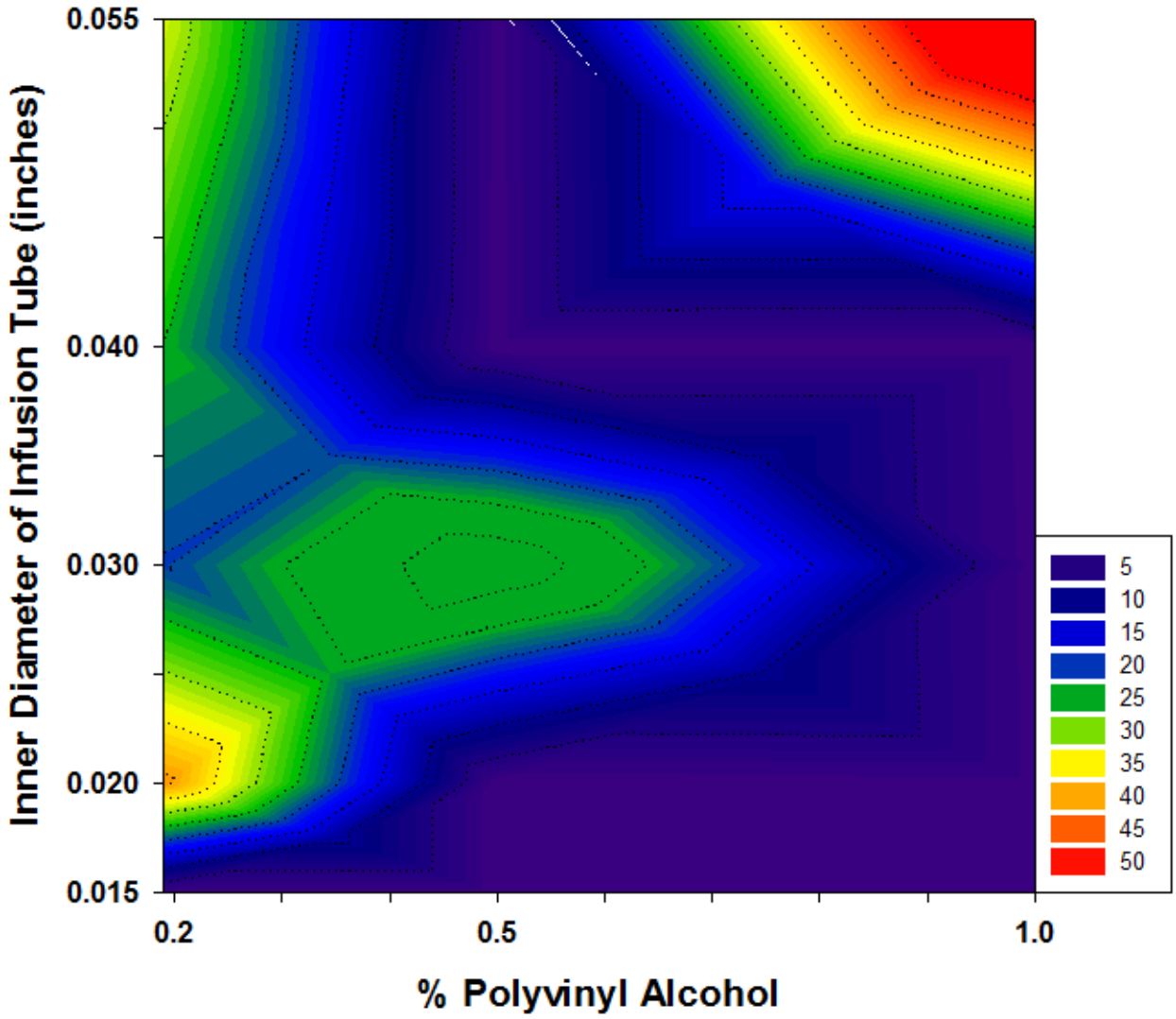
$$
\begin{aligned}
{}^{1}\!/_{P_V\,(\mu m)} = &-0.05\,\mu m + \left(0.04\,{}^{\mu m}\!/_{\%}\right)\cdot PVA - \left(7.38\,{}^{\mu m}\!/_{inches}\right)\cdot ID \\
&- \left(5.45\,{}^{\mu m}\!/_{\% \cdot inches}\right)\cdot PVA\cdot ID + \left(0.02\,{}^{\mu m}\!/_{\%^2}\right)\cdot PVA^2 \\
&- \left(152.28\,{}^{\mu m}\!/_{inches^2}\right)\cdot ID^2 + \left(0.78\,{}^{\mu m}\!/_{\%^2 \cdot inches}\right)\cdot PVA^2\cdot ID \\
&+ \left(60.88\,{}^{\mu m}\!/_{\% \cdot inches^2}\right)\cdot PVA\cdot ID^2 + \left(931.63\,{}^{\mu m}\!/_{inches^3}\right)\cdot ID^3, \\
R^2 = &\ 0.89
\end{aligned}
$$

Figure 2-16. Mean particle diameter based on volume distribution (P_V) of PLA microspheres prepared with variable % of polyvinyl alcohol (PVA) and inner diameter of infusion tube (ID)



$$P_N(\mu m) = 238.59\mu m - \left(41.27\,{}^{\mu m}/_{\%}\right)\cdot PVA - \left(21752.66\,{}^{\mu m}/_{inches}\right)\cdot ID$$
$$+\left(10434.83\,{}^{\mu m}/_{\%\cdot inches}\right)\cdot PVA\cdot ID - \left(56.50\,{}^{\mu m}/_{\%^2}\right)\cdot PVA^2$$
$$+\left(5.53E5\,{}^{\mu m}/_{inches^2}\right)\cdot ID^2 - \left(1387.94\,{}^{\mu m}/_{\%^2\cdot inches}\right)\cdot PVA^2\cdot ID$$
$$-\left(1.24E5\,{}^{\mu m}/_{\%\cdot inches^2}\right)\cdot PVA\cdot ID^2 - \left(4.43E6\,{}^{\mu m}/_{inches^3}\right)\cdot ID^3,$$
$$R^2 = 0.82$$

Figure 2-17. Mean particle diameter based on number distribution (P_N) of PLA microspheres prepared with variable % of polyvinyl alcohol (PVA) and inner diameter of infusion tube (ID)



$$1/P_R = 5.26 - \left(1.99\,\%^{-1}\right)\cdot PVA - \left(467.32\,inches^{-1}\right)\cdot ID + \left(267.53\,(\%\cdot inches)^{-1}\right)\cdot PVA\cdot ID - \left(0.38\,\%^{-2}\right)\cdot PVA^2 + \left(11654.90\,inches^{-2}\right)\cdot ID^2 - \left(54.97\,\%^{-2}\cdot inches^{-1}\right)\cdot PVA^2\cdot ID - \left(2880.55\,\%^{-1}\cdot inches^{-2}\right)\cdot PVA\cdot ID^2 - \left(92505.10\,inches^{-3}\right)\cdot ID^3,\ R^2 = 0.80$$

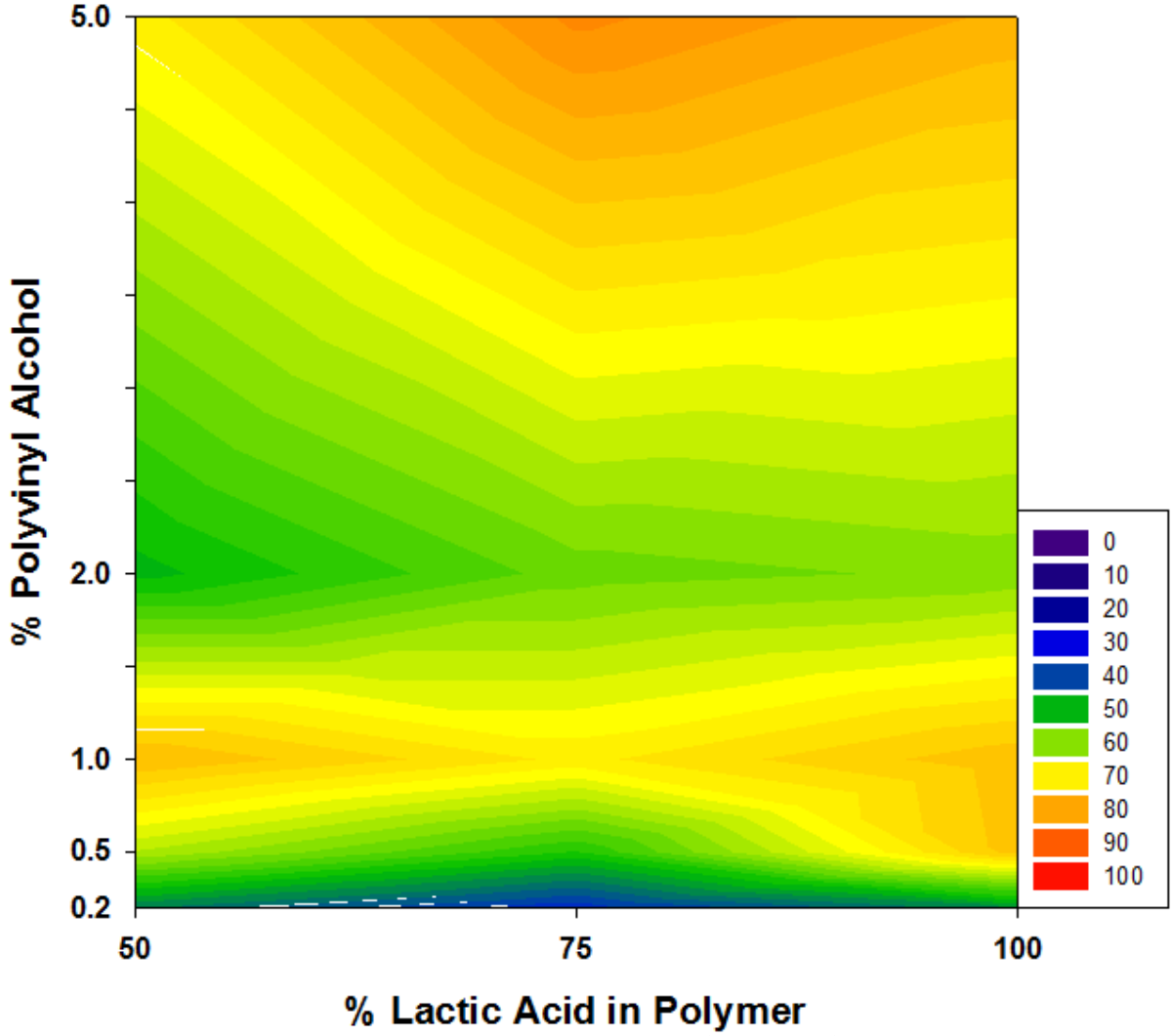
Figure 2-18. Mean particle diameter ratio (P_R) of PLA microspheres prepared with variable % of polyvinyl alcohol (PVA) and inner diameter of infusion tube (ID)

Table 2-5. Experimental design (LA vs. PVA)

Factor	Levels				
	1	2	3	4	5
Lactic Acid composition (%)	50	75	100	--	--
Polyvinyl Alcohol (%)	0.2	0.5	1.0	2.0	5.0

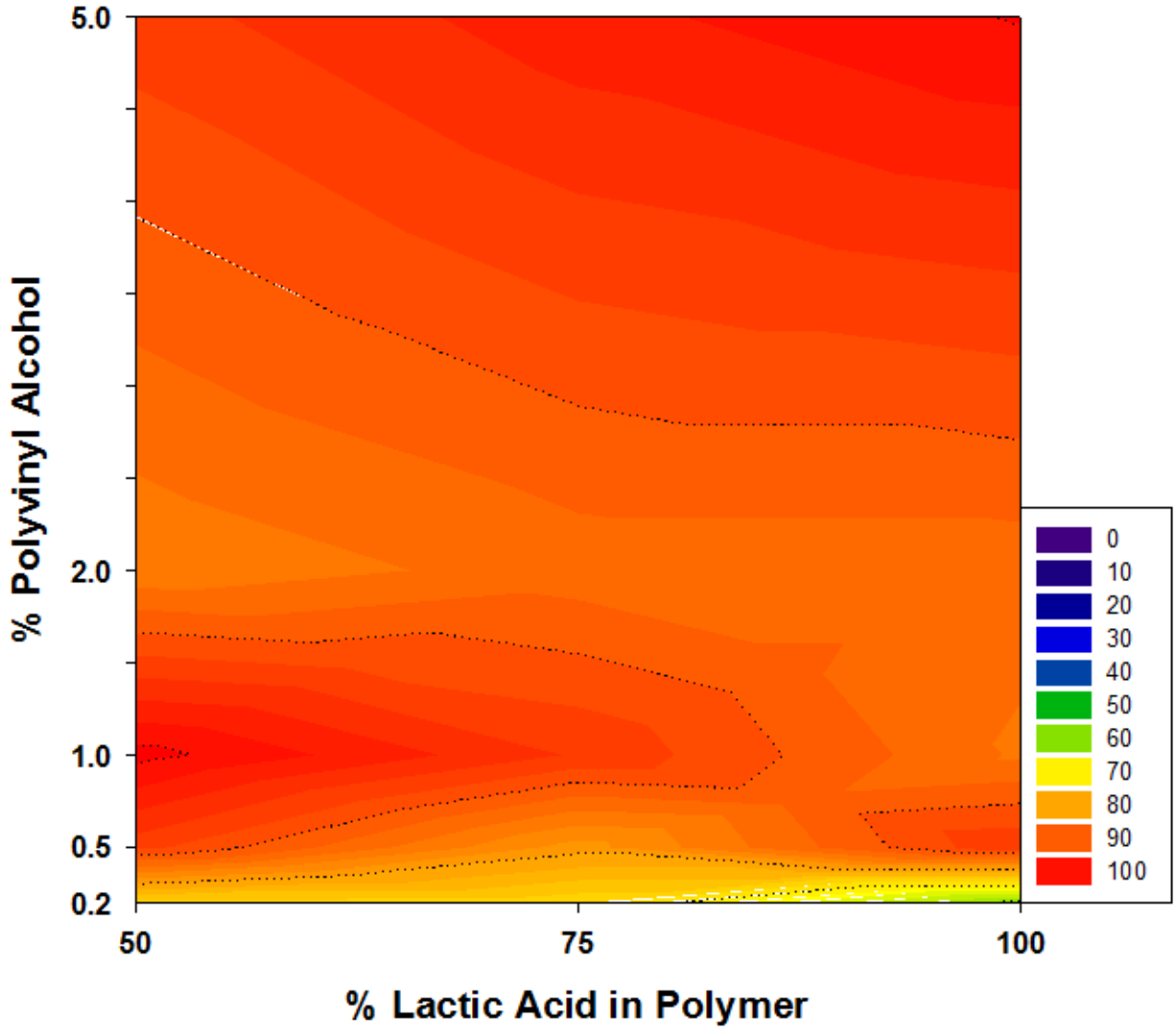
Table 2-6. Microsphere formulation variables and physical parameters (LA vs. PVA)

#	LA (%)	PVA (%)	EE (%)	LE (%)	P_V (μm)	P_N (μm)	P_R
1	50	0.2	43.6	73.0	37.7	16.1	2.4
2	50	0.5	61.9	92.9	37.8	20.2	1.9
3	50	1.0	76.2	100.9	40.9	27.4	1.5
4	50	2.0	49.1	84.5	27.9	7.1	3.9
5	50	5.0	69.0	93.1	8.0	2.8	2.8
6	75	0.2	35.7	72.2	32.2	0.8	41.1
7	75	0.5	51.8	81.0	38.2	27.2	1.4
8	75	1.0	69.4	93.7	38.9	22.4	1.8
9	75	2.0	57.0	86.9	31.3	10.4	3.0
10	75	5.0	82.7	97.3	11.5	3.5	3.2
11	100	0.2	44.8	58.3	44.5	37.7	1.2
12	100	0.5	75.0	93.9	49.3	42.6	1.2
13	100	1.0	75.6	85.7	40.7	27.6	1.5
14	100	2.0	58.6	86.9	22.2	0.9	25.0
15	100	5.0	77.5	100.2	17.0	4.6	3.8



