Aqueous Remote Loading of Peptides in PLGA Microspheres

by

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Dedication

To my family,

Bennie, Linda, Garner and Hazel.

Thank you for your continual love, support and encouragement.

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Abstract

Poly (lactic-*co*-glycolic) acid (PLGA) microspheres are commonly used in long acting release (LAR) products due to their biodegradable and biocompatible nature. Traditional techniques for peptide drug encapsulation in PLGA microspheres such as the double-emulsion-solvent evaporation method, expose peptides and proteins to harsh conditions such as organic solvents, sheer forces, and temperature fluctuations, which can result in degradation and aggregation of the drug. Traditional encapsulation methods are also costly owing to the need for aseptic processing, the difficulty to scale-up to large-scale manufacture, suboptimal yields, and the possibility for batch failure. An alternative method for microsphere formulation is aqueous remote loading, which is performed by placing the aqueous peptide solution in the presence of sterile drug-free PLGA microspheres, thereby minimizing peptide exposure to potential degradants and simplifying manufacturing. This work investigated aqueous remote loading to encapsulate cationic peptides in acid terminated PLGA microspheres as a means to achieve high encapsulation efficiency and peptide loading with desirable release kinetics *in vitro* and *in vivo*.

Free carboxylic acid terminated PLGA (PLGA-COOH) microspheres of various molecular weight (13 kDa- 38 kDa) and lactic acid to glycolic acid ratios (50/50 and 75/25) were prepared to encapsulate the model cationic peptide, leuprolide. Cationic peptides, such as leuprolide, are able to interact with PLGA-COOH via peptide

absorption in the polymer to achieve elevated loading and encapsulation after incubation for only 24 hours at 37 ºC. Leuprolide was encapsulated in PLGA-COOH microspheres by this aqueous remote loading technique. Initial studies of leuprolide absorption to PLGA microspheres achieved loading (~ 9.8 %) comparable to the commercial 1-month product Lupron Depot®. However, these microspheres did not encapsulate leuprolide at high efficiency (< 40%). Using the sorption isotherms of leuprolide, a model for the prediction of leuprolide encapsulation was developed in order to select formulation conditions to achieve elevated encapsulation efficiencies and loading. Using the model predicted conditions, encapsulation efficiency was improved to > 55%. Optimized loading conditions were then applied to low molecular weight 75/25 PLGA, the polymer used in the commercial 1-month leuprolide/PLGA product, the Lupron Depot®.

Low MW 75/25 PLGA microspheres loaded at 180 mg/mL and 240 mg/mL microsphere concentrations encapsulated > 80% leuprolide and 6.5%- 8.4% loading, and exhibited controlled release over 49 days *in vitro.* Gamma irradiation sterilization of preformed microspheres did not significantly affect peptide loading and release kinetics. These microspheres demonstrated strikingly similar efficacy to the Lupron Depot® in terms of sustained testosterone suppression of rats when dosed monthly for 3 months. The generality of remote loading was tested in 5 peptides using loading parameters optimized for leuprolide. Medium to large basic peptides, octreotide, vasopressin, and salmon calcitonin, exhibited > 65% encapsulation efficiency with elevated loading. However, low levels of encapsulation (< 30% encapsulation efficiency) were observed for acidic and small peptides, exenatide and protirelin, respectively. Hence, this thesis

develops a new and simple means to encapsulate peptides in PLGA microspheres under mild, and less complex conditions, and may be generally applicable to long-acting release microspheres for basic peptides and generic product development.