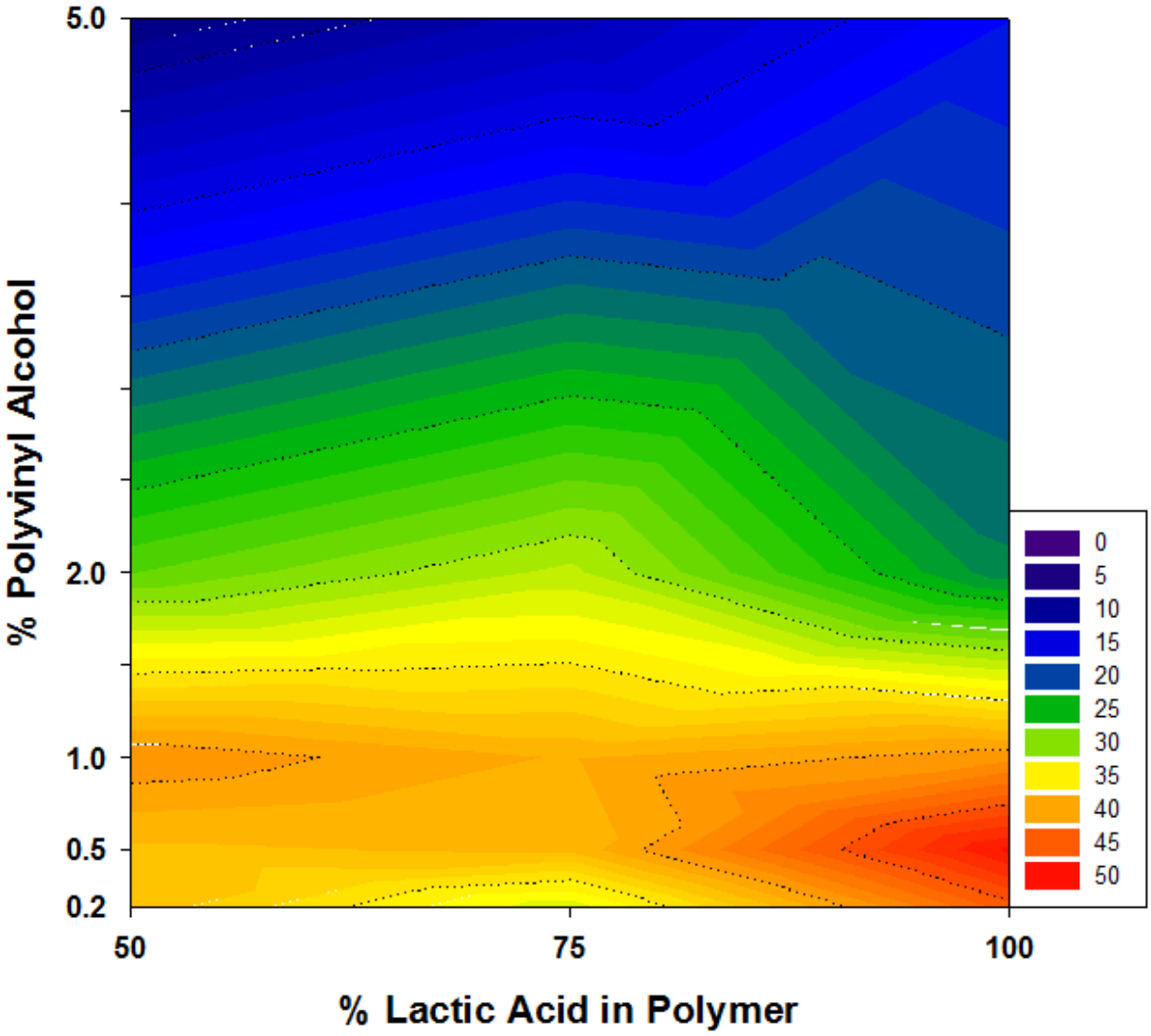
$$EE(\%) = 116.86\% - 2.77 \cdot LA + 61.03 \cdot PVA + \left(1.16\%^{-1}\right) \cdot LA \cdot PVA + \left(0.02\%^{-1}\right) \cdot LA^2 - \left(60.37\%^{-1}\right) \cdot PVA^2 - \left(0.741E-3\%^{-2}\right) \cdot LA^2 \cdot PVA - \left(5.88E-3\%^{-2}\right) \cdot LA \cdot PVA^2 + \left(8.45\%^{-2}\right) \cdot PVA^3, R^2 = 0.95$$

Figure 2-19. Encapsulation efficiency (EE) of PLA microspheres prepared with variable % of lactic acid in the PLGA polymer (LA) and % of polyvinyl alcohol (PVA) in the aqueous phase



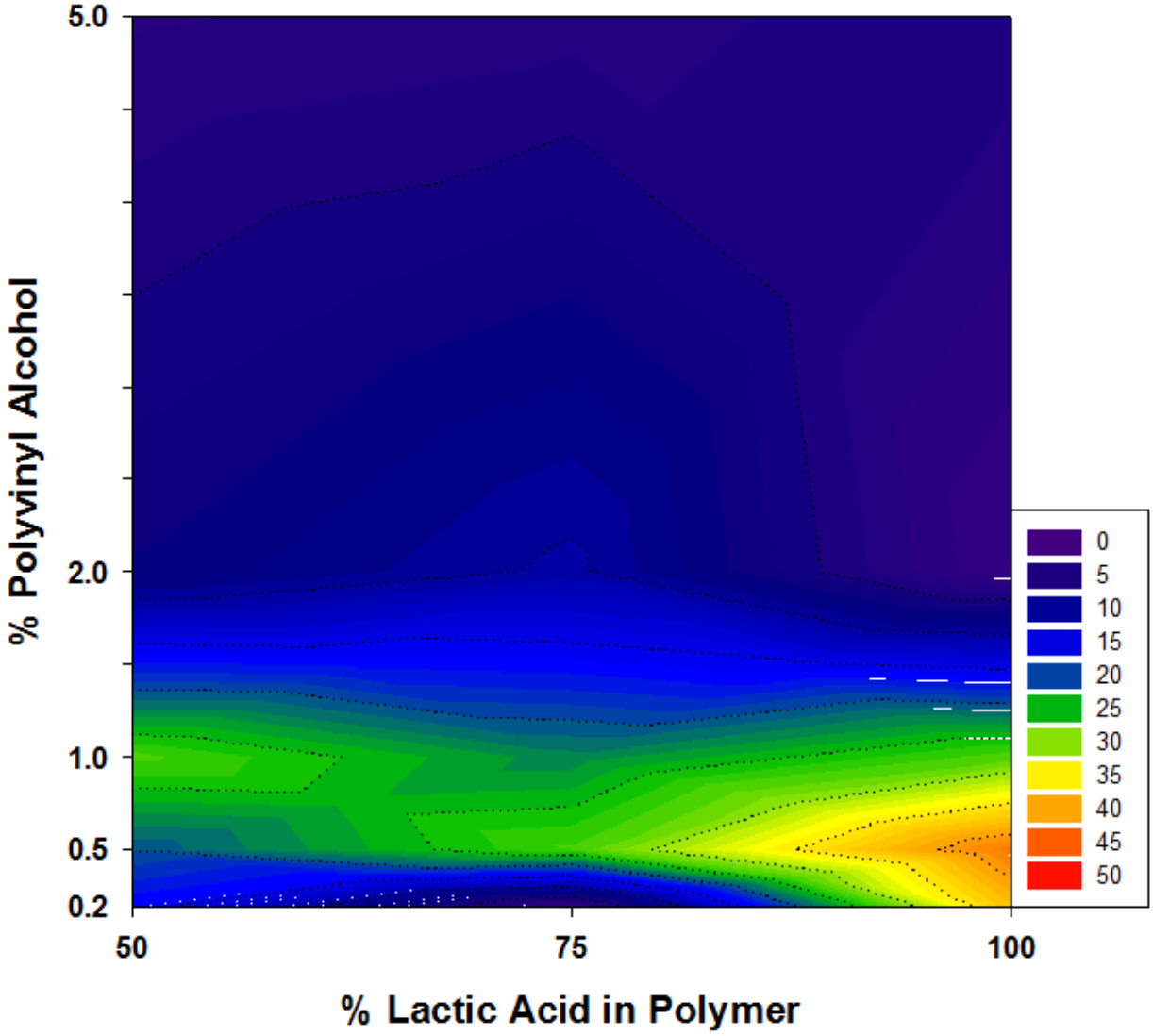
$$LdE(\%) = 88.27\% - 0.63 \cdot LA + 61.80 \cdot PVA + \left(0.26\%^{-1}\right) \cdot LA \cdot PVA + \left(2.52E-3\%^{-1}\right) \cdot LA^2 - \left(41.72\%^{-1}\right) \cdot PVA^2 - \left(7.84E-4\%^{-2}\right) \cdot LA^2 \cdot PVA - \left(0.01\%^{-2}\right) \cdot LA \cdot PVA^2 + \left(5.79\%^{-2}\right) \cdot PVA^3, R^2 = 0.83$$

Figure 2-20. Loading efficiency (LdE) of PLA microspheres prepared with variable % of lactic acid in the PLGA polymer (LA) and % of polyvinyl alcohol (PVA) in the aqueous phase



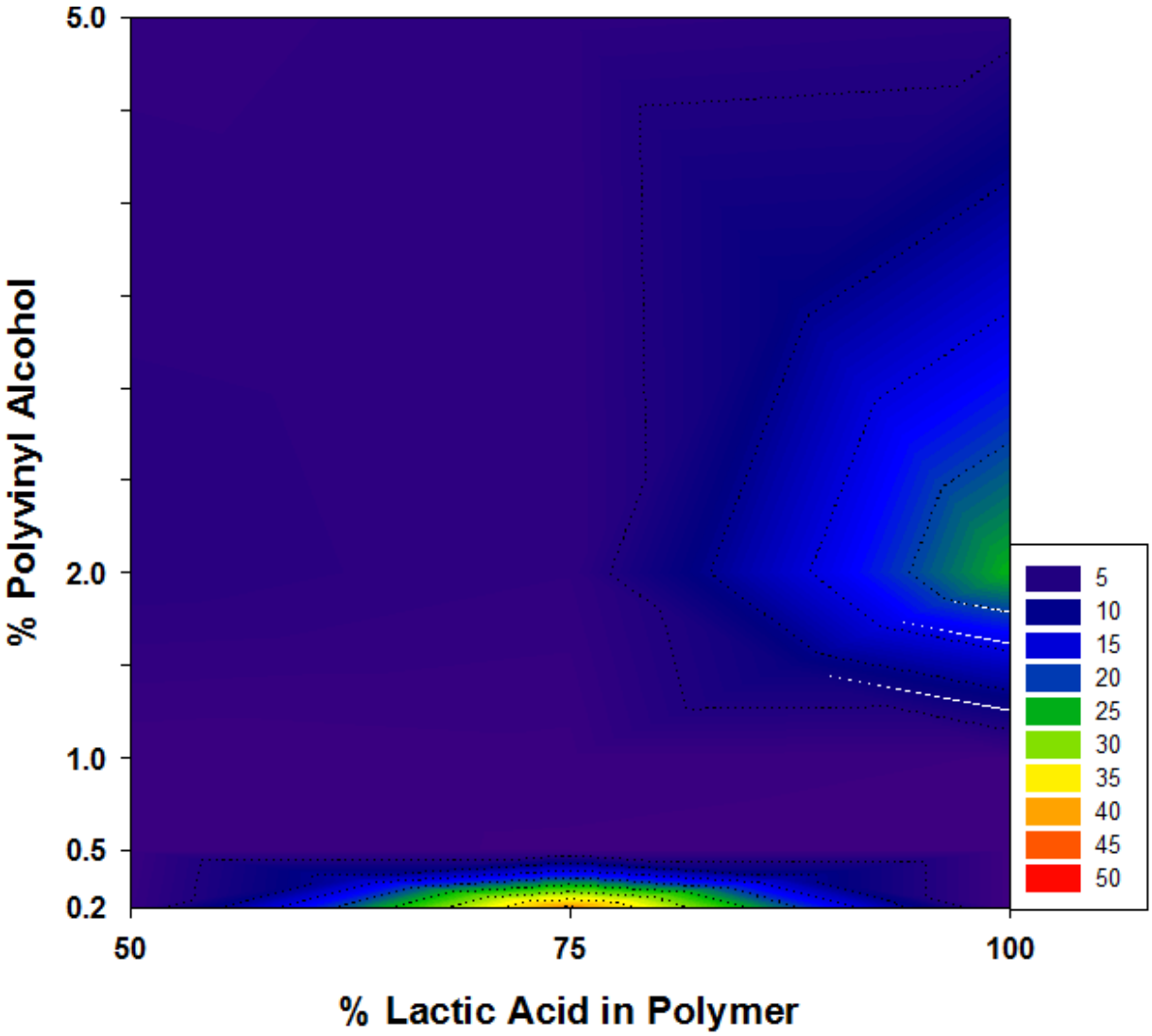
$$P_V(\mu m) = 0.05\mu m - \left(2.51E-4\,{}^{\mu m}/_{\%}\right)\cdot LA - \left(0.05\,{}^{\mu m}/_{\%}\right)\cdot PVA$$
$$+\left(4.57E-4\,{}^{\mu m}/_{\%^2}\right)\cdot LA\cdot PVA + \left(0.02\,{}^{\mu m}/_{\%^2}\right)\cdot PVA^2$$
$$-\left(1.34E-4\,{}^{\mu m}/_{\%^3}\right)\cdot LA\cdot PVA^2 - \left(1.49E-3\,{}^{\mu m}/_{\%^3}\right)\cdot PVA^3, R^2 = 0.99$$

Figure 2-21. Mean particle diameter based on volume distribution (P_V) of PLA microspheres prepared with variable % of lactic acid in the PLGA polymer (LA) and % of polyvinyl alcohol (PVA) in the aqueous phase



$$P_N(\mu m) = 65.62\mu m - \left(2.21\,{}^{\mu m}/_{\%}\right)\cdot LA + \left(57.58\,{}^{\mu m}/_{\%}\right)\cdot PVA + \left(0.25\,{}^{\mu m}/_{\%^2}\right)\cdot LA\cdot PVA$$
$$+\left(0.02\,{}^{\mu m}/_{\%^2}\right)\cdot LA^2 - \left(40.06\,{}^{\mu m}/_{\%^2}\right)\cdot PVA^2 - \left(5.30E-3\,{}^{\mu m}/_{\%^3}\right)\cdot LA^2\cdot PVA$$
$$+\left(0.09\,{}^{\mu m}/_{\%^3}\right)\cdot LA\cdot PVA^2 + \left(4.86\,{}^{\mu m}/_{\%^3}\right)\cdot PVA^3,\ R^2 = 0.86$$

Figure 2-22. Mean particle diameter based on number distribution (P_N) of PLA microspheres prepared with variable % of lactic acid in the PLGA polymer (LA) and % of polyvinyl alcohol (PVA) in the aqueous phase



$$1/P_R = 1.28 - \left(0.04\%^{-1}\right)\cdot LA + \left(1.33\%^{-1}\right)\cdot PVA + \left(5.01E-3\%^{-2}\right)\cdot LA\cdot PVA + \left(3.44E-4\%^{-2}\right)\cdot LA^2 - \left(0.90\%^{-2}\right)\cdot PVA^2 - \left(1.08E-4\%^{-3}\right)\cdot LA^2\cdot PVA + \left(1.73E-3\%^{-3}\right)\cdot LA\cdot PVA^2 + \left(0.11\%^{-3}\right)\cdot PVA^3, R^2 = 0.77$$

Figure 2-23. Mean particle diameter ratio (P_R) of PLA microspheres prepared with variable % of lactic acid in the PLGA polymer (LA) and % of polyvinyl alcohol (PVA) in the aqueous phase