Chapter 1: Introduction

1.1 Background and Motivation

1.1.1 Protein and Peptide Therapies

Peptides and proteins are a rapidly growing sector of pharmaceutical drug development, with an estimated 10% of the market in 2013 [1-3]. For peptides, in particular, it is estimated that the global market will increase to $25.4 billion by 2018, from $14.1 billion in 2011 [3]. The development of peptide protein therapeutics, starting with the discovery of insulin in 1922, has allowed for the potential to target and treat previously untreatable diseases [4]. Peptide and protein therapeutics are primarily developed for metabolic, oncology, and vaccine indications [1, 3]. Small molecules (<500 Da) have long made up the majority of the pharmaceutical market due to their favorable oral bioavailability which allows for easier administration and reduces the potential for patient non-compliance [5]. However, peptides and proteins can bind targets with high specificity, improving potency while minimizing side effects, and potentially even lowering toxicity as compared to small molecules [5].

Development of peptide and protein therapeutics has historically been slow due to their poor solubility, low oral bioavailability, physical and chemical instability, and/or short *in vivo* half-lives, which creates the necessity for frequent drug injections to maintain efficacy, often leading to patient non-compliance [1, 6]. Alternative, non-

invasive delivery routes such as oral, nasal, and transdermal have been explored for administration of large molecules, but with limited commercial success [1, 2, 7]. The development of delivery vehicles such as liposomes, particles, and micelles provide an alternative to help improve *in vivo* half-lives, maintain molecule stability and therapeutic levels, and decrease the overall amount of expensive drug material used [7, 8]. Delivery vehicles can provide additional benefits including controlling drug release rates. These systems will be discussed in greater detail in the next section.

1.1.2 Controlled Release Delivery

The use and development of controlled release drug delivery systems (e.g. polymeric micro/nanoparticles, liposomes, implants, micelles, and *in situ* forming systems [9]) is becoming more common as a delivery method for proteins since they can potentially overcome the challenges of traditional injections [10]. Controlled release systems (CRS) have also been explored to help improve *in vivo* half-life, maintain therapeutic levels, and decrease the amount of expensive drug material needed for delivery [7, 8]. Encapsulation in CRS can also protect peptides and proteins which are susceptible to protease degradation, aggregation, and denaturation [1].

Controlled release polymer systems have been used for both systemic and local delivery of small and large molecules at a constant rate on the time scale of days to months after administration [9]. CRS offer several advantages [11, 12] including: stable blood therapeutic concentrations and increased compliance and convenience. Maintenance of stable drug levels within the therapeutic window leads to improved efficacy by decreasing blood-plasma concentration variability, thus decreasing the potential for toxicity or inefficacy. Due to their high instability and short *in vivo* half-lives,

peptide and protein therapies are usually administered as intramuscular or subcutaneous injections which have a high rate of patient non-compliance since multiple, possibly painful, injections are often needed [13, 14]. Improved patient compliance is achieved from decreased dosing frequency to minimize painful injections and is more convenient for the patient [8, 12, 15].

Controlled release delivery also allows for easier administration of peptides or proteins with short *in vivo* half-lives or poor bioavailability [8, 10, 16, 17]. Issues of solubility/degradation are mitigated since the polymer acts as a protective shell. Bioavailability limitations can be overcome by local delivery without the aforementioned toxicity concerns. Biodegradable polymers are of particular interest for controlled release drug delivery due to the polymer's biodegradable nature; the need to remove the delivery vehicle after administration is eliminated [9]. Therefore, controlled release via biodegradable polymeric systems can be especially beneficial in the case of peptide and protein delivery as they can overcome these challenges.

1.2 PLGA for Long Acting Release (LAR)

1.2.1 Chemistry

One commonly used polymer for controlled release delivery of peptides and proteins in Food and Drug Administration (FDA) approved products is poly(lactic-*co*-glycolic) acid (PLGA) [18]. PLGA is biocompatible and biodegradable [19]. Additionally, PLGA is used for long acting release (LAR) drug delivery, on the order of days to weeks; few other systems are generally able to achieve such extended release durations making them less desirable in terms of administration frequencies [6]. Another

desirable attribute of LAR formulations of PLGA is that they can deliver drugs to a specific target without large fluctuations in drug concentration, which can occur with repeated bolus injections [11, 15, 20], and thereby improve the safety profile (Figure 1.1).

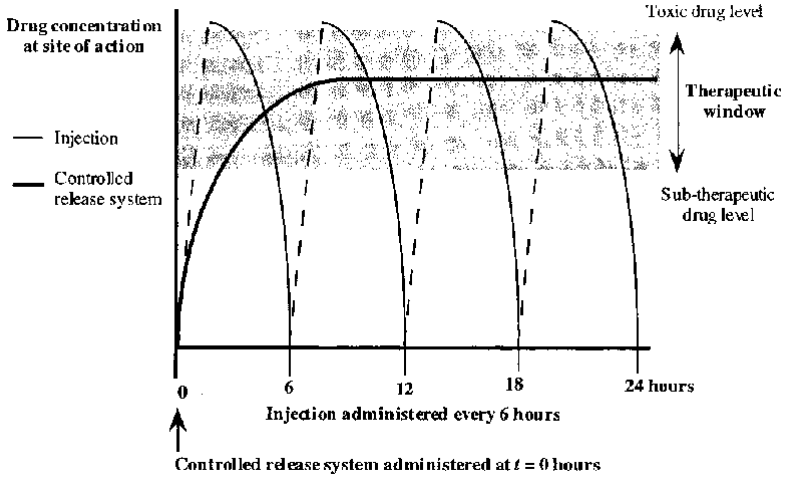

Figure 1.1 Drug concentrations at site of therapeutic action after delivery as a conventional injection (thin line) and as a controlled release system (bold line). Adapted from [15].

PLGA is a copolymer of the biocompatible monomers lactic acid (LA) and glycolic acid (GA) (Figure 1.2). PLGA can be tuned to obtain desired degradation and drug release rates by altering the physical properties such as molecular weight, lactic acid to glycolic acid ratio and terminal groups (ester or free carboxylic acid) [18]. For example, free acid terminated PLGA is more hydrophilic and thus undergoes hydrolysis and degradation at a faster rate than ester terminated PLGA [21]. As any of these properties are changed the process by which the polymer erodes changes therefore affecting the drug release rate [22].

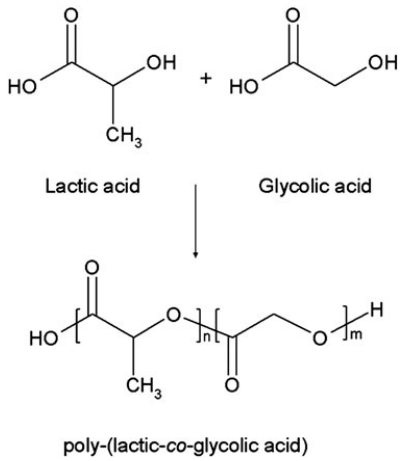

Figure 1.2 Structure of lactic acid and glycolic acid which form the monomeric backbone of PLGA. Adapted from [11].

1.2.2 Attributes of PLGA

Some key attributes of PLGA that can influence degradation and release rates from PLGA are the lactic acid to glycolic acid ratio (LA:GA), molecular weight, water uptake, crystallinity, glass transition temperature (T_g) and shape of the drug delivery system (e.g. microspheres, cylinders).

The interplay between these factors and how they alter the degradation rate of the polymer and consequently the release rate of the encapsulated drug can be controlled is fairly complex [18, 19]. For LA:GA, due to the more hydrophilic nature of GA, PLGA with a higher GA monomer content degrades faster due to greater hydrophilicity since increased hydrophilicity causes more water uptake and hydrolysis of the polymer backbone [18]. Faster degradation of the polymer chains results in faster bulk PLGA degradation and drug release rates [22]. Conversely, PLGA of higher molecular weight has a decreased degradation rate, since the polymer chains are larger and slower to breakdown, thus drug release is slower for these formulations [22].