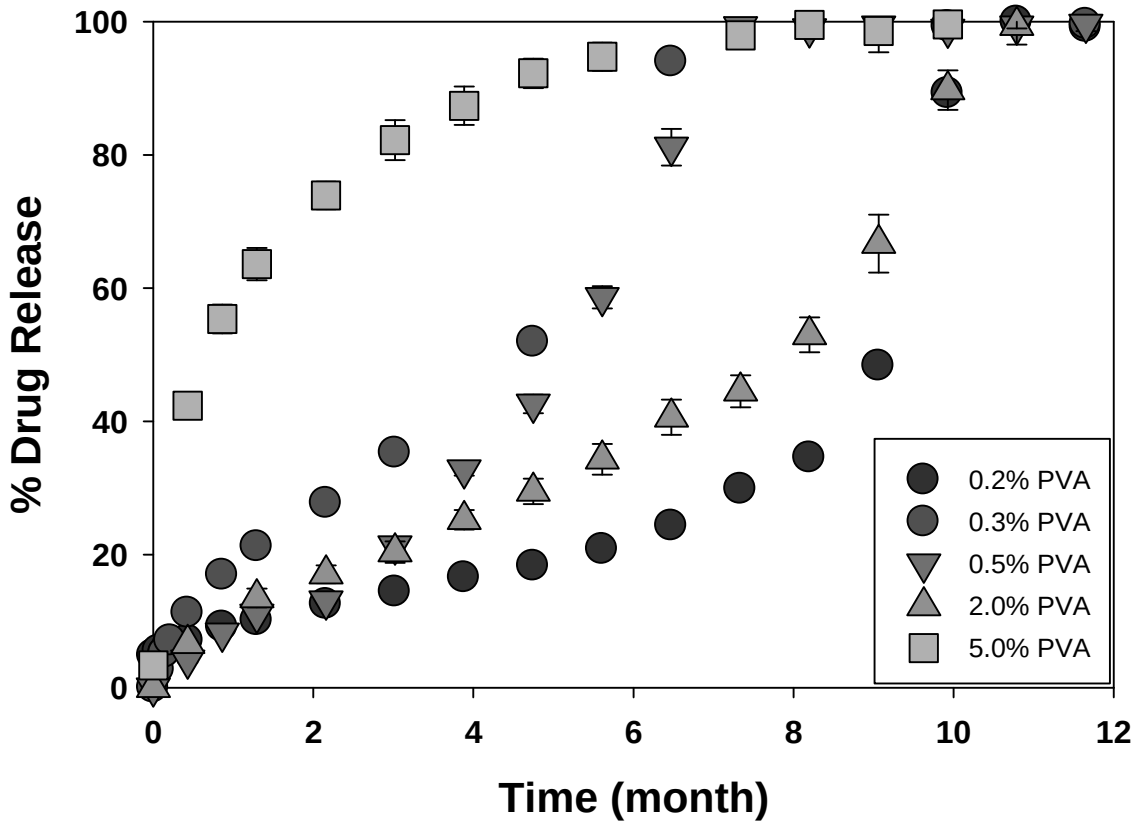


Figure 2-24. *In vitro* drug release profile for LE-PLGA (50:50) microspheres prepared with variable % of polyvinyl alcohol (PVA)

Table 2-7. Particle size properties of LE-PLGA (50:50) microspheres

Formulation	P_V (μm)	P_N (μm)	P_R
0.2% PVA	37.7 (± 1.3)	16.1 (± 2.1)	2.4 (± 0.2)
0.3% PVA	42.4 (± 0.2)	27.5 (± 0.4)	1.5 (± 0.0)
0.5% PVA	37.8 (± 0.3)	20.2 (± 0.9)	1.9 (± 0.1)
2.0% PVA	27.9 (± 2.0)	7.1 (± 0.8)	3.9 (± 0.2)
5.0% PVA	8.0 (± 0.1)	2.8 (± 0.0)	2.8 (± 0.0)

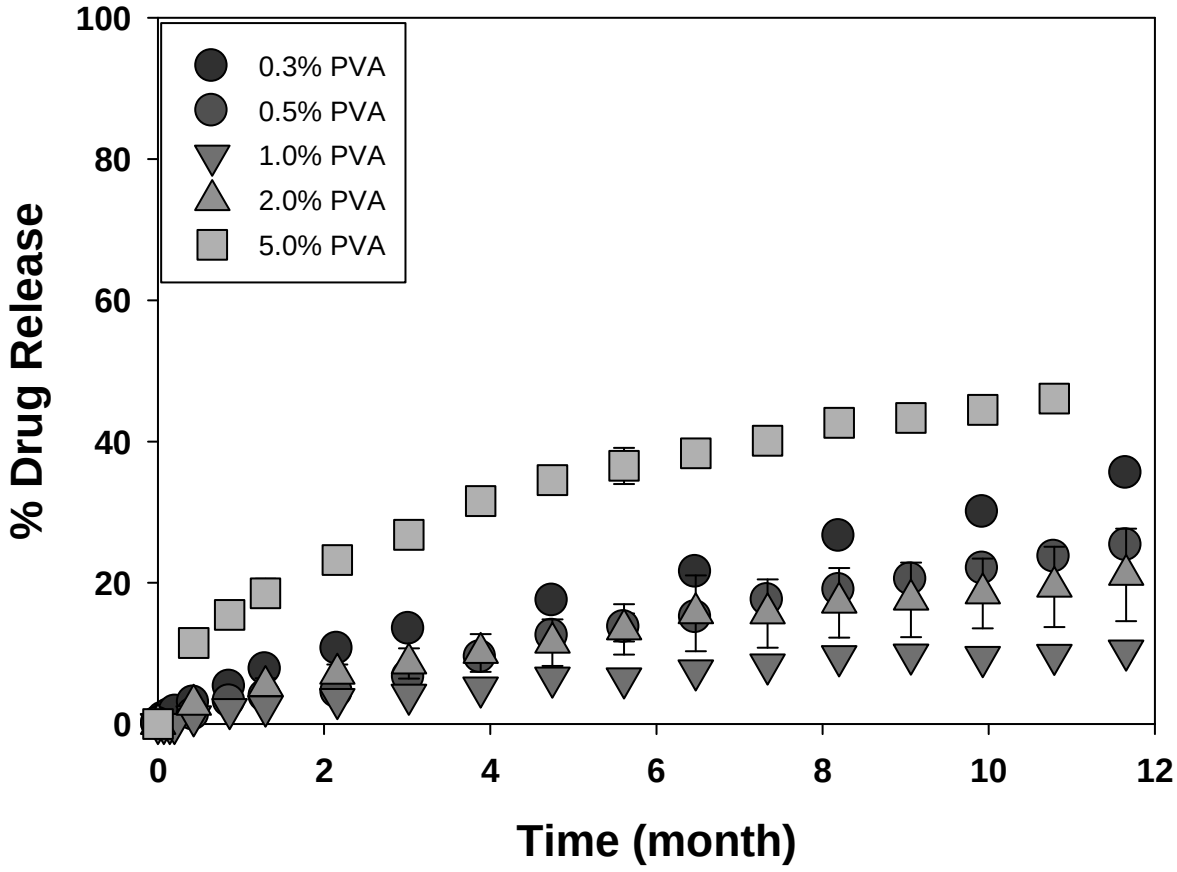


Figure 2-25. *In vitro* drug release profile for LE-PLGA (75:25) microspheres prepared with variable % of polyvinyl alcohol (PVA)

Table 2-8. Particle size properties of LE-PLGA (75:25) microspheres

Formulation	P_V (μm)	P_N (μm)	P_R
0.3% PVA	27.3 (± 0.4)	12.5 (± 0.4)	2.2 (± 0.0)
0.5% PVA	38.2 (± 0.4)	27.2 (± 0.8)	1.4 (± 0.0)
1.0% PVA	38.9 (± 0.4)	22.4 (± 5.1)	1.8 (± 0.4)
2.0% PVA	31.3 (± 1.4)	10.4 (± 1.2)	3.0 (± 0.2)
5.0% PVA	11.5 (± 0.1)	3.5 (± 0.0)	3.2 (± 0.0)

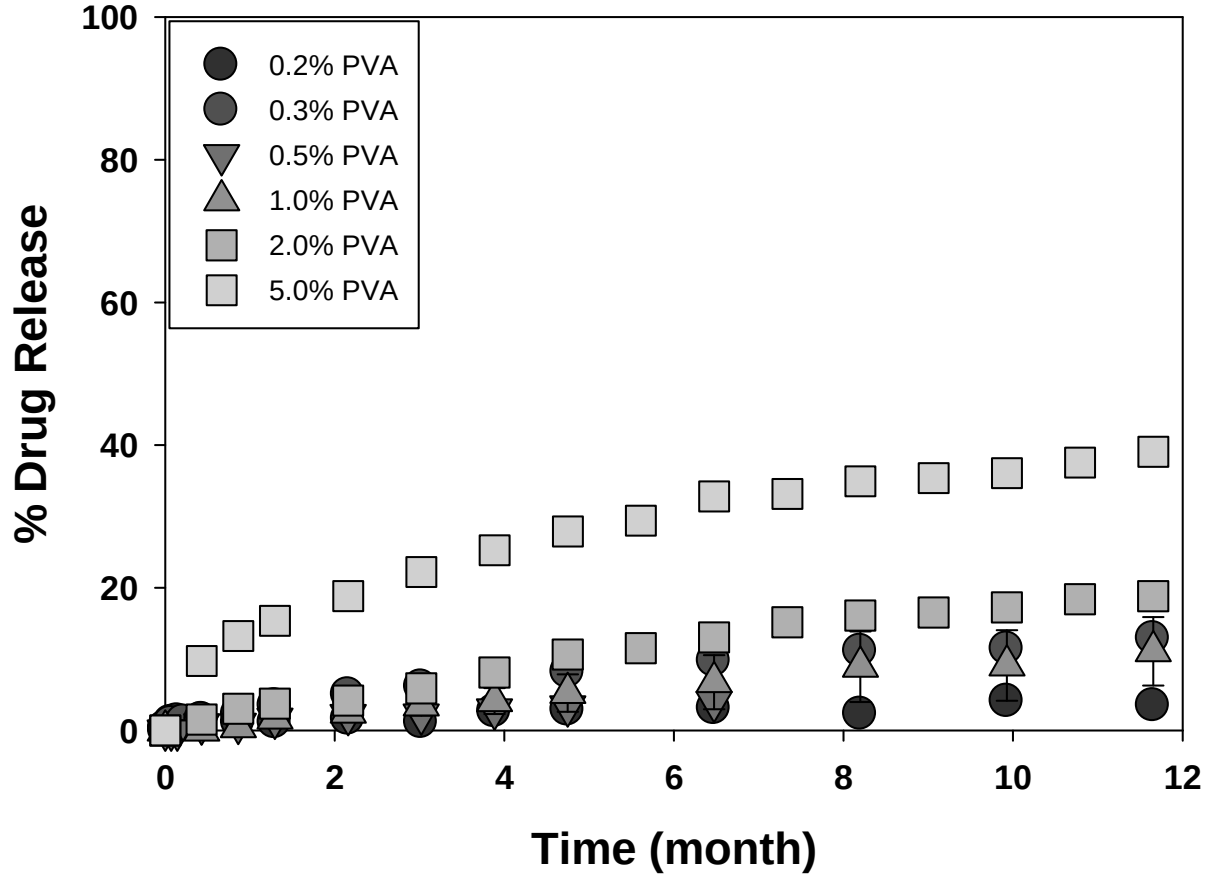


Figure 2-26. *In vitro* drug release profile for LE-PLA microspheres prepared with variable % of polyvinyl alcohol (PVA)

Table 2-9. Particle size properties of LE-PLA microspheres

Formulation	P_V (μm)	P_N (μm)	P_R
0.2% PVA	44.5 (± 0.9)	37.7 (± 3.1)	1.2 (± 0.1)
0.3% PVA	55.9 (± 0.1)	49.8 (± 0.1)	1.1 (± 0.0)
0.5% PVA	49.3 (± 0.2)	42.6 (± 0.3)	1.2 (± 0.0)
1.0% PVA	40.7 (± 0.2)	27.6 (± 0.2)	1.5 (± 0.0)
2.0% PVA	22.2 (± 0.5)	0.9 (± 0.0)	25.0 (± 0.8)
5.0% PVA	17.0 (± 0.8)	4.6 (± 0.1)	3.8 (± 0.2)

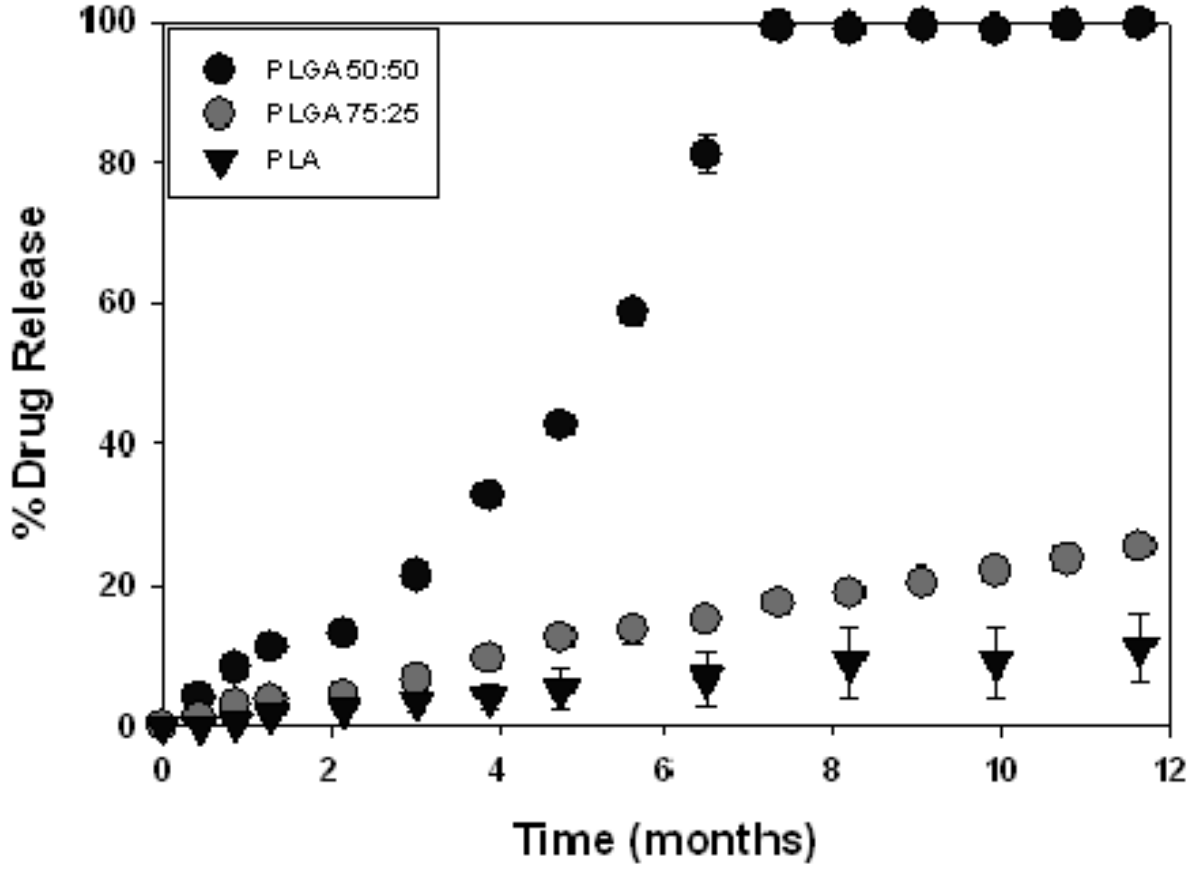


Figure 2-27. *In vitro* drug release profile for LE-PLGA microspheres prepared with variable % of polyvinyl alcohol (PVA)