PLGA hydration can affect the drug release rate by causing both polymer degradation and polymer swelling, which creates pores in the polymer matrix. In terms

of polymer degradation, water uptake causes both hydrolysis and acidic auto-catalysis of the polymer chains [23]. Water uptake by the polymer also results in breaking of the polymer ester bonds creating free acids which can further catalyze hydrolysis of other ester bonds. This causes a catalytic, heterogeneous degradation of the polymer where the polymer rich center degrades faster than the surface [23, 24].

Other factors that can affect degradation and release are the crystallinity and shape of the delivery system. More crystalline PLGA has been shown to have a slower rate of water uptake which results in a decreased rate of degradation [20, 25]. The shape/size of the drug delivery system can also affect degradation as a larger delivery vehicle has a larger gradient of auto-catalysis and heterogeneous degradation which manifests in a faster rate of degradation [23]. In addition, the shape of the delivery vehicle can alter how the drug and degradation products (LA and GA) are distributed in the polymer and thus the release rate from PLGA [23]. Overall the degradation rate of PLGA is a very complex interplay of several factors that can work to accelerate or decelerate drug release from the polymer matrix.

1.2.3 Traditional Microsphere Formulation Methods

1.2.3.1 Solvent Evaporation

There are several methods by which drugs can be encapsulated in PLGA including solvent evaporation of a single or double emulsion, coacervation/phase separation, spray drying, and implants [26]. In the solvent evaporation method, the drug can be encapsulated in an oil-in-water (o/w) single emulsion, solid-in-oil-in-water (s/o/w) double emulsion, or water-in-oil-in-water (w/o/w) double emulsion depending on the drug properties [17]. The w/o/w method is ideal for encapsulating water-soluble

peptides, proteins, or vaccines [17, 27]. In this method, an aqueous solution of the drug/peptide, also known as the inner water phase, is added to the PLGA dissolved in an organic solvent or “oil phase”, such as methylene chloride, and homogenized to generate the inner water-in-oil (w/o) emulsion. The second emulsion is formed by adding an aqueous phase, generally containing an emulsifier, such as poly vinyl alcohol (PVA), to the first emulsion and homogenizing. This double emulsion is then transferred to a large volume “hardening” bath, generally containing a low concentration of PVA to further help stabilize the emulsion, and is stirred for several hours to allow for evaporation of the organic solvent which subsequently hardens the polymer [27, 28]. Microspheres are then washed and collected in the desired size range, 20 μm – 100 μm is ideal for injectable LAR formulations, and lyophilized to produce the final product.

This double emulsion technique can produce high loading efficiencies of water soluble drugs but each step of the process may need to be optimized as it will impact the final product. For example, the rate and extent at which the solvent is removed from the microspheres can result in cracks in the polymer which will adversely affect the drug release [29]. The various processing steps can also cause protein or peptide denaturation, aggregation, oxidation and/or cleavage due to the thermal stress and shear forces of homogenization and exposure to the aqueous-organic interface [28]. This instability can lead to a decrease or loss of activity of the encapsulated protein/peptide. Once parameters are optimized it is important to control processes to prevent batch-to-batch variation in microspheres which is important for maintaining syringeability and final microsphere characteristics [6, 30].

1.2.3.2 Phase Separation and Spray Drying

Phase separation, or coacervation, is the process by which water-in-oil-in-oil (w/o/o) microspheres can be produced. In this method, PLGA is dissolved in an organic solvent and the solubilized drug is dispersed in this polymer solution to create the primary w/o emulsion. A solvent in which PLGA is not soluble (e.g. silicon oil) is then added to the polymer-drug solution. The polymer then undergoes phase separation to produce a coacervate phase which forms droplets entrapping the drug. The final steps are microsphere hardening by adding the coacervate to a hardening agent such as hexane to extract the organic solvents, followed by washing and lyophilization steps [26, 31]. However, this method can often lead to microsphere aggregation as they may agglomerate prior to the hardening phase [26].

Unlike the double emulsion and phase separation methods, spray drying is a rapid technique with mild conditions and is not dependent on polymer solubility [26]. In this method, a protein-polymer solution/emulsion is atomized in hot air allowing for volatile solvent evaporation [28, 31]. The final properties of the microsphere are however, highly dependent on the parameters used during development and can result in low microsphere yield [31].

1.2.3.3 PLGA Implant Systems

Implantable devices were the first to utilize controlled release drug delivery and used a rate controlling membrane for ophthalmic or intrauterine delivery of drugs [32]. Since, PLGA implants have been successfully developed and commercialized for the delivery of several drugs, including goserelin (Zoladex®) and dexamethasone (Ozurdex®) which are cylindrical PLGA implants [33, 34]. One method for development

of cylindrical PLGA implants (“millicylinders”) includes extruding a polymer solution containing dispersed drug powder. In this method PLGA is dissolved in acetone and mixed with the drug particles. The solution is then extruded, dried, and cut to the desired length [35, 36]. PLGA implants are promising drug delivery system as they allow for site specific delivery and have shown promising application for ocular and brain drug delivery; however, these are more invasive then microspheres since they require surgical intervention [6].

1.2.4 Determination of Drug Loading in PLGA

Efficient drug encapsulation is important to ensure the correct dosage is delivered to maintain therapeutic efficacy *in vivo* throughout the desired period of administration. Efficient drug loading in PLGA microspheres also ensures minimal drug is wasted during formulation of microspheres [20]. The amount of drug in microspheres can be quantified by encapsulation efficiency and weight-by-weight (w/w) loading efficiency. Encapsulation efficiency (*EE*) is defined as the amount of loaded or encapsulated drug divided by the total drug (encapsulated and un-encapsulated), i.e $EE = Encapsulated\ Drug/Total\ Drug$. Loading efficiency is determined by the encapsulated drug divided by the total weight of the polymer and peptide, i.e $w/w = Encapsulated\ Drug/Encapsulated\ Drug\ Mass + PLGA\ Mass$.

1.2.5 Release Mechanisms from PLGA

Generally, drug release from PLGA LAR formulations occurs in a multi-phasic manner through both diffusion and degradation mediated mechanisms [37] (Figure 1.3). However, the underlying mechanisms that control release rate are complex and depend on several properties of the polymer (Figure 1.4).

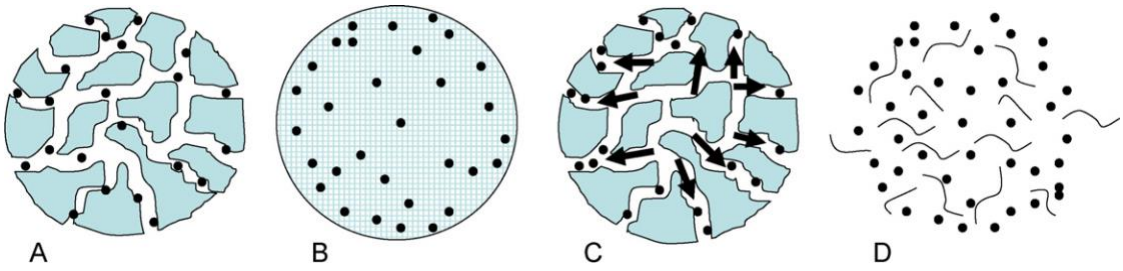

Figure 1.3 Drug release mechanism: Diffusion through water filled pores (A), diffusion through the polymer (B), osmotic pumping (C), and erosion (D). Adopted from [23].