Table 2-10. Particle size properties of LE-PLGA microspheres

Formulation	P_V (μm)	P_N (μm)	P_R
PLGA 50:50	37.8 (± 0.3)	20.2 (± 0.9)	1.9 (± 0.1)
PLGA 75:25	38.2 (± 0.4)	27.2 (± 0.8)	1.4 (± 0.0)
PLA	38.6 (± 0.2)	26.7 (± 0.6)	1.4 (± 0.0)

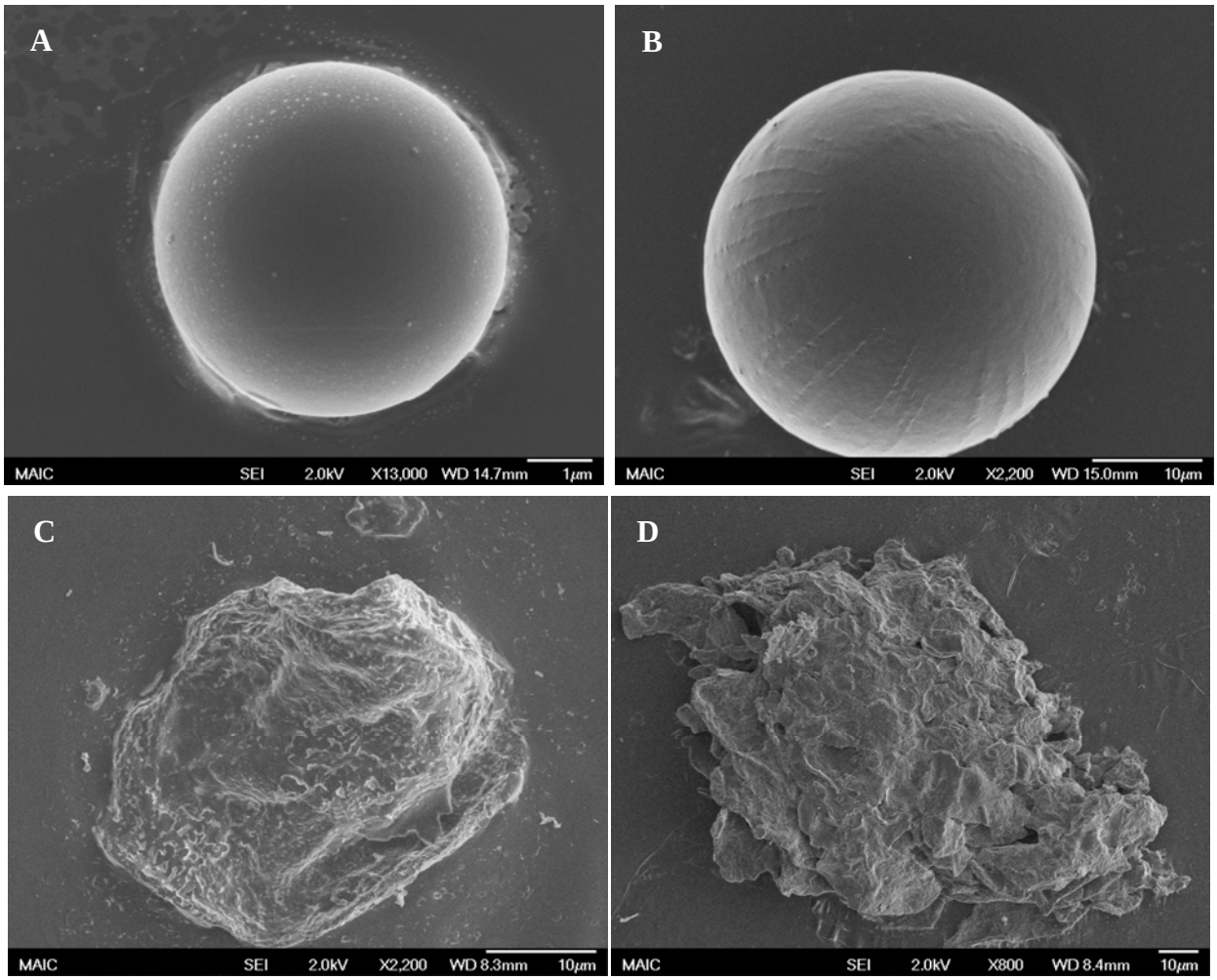


Figure 2-28. Particle morphology of the LE-PLGA 50:50 microspheres during the *in vitro* drug release studies at (A) 0, (B) 4, (C) 8, and (D) 12 months

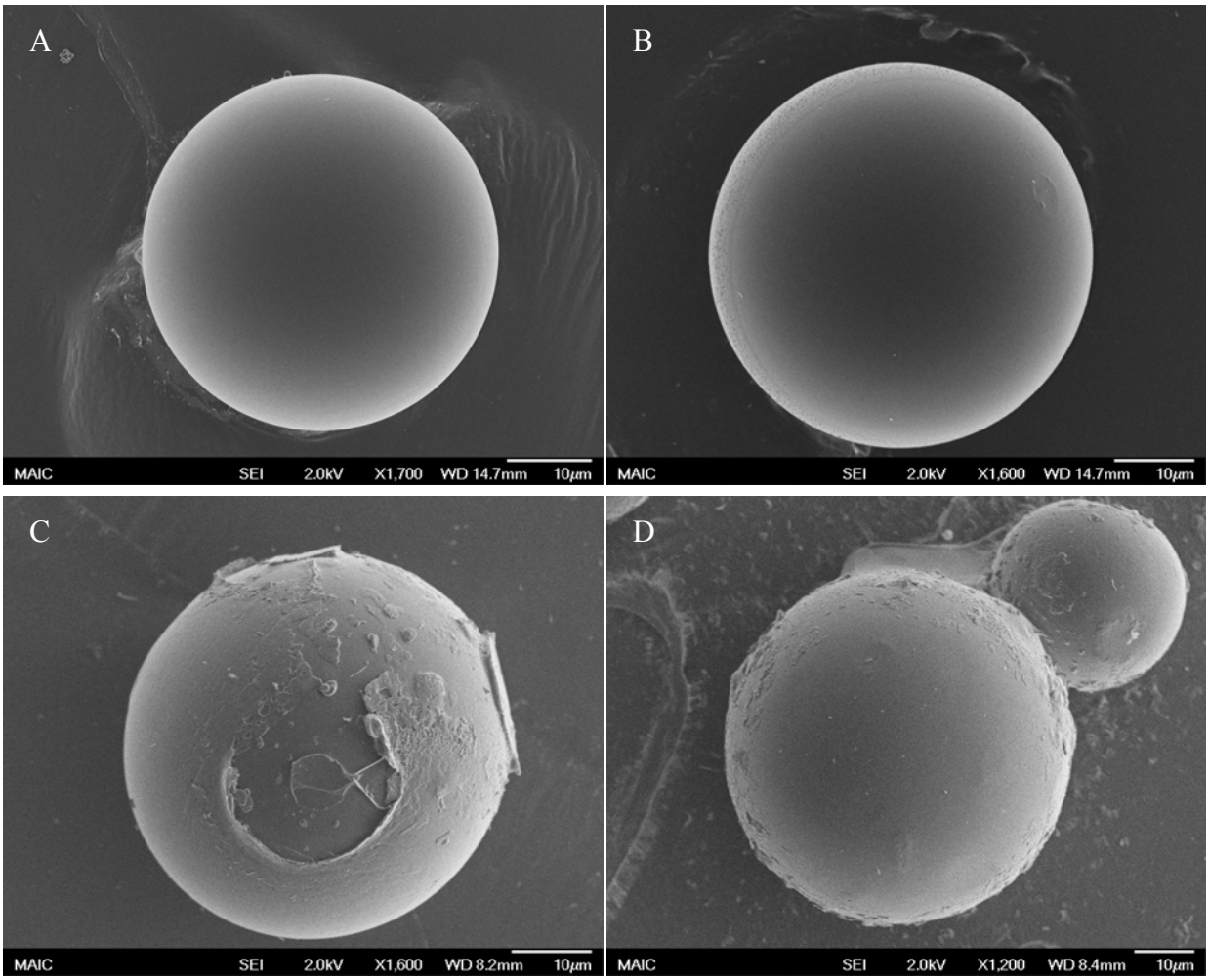


Figure 2-29. Particle morphology of the LE-PLGA 75:25 microspheres during the *in vitro* drug release studies at (A) 0, (B) 4, (C) 8, and (D) 12 months

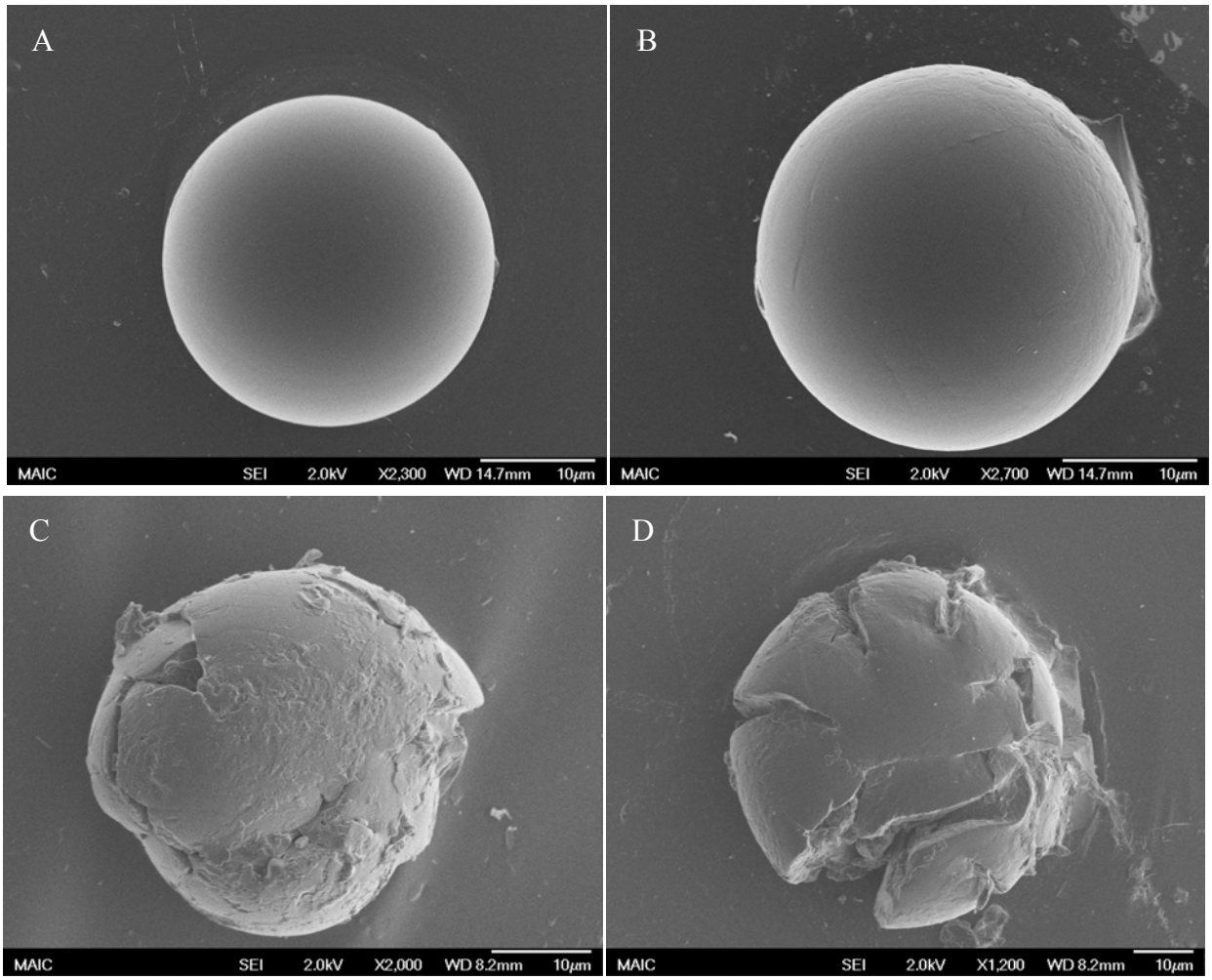


Figure 2-30. Particle morphology of the LE-PLA microspheres during the *in vitro* drug release studies at (A) 0, (B) 4, (C) 8, and (D) 12 months

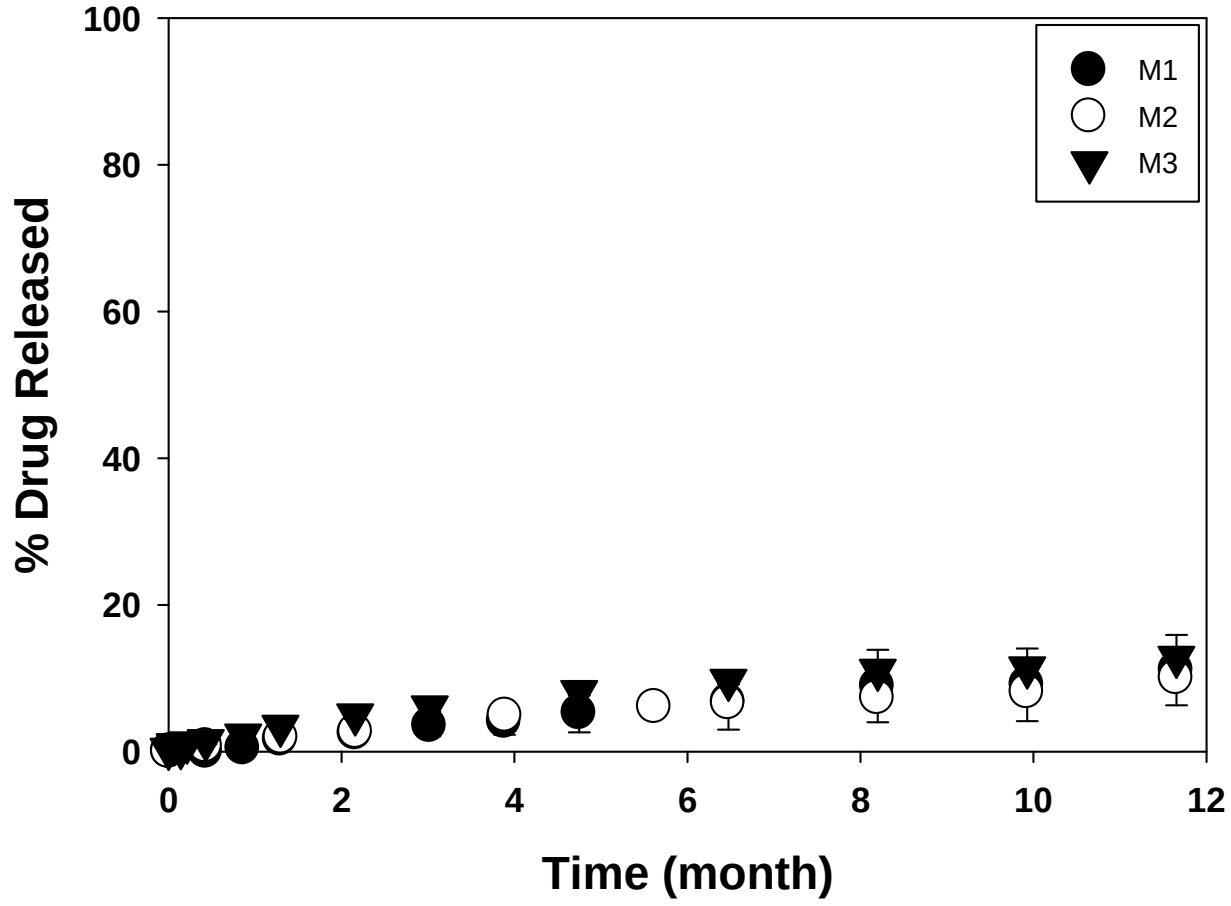


Figure 2-31. *In vitro* drug release profile for LE-PLA microspheres with varying mean volume (P_V) and number (P_N) diameter and same polydispersity (P_R)