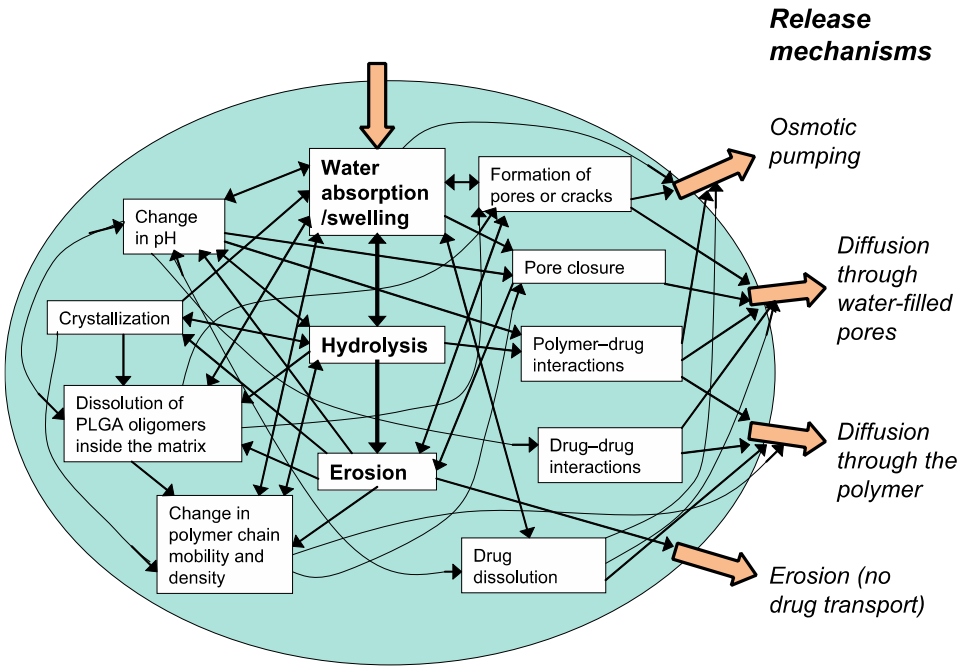

Figure 1.4 The complex physio-chemical processes occurring within PLGA matrices, leading to drug release. Arrows indicate the influences of processes on drug release and on other processes. Adapted from [23].

There are three phases of drug release from PLGA: an initial burst phase, a lag or induction phase, and an erosion phase. During the initial burst phase, release of drug that is not physically bound to the polymer or is a free solid occurs as the drug diffuses out of the microspheres over the course of days to weeks. During the “lag” or “induction phase” there is very little drug released as small amounts of polymer erode from the inside-out. Lastly, the active erosion phase, occurs during bulk degradation of the PLGA in which polymer mass is continuously lost resulting in continuous release of the

encapsulated cargo [6]. The interplay of drug diffusion and polymer erosion as well as microsphere porosity and size can affect the drug release. The effects of these parameters are more closely examined next.

1.2.5.1 Effect of Polymer Erosion and Drug Diffusion on PLGA Release Kinetics

Biodegradable polymers such as PLGA can undergo either surface or bulk erosion, both of which can influence the drug release rate. PLGA erosion is dependent on the amount of water that diffuses into the bulk polymer and the degradation rate of the polymer backbone by hydrolysis [30, 38]. As the PLGA backbone is hydrolyzed, soluble oligomers are formed, increasing the polymer permeability leading to continuous or pulsatile drug release [39].

Diffusion of the drug, particularly during the initial burst phase, can also have significant impact on the overall drug release kinetics. The primary contributor to initial burst is un-encapsulated or surface associated drug molecules which are not physically bound to the polymer. Drugs that are located within the polymer matrix can also contribute to the initial burst via diffusion through pores and cracks, which result from the initial microsphere formulation process [29, 40].

Drug diffusion can occur before erosion mediated drug release and the balance between diffusion and erosion controlled release can result in different observed shapes for the release profiles. For example, a tri-phasic release occurs when diffusion ends prior to the start of erosion and continuous release occurs when diffusion and erosion overlap with each other [30]. The different release profiles are shown in Figure 1.5 [23].