Table 2-11. Particle size properties of LE-PLA microspheres with varying P_V and P_N and constant P_R

Formulation	P_V (μm)	P_N (μm)	P_R
M1	38.6 (± 0.2)	26.7 (± 0.6)	1.4 (± 0.0)
M2	43.4 (± 0.0)	40.2 (± 0.0)	1.1 (± 0.0)
M3	55.9 (± 0.1)	49.8 (± 0.1)	1.1 (± 0.0)

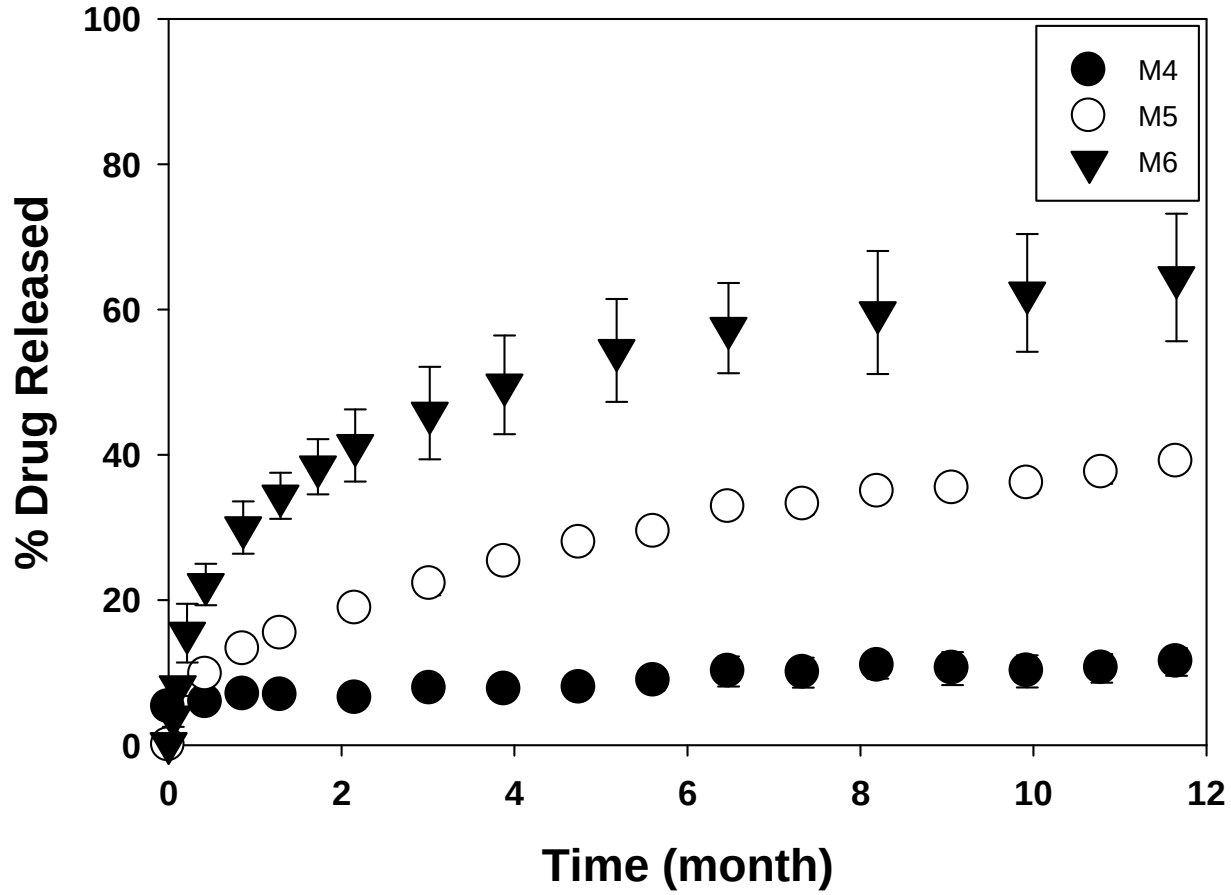


Figure 2-32. *In vitro* drug release profile for LE-PLA microspheres with the same mean volume diameter (P_V) but varying ratio (P_R)

Table 2-12. Particle size properties of LE-PLA microspheres with same P_V but varying P_R

Formulation	P_V (μm)	P_N (μm)	P_R
M4	30.4 (± 0.8)	21.5 (± 2.1)	1.4 (± 0.1)
M5	17.0 (± 0.8)	4.6 (± 0.1)	3.8 (± 0.2)
M6	24.1 (± 0.4)	0.9 (± 0.0)	28.1 (± 0.4)

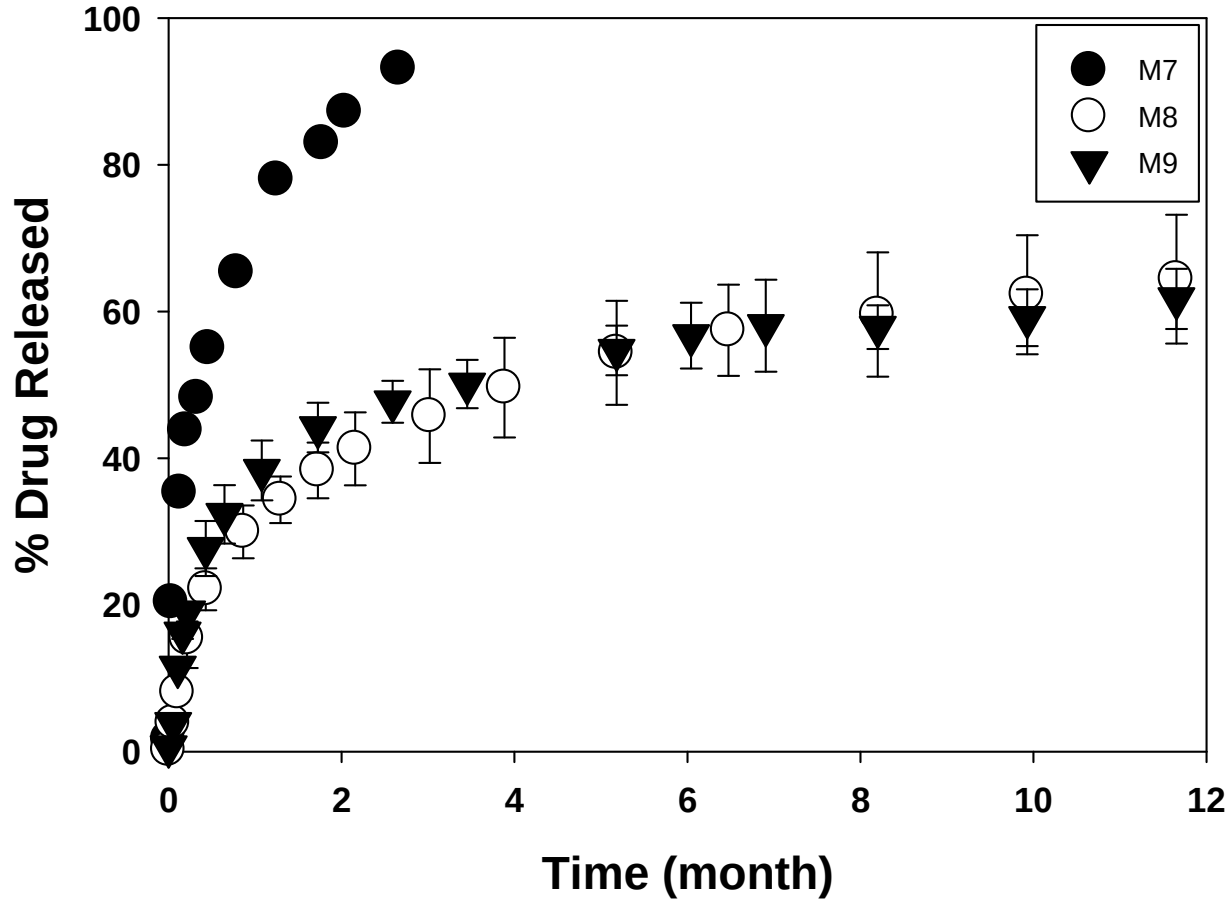


Figure 2-33. *In vitro* drug release profile for LE-PLA microspheres with the same mean number diameter (P_N) and varying ratio (P_R)

Table 2-13. Particle size properties of LE-PLA microspheres with same P_N and varying P_R

Formulation	P_V (μm)	P_N (μm)	P_R
M7	5.4 (± 0.4)	1.0 (± 0.0)	5.4 (± 0.4)
M8	24.1 (± 0.4)	0.9 (± 0.0)	28.1 (± 0.4)
M9	37.4 (± 0.1)	0.8 (± 0.0)	46.3 (± 0.2)

CHAPTER 3
CYTOTOXICITY OF SUSTAINED RELEASE MICROSPHERES

Introduction

Glucocorticoids can induce apoptosis in β-cells by the mitochondrial apoptotic pathway [118]. Glucocorticoids activate the glucocorticoid receptor by releasing the molecular chaperones heat-shock protein (HSP90) and FK-binding protein from the receptor complex [119]. HSP90 activates PP-2B (calcineurin) which increases the [Ca^{2+}] leading to the dephosphorylation of the proapoptotic Bcl-2-associated death promoter (BAD) protein [120, 121]. The dephosphorylated BAD leads to mitochondrial pore formation resulting in the release of cytochrome c into the cytosol [122]. Cytosolic cytochrome c activates the caspase cascade causing cleavage of various proteins, degradation of nuclear DNA, and eventually cell death [123, 124]. Glucocorticoids can also slow down cell growth [125]. Dexamethasone was shown to diminish cell proliferation by arresting the G_1 cell cycle [126] and down-regulating growth-promoting factors [127].

Since preservation of the transplanted β–cells is a major concern, it is important to know the β-cell cytotoxic dose of loteprednol etabonate in the therapeutic range necessary with the LE-PLA microspheres (~50 μg for 3 month release). It is also important to know if the PLA microspheres will negate the cytotoxic effect of loteprednol etabonate or enhance it. Previous studies performed on PLA/PLGA microspheres showed that the microspheres could induce a different degree of cytotoxicity on different types of cells. Gomes et al. investigated the cytotoxicity of blank PLGA (50:50) microspheres on rat peritoneal exudate cells in the concentration range of 0.1 to 1.0 mg/mL polymer [128]. There was no significant difference in the percent cell viability as compared to the control containing no polymer. Xie et al. investigated the cytotoxicity of cisplatin loaded PLA/PLGA microspheres on C6 glioma cell line

in the concentration range of 0.125 to 2 mg/mL polymer [129]. It was observed that the cisplatin loaded polymer microspheres had a 1.6 to 2 fold higher cytotoxicity as compared to the unencapsulated drug. In addition, longer exposure to cells (3 days as opposed to 1), further decreased the cell viability. Manca et al. looked at the rifampicin loaded PLGA (75:25) cytotoxicity on the human A549 alveolar cells [130]. Incorporating the drug into the polymer matrix did significantly reduce the cytotoxicity of the drug. However, there was an increase in the cytotoxicity with an increase in the formulation concentration.

Unfortunately, no studies have been performed on the PLA microsphere toxcity on the β-cells. This project investigated the cytotoxicity of loteprednol etabonate loaded PLA microspheres on the MIN-6 mouse insulinoma cell line. Cytotoxicity was determined by a colorimetric method based on the MTT assay. Viable cells are unable to induce MTT to colored formazan serving as an indirect measurement of cell viability [131].

Hypothesis

We hypothesized that by encapsulating the glucocorticoid loteprednol etabonate (LE) within the polymer poly(D,L-lactic) acid (PLA) microspheres, the cell toxicity to the beta cells will be decreased as compared to the unencapsulated LE formulation at equivalent drug concentrations.

Materials and Methods

Chemicals

The MIN-6 mouse insulinoma cell line was obtained from Dr. Sihong Song's lab. The Dulbecco's modified Eagle's medium (Cat. # 10-013-CV), Trypsin EDTA (Cat. # 25-052-Cl), phosphate buffer saline solution (1X PBS), fetal bovine serum, penicillin and streptomycin solution (Cat. # 30-002-CI) were purchased from Cellgro (Manassas, VA). Dimethyl sulphoxide

(DMSO), Isopropanol, and Hydrochloric acid (HCl) were purchased from Fisher Scientific Inc. (Suwanee, GA). Tween 80 was purchased from Sigma Chemical Co. (St. Louis, MO). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was purchased from Calbiochem (EMD Chemicals, Inc., Gibbstown, NJ).

MIN-6 Cell Culture

The MIN-6 cells were cultured in 10 cm plates in 4.5–g/L glucose Dulbecco's modified Eagle's medium (DMEM) containing 15% fetal bovine serum (FBS) and 1% penicillin and streptomycin. The cells were incubated at 37^{o}C under conditions of 5% CO_2. The medium was changed every 2 to 3 days and subcultured when the plate was confluent (~weekly).

MIN-6 Cell Viability Determination

The MTT assay was used to estimate cell viability. The MIN-6 cells (10,000 cells/well) were seeded in a 96-well flat-bottom plate (Corning Inc., Corning, NY) in 4.5-g/L glucose DMEM for 24 hours. The medium was removed and replaced with DMEM containing unencapsulated drug or LE-PLA microspheres at the following concentrations of LE: 100, 10, 1, 0.1, and 0.01 μM. The control used was a blank having 0 μM LE in DMEM media. After incubation for 1 and 4 days, the medium was removed and replaced with filtered MTT (100 μL, 0.5 mg/mL in DMEM) and incubated for 3 hours at 37^{o}C. The cells were treated with 100 μL isopropyl alcohol containing 0.04 mol/L HCl for 30 minutes under dark at room temperature. The absorbance was measured using the Dynex Technologies microplate spectrophotometer model MRX^{TM} (Chantilly, VA) at a wavelength of 550 nm. The percent cell viability was calculated to be:

$$\%\ Cell\ Viability = \frac{Absorbance\ of\ Sample}{Avg.Absorbance\ of\ Control} * 100\%$$

The percent cell viability of the samples was presented as mean ± SE. Statistical significance of the difference in the percent cell viability between each concentrations of unencapsulated LE or LE-PLA microspheres were tested using the Student t-test (SigmaPlot for Windows Version 10.0, Systat Software, Inc., San Jose, CA).

Results and Discussion

The MIN-6 cells were exposed to varying concentrations (0.01, 0.1, 1, 10, and 100 μM) of LE. After one day, the unencapsulated drug decreased the cell viability of the MIN-6 cells with a threshold concentration of 10 μM LE (Figure 3-1a). After 4 days, the threshold concentration was still 10 μM LE but the decrease in the cell viability was more pronounced (Figure 3-1b). The half maximal inhibitory concentration (IC_{50}), which could only be determined at day 4, was 20 μM LE.

The blank PLA microspheres at 0.1, 1, 10, 100, and 1000 mg/mL concentrations were also exposed to the MIN-6 cells for 1 and 4 days. There was no decrease in the cell viability at day 1 (Figure 3-2a) and day 4 (Figure 3-2b) indicating that there was no β-cell cytotoxicity. Interestingly, there was an increase in the cell viability with a threshold concentration of 100 mg/mL at day 1 and 1000 mg/mL at day 4. A possible explanation for the increase in cell viability was due to the degradation product of the PLA polymer (lactic acid) feeding the citrate cycle by producing pyruvate through the lactate dehydrogenase reaction [132]. The citrate cycle (also known as the Krebs cycle) is a metabolic pathway that produces energy in the mitochondria of living cells. The MTT assay is based on cell proliferation by determining the formazan production from active mitochondria (only found in living cells). The higher concentration of lactic acid, from a higher concentration of polymer, would produce more pyruvate. A higher concentration of pyruvate could enhance the mitochondria activity hence producing more

formazan resulting in an increase in the cell viability. Ignatius et al. noticed an increase in the succinate dehydrogenase activity, a mitochondrial enzyme used in the citrate cycle, when exposing Clone L929 mouse fibroblast cells to the degradation products of PLGA (70:30) and PLGA (90:10) [132]. This observation suggests that PLA could increase mitochondria activity.

By incorporating the drug into the PLA microspheres, there was no significant decrease in the cell viability even with an increase in the formulation concentration at day 1 and day 4 (Figure 3-3). The insignificant decrease in cell viability could be due to minimal crystalline drug on the surface on the PLA microspheres and hence a low initial burst. Figure 2-7 shows the PXRD analysis of LE-PLA microspheres. There are small crystalline peaks at the 16, 17, 19, and 24 2θ angle for the 5% loteprednol etabonate and blank PLA microsphere mixture which correlated to the prominent peaks for the unencapsulated drug at the same angles. Note that the blank PLA microspheres are amorphous hence there were no crystalline peaks. However, with the LE-PLA microspheres containing 5% loteprednol etabonate, no crystalline peaks were observed indicating that the drug was incorporated within the microspheres and not residing on the surface. The initial burst of the tested formulation is 1.7% at 0 hours which correlates to 1.7 μM of LE being exposed to the β-cells. This concentration is below the 10 μM threshold concentration. Murillo et al. also observed a similar trend when investigating the cytotoxicity of PLGA (50:50) microspheres containing Hot Saline antigenic extract on the macrophage cell line J774.2 [133]. The formulation had 24.4 (±6.3) % cell viability and 40.3 (±2.7) % initial burst. The low cell viability was due to the high initial burst of the formulation which meant an exposure of a higher dose of the toxic drug to the cells.

Conclusion

- LE was cytotoxic to the MIN-6 insulinoma cells at a threshold concentration of 10 μM and with an IC_{50} of 20 μM (only observed at day 4).

- The blank PLA microspheres had no β-cell cytotoxicity in the 0.01 to 1 mg/mL concentration range. In fact, the blank microspheres increased the cell viability of the MIN-6 insulinoma cell line indicating that PLA could enhance the mitochondrial activity of the β-cells.

- Since the LE-PLA microspheres had no crystalline drug on the surface of the microspheres and a low initial burst, there was no observed cytotoxicity indicating that the LE-PLA microspheres can prevent β-cell cytotoxicity induced by the drug.

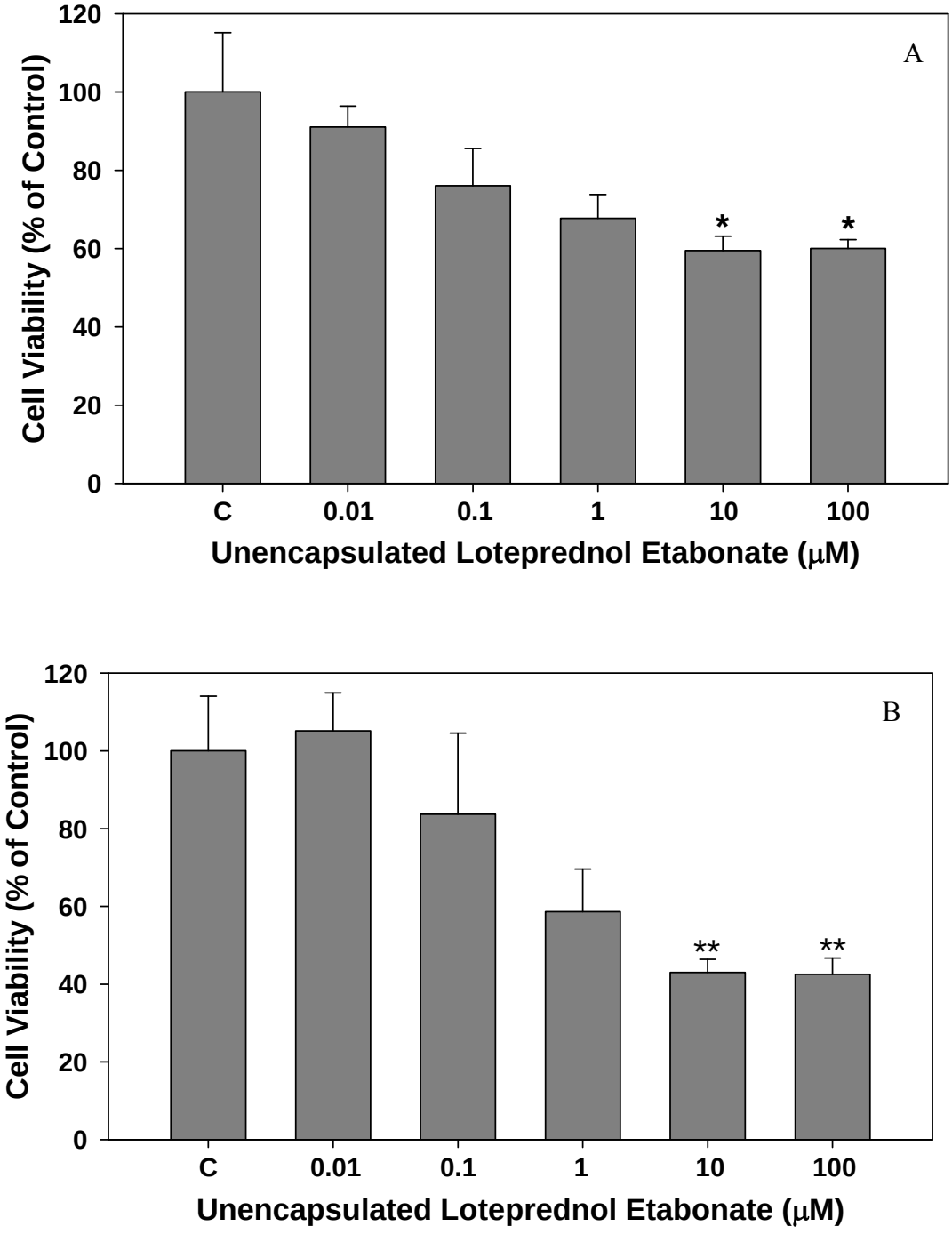
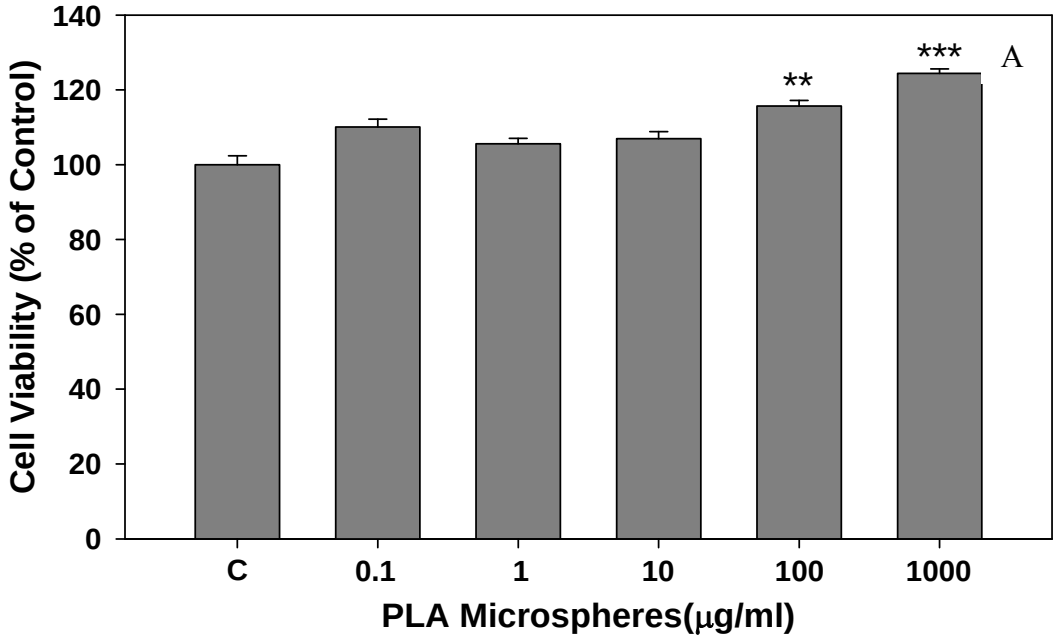


Figure 3-1. Effect of loteprednol etabonate on MIN-6 cell viability after 1 day (a) and 4days (b) of incubation (n=4). (*P<0.05, **P<0.01)



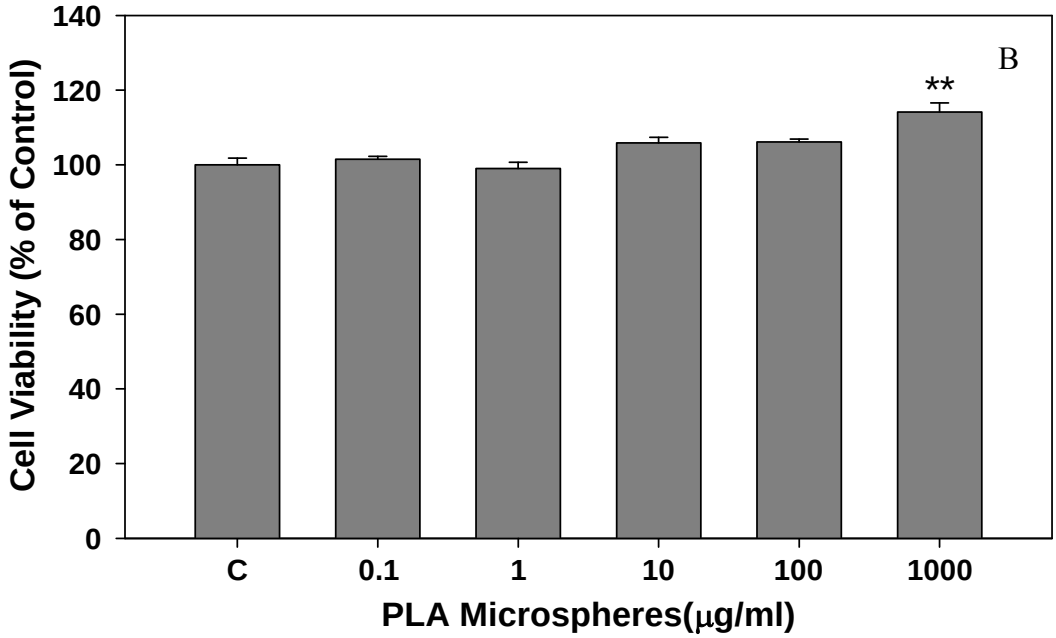


Figure 3-2. Effect of PLA microspheres on MIN-6 cell viability after 1 day (a) and 4 days (b) of incubation (n=4). (**P<0.01; ***P<0.001)

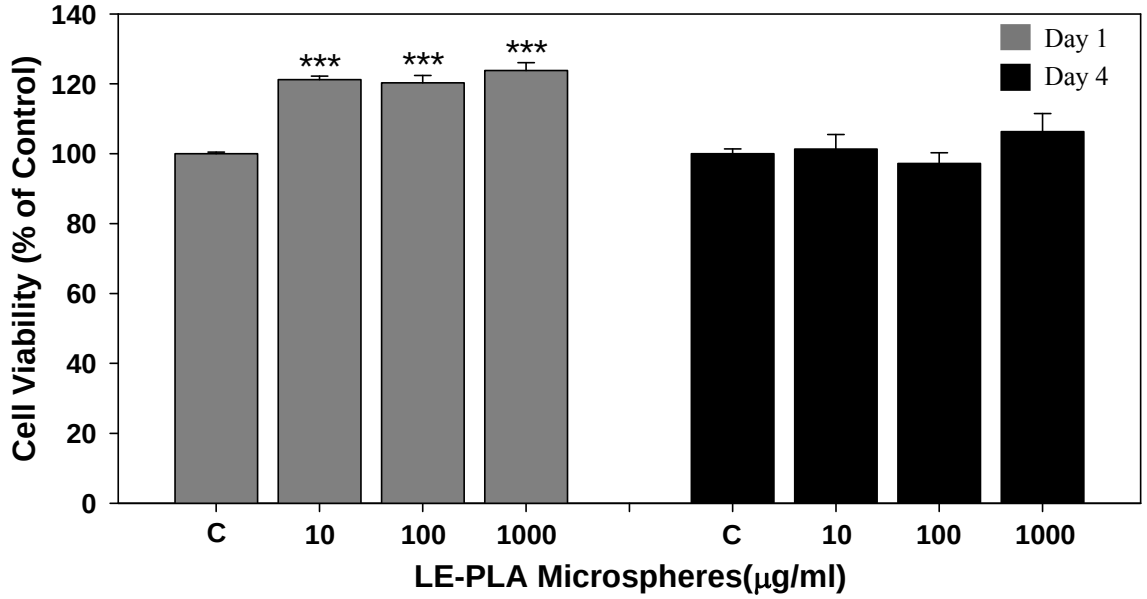


Figure 3-3. Effect of PLA microspheres on MIN-6 cell viability after 1 and 4 days of incubation (n=4). (***P<0.001)

CHAPTER 4
IN VIVO CHARACTERIZATION OF SUSTAINED RELEASE MICROSPHERES

Introduction

Life-long immunosuppression is required for patients receiving islet transplantation. Current regimens are associated with a lot of complications lowering a patient's quality of life [47, 48, 55, 134-140]. Localized, versus systemic, delivery of immunosuppressants reduce systemic toxic effects while preventing rejection of transplanted organs. Wang et al. looked at the intraportal delivery of immunosuppressants in a rat model receiving intrahepatic islet allografts [141]. Intraportal delivery of tacrolimus significantly increased the mean survival duration as compared to the control (no immunosuppressant) and intravenously administered tacrolimus. In addition, the intraportal administration of tacrolimus, as opposed to intravenous administration, had lower mean systemic levels possibly resulting in lower systemic toxicity. Ruers et al. observed the same results when comparing cardiac allograft survival times and systemic drug levels in rats using systemic (jugular vein) and local (carotid artery) routes of administration of budesonide [142].

Localized delivery of immunosuppressants could be possible by many drug delivery systems (DDS) ranging from liposomes to drug-eluting stents to hydrogels [143-147]. Bocca et al. analyzed the possibility of applying of three DDS for localized immunosuppression long-term delivery within the neovascularized islet biohybrid device: (1) sustained-release implant (similar to Retisert™); (2) infusion using an Alzet® implantable osmotic minipump; and (3) sustained-release microspheres [53]. Retisert™ is a nonbiodegradable implant (3 x 2 x 5 mm) containing a fluocinolone acetonide microcrystalline cellulose tablet [148]. As the tablet breaks down, the drug is released through an orifice in the implant into the surrounding environment. The drug release profile has an initial release rate of 0.6 μg/day over the first month followed by

0.3 to 0.4 μg/day over 2.5 years. Implantable infusion pumps have been used for localized delivery of various therapeutics including 6-mercaptopurine, prostaglandin E (PGE1), heparin, tacrolimus, and 15-deoxyspergualin into transplanted organs [51, 149-152]. The Alzet® minipumps are filled with the drug solution and usually implanted subcutaneously with the catheter inserted into the nearby artery or into the transplant. Release profiles range from 1 day to 6 weeks with delivery rates between 0.11 to 10 μL/hr [153]. Sustained-release microspheres have been previously used as a localized DDS of immunosuppressants for preventing transplant rejection [144, 154, 155].

In this project, we studied the feasibility of using biodegradable PLA microspheres for localized sustained-release delivery of loteprednol etabonate (LE). Using various considerations, a drug release rate of approximately 1 nmol/day or 0.5 μg/day was obtained as a first estimate for the local dose needed to provide sufficient immunosuppression by maintaining a therapeutic concentration of 5 – 500 nM within the biohybrid device [53]. The LE-PLA microspheres DDS was compared to the 2 week Alzet osmotic minipump DDS. The animal studies were performed at the Diabetes Research Institute at the Miller School of Medicine, University of Miami in collaboration with Dr. Peter Buchwald's Drug Discovery group and Dr. Antonello Pileggi's Translational Cell Processing and Transplant Models Core.