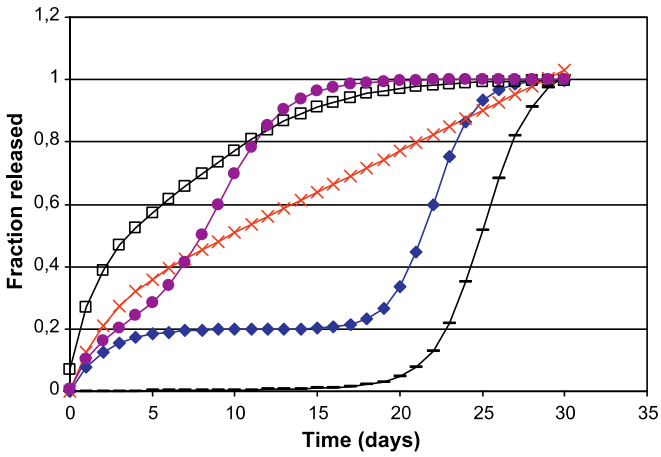

Figure 1.5 Release profiles of different phases. Open Squares: burst and a rapid phase II. Filled circles: tri-phasic release with a short phase II. Crosses: burst and zero-order release. Filled diamonds: tri-phasic release. Dashes: bi-phasic release, similar to tri-phasic but without the burst release. Adapted from [23].

The lag/induction phase of release is characterized as the period over which little to no drug diffusion out of the polymer matrix and the polymer mass loss is minimal due to decreased presence of water soluble oligomers [6]. Another reason for the decrease in drug diffusion during the lag phase is the collapse of microspheres surface pores when exposed to aqueous environments resulting in polymer chain rearrangement and healing of the surface pores, thus slowing drug diffusion from the microspheres [29, 41, 42]. The final phase is characterized by complete solubilization of the drug due to polymer erosion which results in faster rates of drug release [23, 26]. During this phase the polymer mass continually decreases resulting in continuous release of drug as it's freed from the remaining polymer matrix [6].

1.2.5.2 Effect of PLGA Microsphere Characteristics on Release Kinetics

In addition to the polymer characteristics, several properties of the microspheres can influence drug release kinetics including the porosity, particle size and formulation method.

Control over particle size is not only important when considering syringeability but also for drug diffusion from the polymer matrix. For example, larger particles present a challenge to drug diffusion as the path length for diffusion of a drug out of the microspheres is increased while the drug concentration gradient is decreased [43]. The method of microspheres formation can affect porosity and therefore drug release as variable pore networks are created during the process. For example, during solvent evaporation, if solvent removal occurs quickly, microsphere porosity increases since the particle exterior hardens prior to oil droplets completely shrinking, leaving behind large porous cavities in the microspheres. This causes a decrease in polymer density and therefore can cause a higher initial burst release of the drug [29, 44]. Slow solvent removal allows time for the particle volume to decrease, slowly decreasing the particle surface area, and for oil droplets to sufficiently shrink before hardening occurs, maximizing the density of the polymer core and thus slowing the initial burst release [29].

The aforementioned factors can all affect the drug release kinetics from PLGA in different ways, therefore, it is important to ensure that the formulation methods and type of PLGA chosen are ideal for achieving the desired release kinetics. Overall, developing a LAR PLGA formulation is challenging and fraught with complexities. Despite the advantages of LAR formulations, the inability to obtain acceptable release profiles, as well as the difficulty of reproducibility, have been the main contributors to the slow development and approval of marketed LAR products [44].