Hypothesis

We hypothesized that the 3-month release LE-PLA microsphere formulation will prevent the rejection of the islet allografts (as indicated by the mean survival duration of the chemically diabetic rats) for around a 3-month period.

Materials and Methods

Islet Transplantation

Islet transplantation using the neovascularized islet biohybrid device was performed at the Diabetes Research Institute using the method developed by Pileggi et al. [30]. The project and animal care was conducted under protocols approved by the Institutional Animal Care Committee.

To summarize the procedure (Figure 4-1), the rats were put under general anesthesia using isoflurane USP (Baxter, Deerfield, IL) prior to the surgery. The sterilized islet biohybrid device was subcutaneously implanted into the dorsal region of the rats. The stainless steel device was 2 cm long with a diameter of 0.6 cm and pore size of 450 μm with a polytetrafluoroethylene (PTFE) plug and inserted plunger (which filled the inner void of the device). After forty days, the biohybrid device was neovascularized and preparations for islet transplantation were started.

Donor islets were obtained from male Wistar Furth rats (Indianapolis, IN) and purified by enzyme digestion using Liberase (Roche; Indianapolis, IN) and density gradient separation (Mediatech; Herndon, VA). Islet grafts were cultured in CMRL-1066 medium (Gibco-Invitrogen; Carlsbad, CA) at 37^{o}C in 5% CO_2. Female Lewis rats of body weights around 200 g were administered 60 mg/kg intravenously the β-cell toxin streptozotocin (Sigma Aldrich, St. Louis, MO) at day one and day two/three prior to transplantation. Only rats having non-fasting glucose levels ≥ 350 mg/dL were used. The PTFE plunger was removed from the biohybrid device through a cutaneous incision. Islet grafts of 3000 islet equivalents (IEQ) were suspended in PBS and inserted into the inner void of the device using a syringe (Hamilton; Reno, NV) and polyethylene catheter. Another PTFE plug was used to seal the device. The skin was then sutured to close the incision.

Animals were then maintained on a two- to three-week regimen of systemic immunosuppression in addition to the localized delivery. The systemic immunosuppression was then gradually withdrawn leaving only the localized one. For the control, no immunosuppressants were inserted into the biohybrid device. For localized delivery of LE by infusion, 10 mg/L LE (in 10% ethanol and 90% 1X PBS) was inserted into an Alzet® Osmotic Pump Model #1002, 0.25 μL/h (Durect Corp., Cupertino, CA). The pump was implanted subcutaneously and catheter inserted into the orifice of one of the PTFE plugs and replaced every two weeks as needed. For localized delivery of LE-PLA microspheres, 4.5 mg loading dose of LE-PLA microspheres (sterilized by γ radiation) were inserted into the biohybrid device prior to sealing the device. The delivery rate was estimated to be around 3.0 μg/day based on a 4% drug content released over approximately 60 day period.

Statistical Analysis

The statistical significance of difference in the percent survival of islet recipients between rats receiving different immunosuppressant treatments was tested using the Student t-test (SigmaPlot for Windows Version 10.0, Systat Software, Inc., San Jose, CA). Curve fitting of the *in vitro* drug release profile was preformed with SigmaPlot 10.0 and Excel software programs.

Results and Discussion

The mean survival duration of the chemically diabetic rats was increased with the use of the sustained release formulation as compared to the control (saline) and infused drug (Figure 4-2). The chemically diabetic rats received two to three weeks weaning of systemic immunosuppression following islet transplantation in conjunction with the localized immunosuppressant therapies. The localized immunosuppression was maintained after weaning of the systemic administration. The localized immunosuppressant therapies used was: no drug

(control), LE solution delivered with 2-week release Alzet® pump, and LE-PLA microspheres inserted into the implant. The mean survival duration was 9.3 ± 3.6 days (n = 10), 13.6 ± 4.9 days (n = 5), and 20 ± 6.6 days (n = 4) for the control, LE infusion, and LE-PLA microspheres respectively. The survival duration for the control was not significantly different from the rats receiving LE infusion but was significantly different from those receiving LE-PLA microspheres ($P < 0.01$). There was no significant difference in the survival durations of the rats receiving the LE infusion and LE-PLA microspheres.

The LE-PLA microspheres tested had a three month *in vitro* drug release profile (Figure 4-3). The *in-vitro* drug release duration did not match the *in vivo* mean survival duration of the recipients. Possible reasons for the differences could be due to (1) the drug release rate from the formulation being below the therapeutic range, (2) the size of the microspheres not being large enough, and (3) the *in vitro – in vivo* drug release time scale not correlating to each other.

In order to investigate if the drug release rate fell below the therapeutic range, the *in vitro* drug release profile of the LE-PLA microspheres tested was compared to various commonly used dissolution models to find one that had the best fit [156]. Table 4-1 shows the best fit equations and the correlation coefficient (R^2) of *in vitro* release profile to the various dissolution models. The Second-order exponential and Korsmeyer-Peppas dissolution models gave the best fit as shown in Figure 4-3. The Korsmeyer-Peppas model was chosen for future analysis due to the simplicity of the model as compared to the second order exponential dissolution model. In taking the differential of the Korsmeyer-Peppas model, we get the drug release rate:

$$\frac{dQ_t}{dt} = Q_\infty K_k n t^{n-1} \tag{4-1}$$

where Q_t is the amount of drug released as a function of time (mg)
Q_∞ is the total amount of drug in the microspheres (mg)
K_k is a constant = 0.696

n is the order of the model = 0.32
t is the time of drug released in months

The drug release rate (Equation 4-1) is plotted as shown in Figure 4-4. Bocca et al. [53] recommended a drug release rate of 0.5 μg/day in order to maintain therapeutic concentrations based on *in vitro* cytotoxicity studies and computational modeling. The release rate was determined under the assumption that no local drug metabolism occurred which may not be true in the case of LE considering that it is a soft drug that hydrolyzes in aqueous solution. LE has a half life of 1.4 days in aqueous solution (data not shown) meaning a minimal drug release rate of l.6 μg/day may be needed to maintain therapeutic concentrations. The baseline 0.5 μg/day (Figure 4-4) indicates that the formulation should last 3 months. However, the baseline 1.6 μg/day indicates that the formulation should last 1.1 months or 33 days which is more representative of the *in vivo* data. Since the device was prevascularized, it is quite possible for the enzymes that promote hydrolysis to enter the device and degrade the drug faster. So the minimal drug release rate is not certain.

Figure 4-5a depicts the particle size distribution of the LE-PLA microspheres based on the % volume (meaning the distribution has been normalized to emphasize the larger particles). Figure 4-5b depicts the particle size distribution based on the % number (the distribution has not been normalized and would represent the distribution observed under SEM). Both have different mean averages implying that the formulation is polydispersed as indicated by a high $mean_{volume}/mean_{number}$ ratio (Table 4-2). The subcutaneous tissue surrounding the device contained arteries, veins, capillaries, and adipose tissue which could carry the smaller microspheres into the blood stream [157]. With a lower amount of microspheres within the device, the total drug concentration would be lower. Also, the drug release rate would be lower

since larger microspheres have slower rates. Future studies will be conducted with larger microspheres.

A good correlation with the *in vitro* dissolution profile and the *in vivo* plasma concentrations can reduce product development cost and time by predicting beforehand the expected *in vivo* duration of drug release [157]. The *in vitro* drug release profile of the LE-PLA microspheres was determined from a dissolution assay performed at 37^{o}C under sink conditions at 30 rpm using a magnetic stirrer. Using low stir rates (as opposed to the standard 100 rpm) is common when determining the drug release of drug loaded microspheres injected into muscle or subcutaneous tissue. The low stir rates would represent the environment in the subcutaneous tissue which is dense and would have low flux. Chu et al. used a bath shaker at 40 rpm to conduct *in vitro* dissolution studies for huperzine A loaded PLGA microspheres [158]. Four different formulations were injected either subcutaneously or intramuscularly. All had an *in vitro – in vivo* correlation (IVIVC) coefficient (R^2) of > 0.96 indicating a great correlation. The enzymatic degradation of the polymers also need to be considered when developing the *in vitro* release study [159].

While the dissolution assay was performed under conditions suitable for drug release within a subcutaneous implant, it would advisable to determine the *in vitro – in vivo* correlation. Unfortunately, the rats receiving islet transplantation undergo a lot of stress from the surgery that obtaining blood samples at sufficient time points for a proper pharmacokinetic study could be detrimental to the rat and hence the study. Other alternatives include determining the drug content remaining in the biohybrid implant device which would tell us how much drug was released. Also determining the cumulative amount of drug and metabolites (as a function of time) excreted from urine and feces could be an indirect correlation to the amount of drug

remaining in the device [160]. The drug content in the device is currently being investigated. We may look at the cumulative drug and metabolites amount in the waste in the future.

Conclusion

- The LE-PLA microspheres was successful in impeding islet transplantation rejection as indicated by the mean survival time of 20 ± 6.6 days. The survival time of the microspheres was significantly different from the control (no drug).
- The LE-PLA microspheres did not maintain therapeutic concentrations over the expected 3 month duration.
- Future studies have to be done with larger, more monodisperse microspheres (> 10 μm and $mean_{volume}/mean_{number}$ ratio < 1.5).
- Future studies have to be done to determine the *in vitro – in vivo* drug release correlation by either analyzing the drug remaining in the device at the end of the study or the feces and urine for the amount of drug and metabolites expelled from the body.

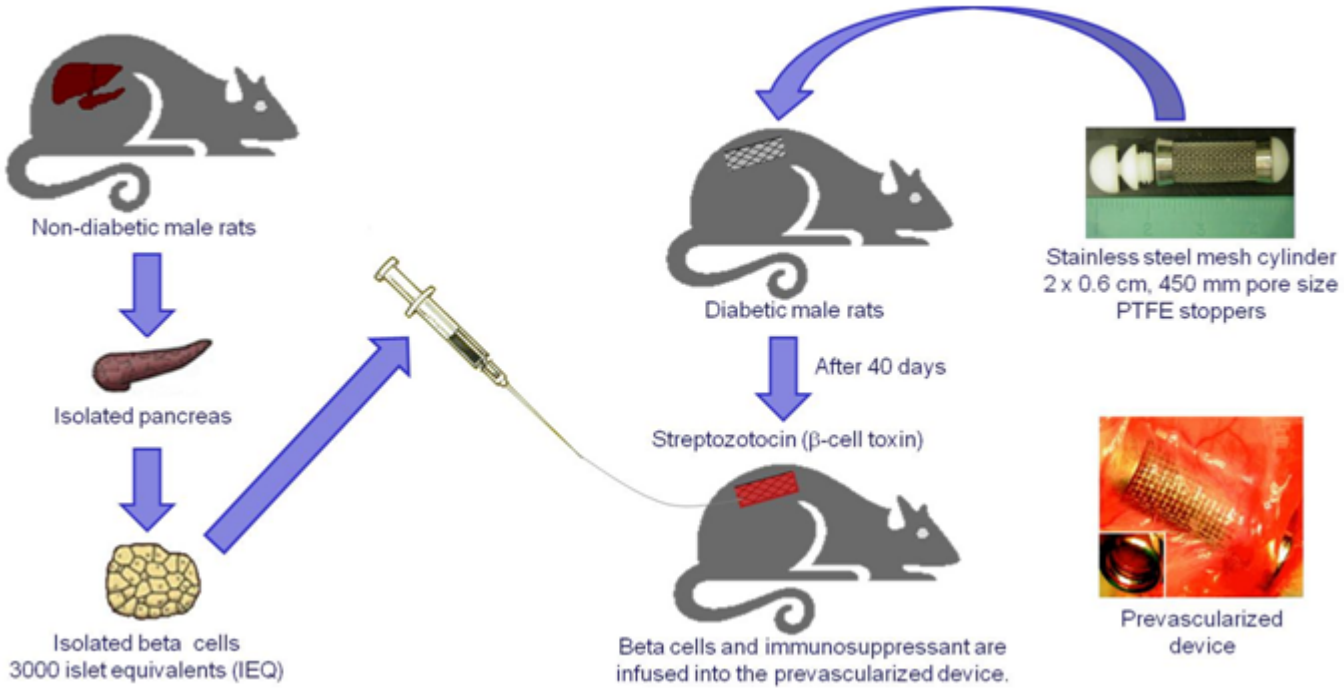


Figure 4-1. Islet transplantation using the novel biohybrid device.

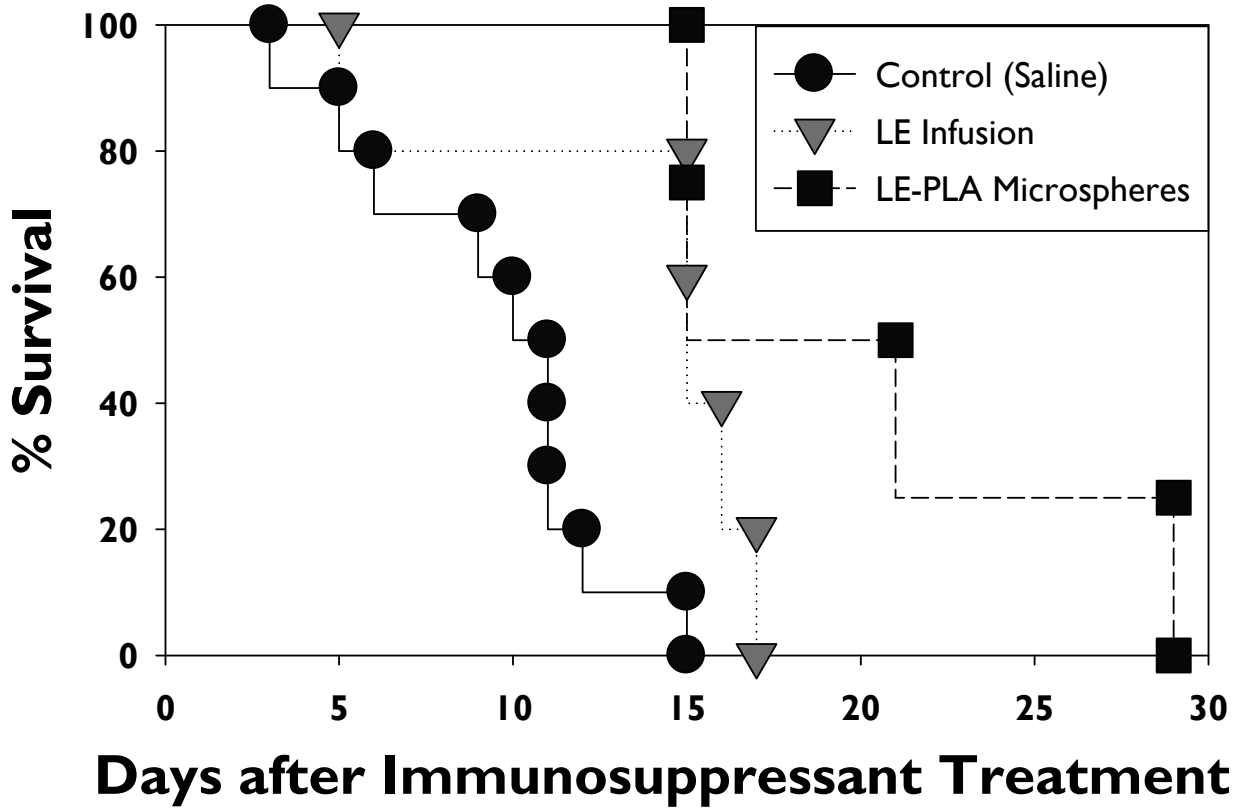


Figure 4-2. Percent survival of chemically diabetic rats receiving islet transplantation in conjunction with the following local immunosuppressant therapy: saline solution with no drug (control), LE solution infused with Alzet® pump (LE Infusion), and LE-PLA microspheres inserted into device (LE-PLA Microspheres)

Table 4-1. Best fit equations of commonly used dissolution models for the *in vitro* drug release of the LE-PLA microsphere formulation

Model	Theoretical Equation	R^2	Best Fit Equation
Higuchi	$Q = \sqrt{D(2C - C_s)C_s t} = K_H\sqrt{t}$	0.87	$Q = 2.06(mg)\sqrt{t}$
First-order	$W = VC_s\left(1 - e^{-kt}\right)$	0.93	$W = 2.49(mg)\left(1 - e^{-2.82/month \cdot t}\right)$
Second-order	$W = a\left(1 - e^{-k_1 t}\right) + b\left(1 - e^{-k_2 t}\right)$	1.00	$W = 0.91(mg)\left(1 - e^{-26.2/month \cdot t}\right) + 1.97(mg)\left(1 - e^{-0.94/month \cdot t}\right)$
Hixson-Crowell	$Q_t^{1/3} = Q_0^{1/3} - K_s t$	0.53	$Q_t = \sqrt[3]{0.91(mg^3) - 0.24 \cdot \left(mg^3/month\right) \cdot t}$
Weibull	$\log\left[-\ln\left(1 - \left(Q_t/Q_\infty\right)\right)\right] = b \times \log t - \log a$	0.99	$Q_t = 2.96(mg)\left(1 - e^{\left(-\frac{t^{0.55}}{0.75month}\right)}\right)$
Korsmeyer-Peppas	$\frac{Q_t}{Q_\infty} = K_k t^n$	1.00	$Q_t = 2.06(mg) \cdot t^{0.32}$
Quadratic	$Q_t = 100\left(K_1 t^2 + K_2 t\right)$	0.33	$Q_t = \frac{-0.46}{month^2} \cdot t^2 + \frac{1.97}{month} \cdot t$
Logistic	$Q_t = \frac{A}{\left[1 + e^{-K(t-y)}\right]}$	0.92	$Q_t = \frac{2.51(mg)}{\left[1 + e^{-3.94/month(t - 0.3month)}\right]}$

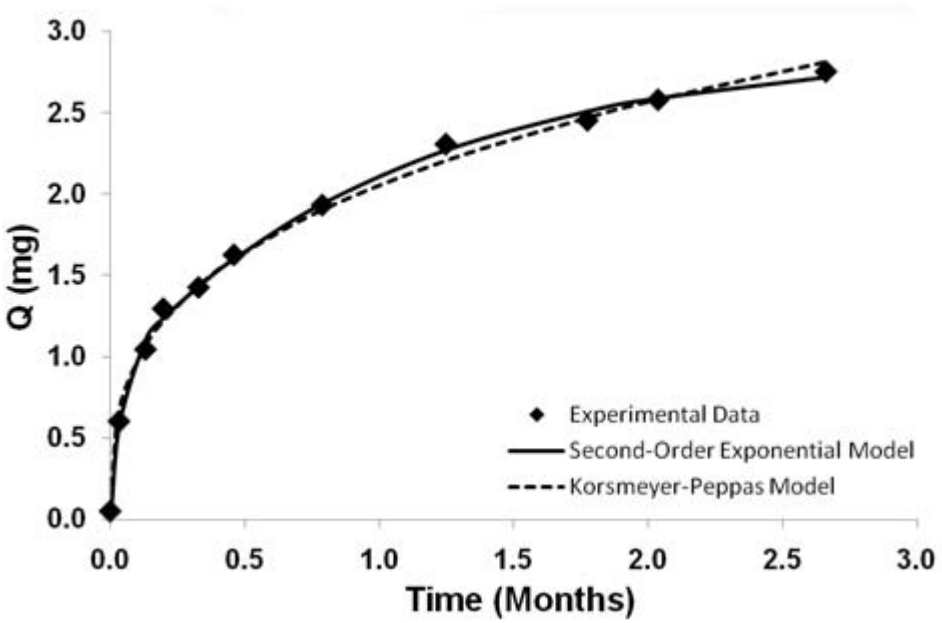


Figure 4-3. Curve-fitting of the *in vitro* drug release (Q) of the LE-PLA microsphere formulation

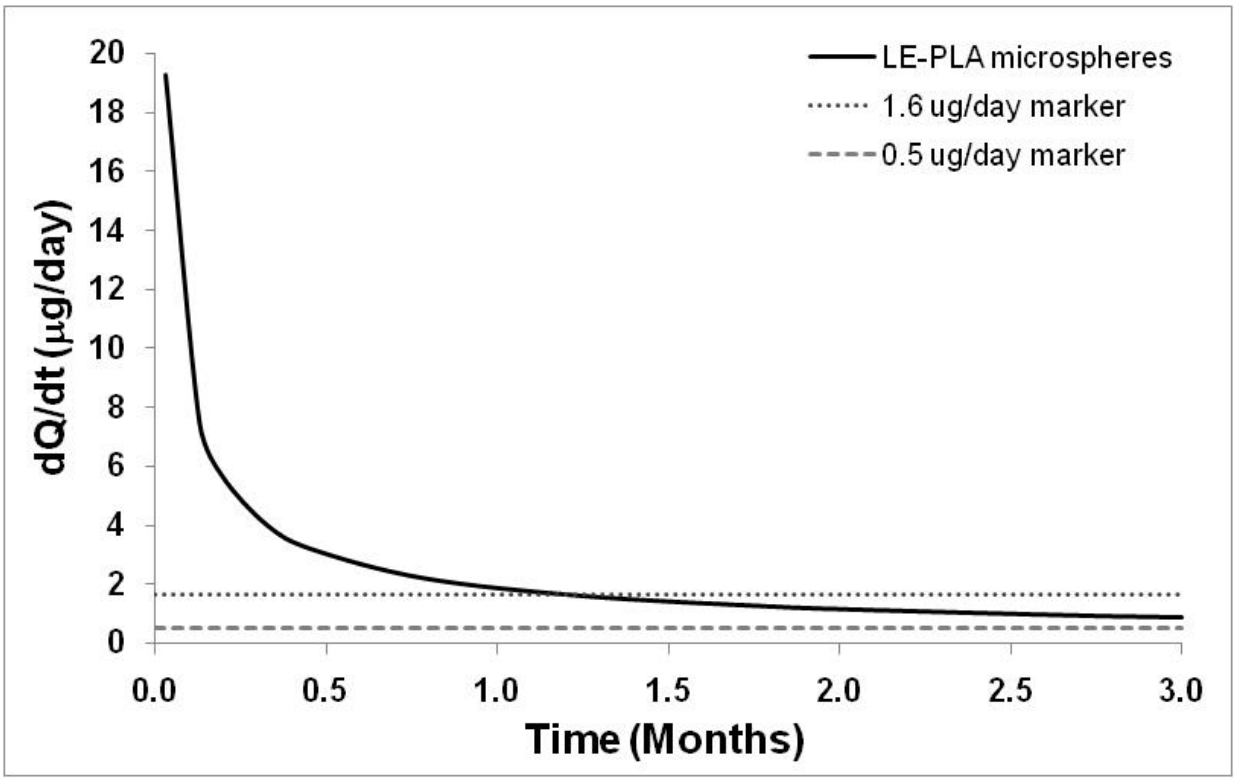


Figure 4-4. Rate of drug released (dQ/dt) from the LE-PLA microsphere formulation

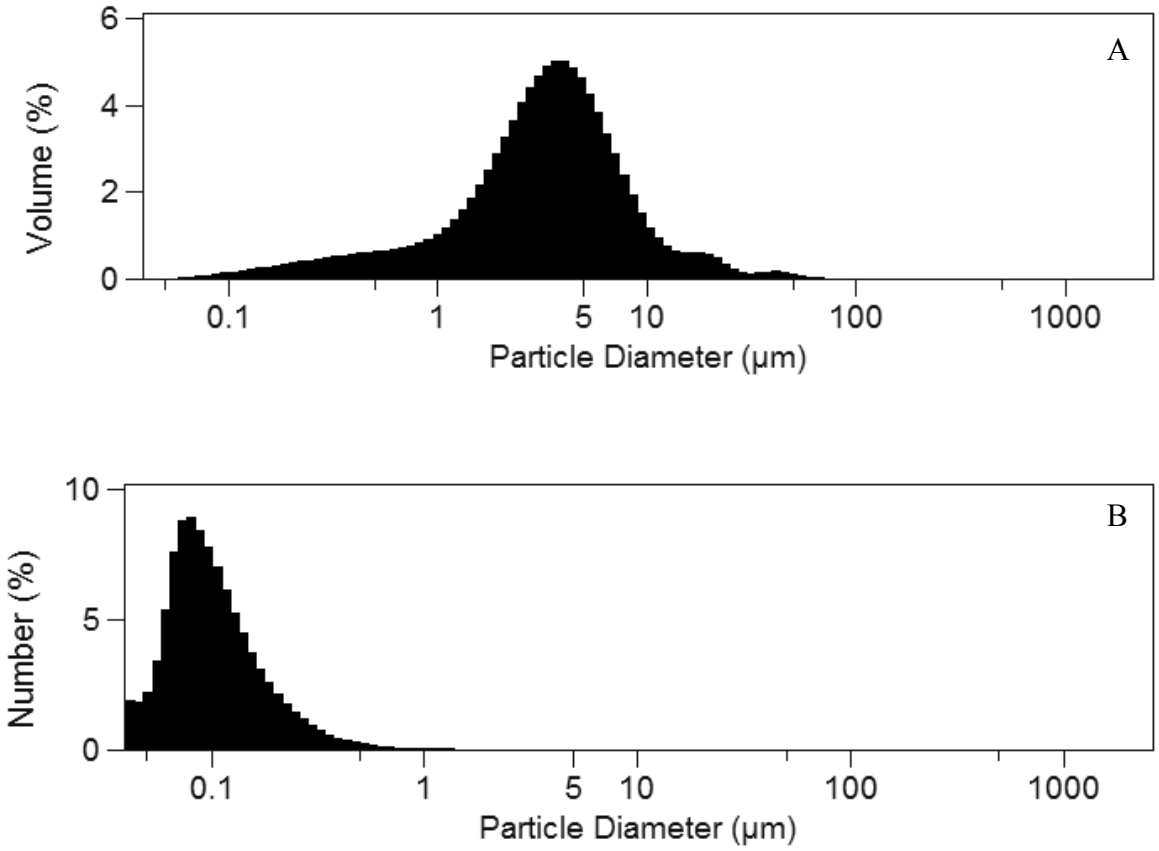


Figure 4-5. Particle size distribution of the LE-PLA microsphere formulation based on the (A) volume and (B) number distribution

Table 4-2. Volume and number distribution statistics of LE-PLA microspheres

	Volume Distribution	Number Distribution
Mean (µm)	5.059	0.124
Median (µm)	3.412	0.094
Standard Deviation (µm)	6.839	0.139
d_{10} (µm)	0.729	0.059
d_{90} (µm)	9.295	0.209
$Mean_{volume}/Mean_{number}$	40.80	

CHAPTER 5
SUMMARY

Poly(D,L-lactic-co-glycolic) acid (PLGA) microspheres developed by solvent evaporation was successful as a sustained drug delivery system for localized delivery of the immunosuppressant loteprednol etabonate (LE) within an implant device. The solvent evaporation process parameters (Chapter 2) were optimized to produce 5 to 50 micron-sized, monodispersed, smooth microspheres having sustained release ranging from three months to a few years. The type of emulsification method used made a difference in the morphology and the release profile of the microspheres produced. The infusion rate of the organic phase into the aqueous phase and the inner diameter of the infusion tube, during the emulsification step, also made a difference in the smoothness, encapsulation efficiency, drug loading, and polydispersity of the microspheres produced. Emulsification using the infusion method produced more monodispersed, spherical microspheres with a more sustained and zero-order release. The drug loading at 5% produced LE-PLA microspheres with the lowest initial burst due to low or no drug adsorbed onto the surface of the microspheres. Using 1% SDS in the wash phase did a great job of removing the drug adsorbed on the microsphere surface. The resultant microspheres had a very low initial burst.

A factorial design analysis study was performed to more thoroughly determine the optimal formulation. The optimal infusion rate (IR) was determined to be 0.8 mL/min. Increasing the inner diameter of the infusion tube (ID) produced larger LE-PLA microspheres but also increased the polydispersity. Increasing the % of polyvinyl alcohol (PVA) in the aqueous solution, produced smaller microspheres with higher polydispersity. The encapsulation and loading efficiency also increased with increasing % PVA. In looking at the *in vitro* drug release of the LE-PLGA microspheres, another parameter beside the size (possibly the density/porosity)

of the microspheres influenced the drug release. Varying the composition of the lactic and glycolic acid in the PLGA polymer had no significant effect on the encapsulation and loading efficiency and the particle size and polydispersity of the microspheres. However, in decreasing the % of the lactic acid monomer, the drug release rates increased. SEM pictures show that the LE-PLGA 50:50 microspheres internal structure collapsed and eroded over time. LE-PLA microspheres ruptured after 4 months of *in vitro* drug release. Monodisperse LE-PLA microspheres had very little difference on the *in vitro* LE release rates when varying the size of the microspheres. However, varying the mean volume diameter, mean number diameter, and the polydispersity made a big difference and should be taken into consideration when predicting the *in vitro* drug release profiles.

Formulation M7 was chosen for the *in vitro* cell toxicity studies (Chapter 3) and the *in vivo* feasibility study (Chapter 4) since it had a desired duration of release of 3 months. The LE-PLA microspheres were analyzed for its *in vitro* cell toxicity using the MTT assay. The drug LE had a threshold concentration of 10 μM and IC_{50} of 20 μM on the MIN-6 insuloma cell line. However, the LE-PLA microspheres had no cytotoxicity. The LE-PLA microspheres were tested to find the immunosuppressive activity in chemically diabetic rats receiving islet transplantation using the novel implant device. The microspheres were successful in preventing islet transplantation by increasing the mean survival duration to 20 ± 6.6 days. The survival duration of the microspheres was twice as much as the control (no drug).

The LE-PLA microspheres did not maintain therapeutic concentrations over the expected 3 month duration. Future studies have to be done with larger, more monodisperse microspheres (> 7 μm and $mean_{volume}/mean_{number}$ ratio < 1.5). Future studies have to also be done to determine the *in vitro – in vivo* drug release correlation by either analyzing the drug remaining in the device at

the end of the study or the feces and urine for the amount of drug and metabolites expelled from the body. However, these results show a great promise in the sustained release LE-PLGA microspheres improving the success rate of islet transplantation by making the way for a more patient compliant and effective immunosuppressant therapy.

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BIOGRAPHICAL SKETCH

Elanor Pinto was born in Kuwait City, Kuwait on September 21, 1980. She graduated St. John's Lutheran High School, Ocala, Florida in 1997. She received her B.S. in Chemical Engineering from University of Florida, Gainesville, Florida in 2002. After her bachelors, Elanor worked for almost a year as a research technician for the Particle Engineering Research Center (PERC), Gainesville, Florida. She entered the pharmaceutical sciences program in 2003 under the supervision of Dr. Günther Hochhaus. She received her Ph.D. in pharmaceutical sciences in December 2008.

While in undergraduate school, Elanor participated in the Particle Engineering Research Center (PERC) Undergraduate Research Assistantship program under the supervision of Dr. Kerry Johanson. In addition, she participated in the selective Integrated Product & Process Design (IPPD) course in which she worked on a Kimberly Clark Co. sponsored project. The feasibility project was successful and resulted in a promising processing technology for the company. While in graduate school, Elanor attended a summer internship at Boehringer Ingelheim in Ridgefield, Connecticut under the supervision of Dr. Xiaohui Mei.

Elanor was an active member of the American Association of Pharmaceutical Scientists (AAPS). During her first year as a member in 2004, she was the Chair of AAPS' University of Florida's Student Chapter. The following year, she was the co-chair and co-founder of the graduate student initiated South East Regional Interdisciplinary Symposium (SERIS) which was held in Gainesville, Florida in 2006. In 2006, she became the first student representative of AAPS' Modified Release Focus Group Steering Team. Also while in graduate school, she was the Pharmaceutical Sciences representative for University of Florida's Graduate Student Council.