1.3 Currently Marketed Products

1.3.1 Lupron Depot®

Lupron Depot® (LD), developed by Takeda Pharmaceutical Company, was approved in the US in 1989 as a PLGA microsphere product for the delivery of leutinizing hormone-receptor hormone (LHRH) analogue, leuprolide [9]. Initially it was approved as a monthly injection for the treatment of prostate cancer; the LD was one of the first PLGA based drug delivery product approved in the US and is still marketed today. Since its initial approval, many new LD formulations using either PLGA or polylactic acid (PLA) as the polymeric matrix have received approval for the treatment of endometriosis, fibroids, and central precocious puberty with injections frequencies ranging from 1 to 6 months [45, 46]. LD is administered by intramuscular injection and functions by releasing leuprolide, a gonadotropin releasing hormone (GnRH) agonist, which acts on the LHRH receptor to stimulate gonadotropin secretion and steroidogenesis when administered in acute doses. However, when administered chronically at high doses, leuprolide results in chemical castration due to overstimulation of the LHRH receptor causing receptor downregulation and inhibition of steroidogenesis [47].

During initial development of LD, scientists were tasked with the development of a new method to formulate small, spherical, microspheres containing large doses of leuprolide acetate, that could be delivered with zero order release kinetics over 1 month. Microspheres of PLGA and PLA of various molecular weights, containing an inner water phase of leuprolide acetate, were prepared by an in-water drying method and sieved for various sizes ranging from 37 μm - 125 μm. To achieve high encapsulation of

leuprolide, several parameters were varied including inner water phase viscosity and the viscosity of the water-in-oil (w/o) emulsion. A higher inner water phase viscosity and w/o emulsion viscosity correlated to a higher encapsulation of leuprolide in microspheres [48]. So, final formulations of PLGA microspheres having an inner water phase of leuprolide acetate in water plus gelatin were used [48, 49].

The next step in the development of LD was to ensure release over 1 month. This was done by testing the effect of additives and modulating polymer molecular weight to alter the release kinetics of leuprolide. The inclusion of additives only resulted in an increased initial burst release but did not increase the release duration. Lowering the molecular weight of the PLA from 22.5 kDa to 12.2 kDa and 6 kDa increased the release rate, an undesirable result. Using PLGA 75/25 with a molecular weight of 14 kDa yielded the optimal result as leuprolide was released over 1 month with near zero-order kinetics and had a low initial burst release [50]. The final product consisted of 14 kDa 75/25 PLGA microspheres of leuprolide emulsified with gelatin, to ultimately yield a peptide core, in which the arginine and histidine residues from leuprolide ionically interacted with the carboxylic acid ends of the PLGA [51].

1.3.2 Slow Market Adoption of PLGA Products

The application of biodegradable polymers, such as PLGA, for sustained release of steroids, peptides and proteins began in the mid to late 1980's. Several of those early products including Decapeptyl® LP, approved in Europe in 1986, and Lupron Depot®, approved in the US in 1989, are still on the market today [32]. However, the approval of polymeric LAR for sustained drug delivery has been slow in the US, as there are approximately 15 approved products on the market to date despite the first US approval

occurring nearly 30 years ago [49]. These marketed LAR formulations include microspheres, implants and *in situ* forming gels (Table 1.1) [52]. The majority of these approved products are PLGA microspheres and implants delivered either intramuscularly or subcutaneously with dose frequencies ranging from once a week to once every 6 months [49].

Table 1.1 List of PLGA and PLGA drug products approved by the US FDA. Adapted from [49].

Product	Active Ingredient	Dosage Form	Route of Administration	Indication (Initial Approval Date)
Vivitrol	Naltrexone	Microsphere	IM	Treatment of alcohol dependence (2006)
Zoladex	Goserelin acetate	Implant	SQ	Management of locally confined prostate carcinoma (1989)
Lupron Depot	Leuprolide acetate	Microsphere	IM	Palliative treatment of prostate cancer (1989)
Lupron	Leuprolide acetate	Microsphere	IM	Treatment of Central Precocious Puberty (1993)
Lupron Depot-PED	Leuprolide acetate	Microsphere	IM	Management of endometriosis (1995)
Sandostatin LAR	Octreotide	Microsphere	SQ	Acromegaly (1998)
Trelstar	Triptorelin pamoate	Microsphere	IM	Palliative treatment of advanced prostate cancer (2000)
Risperdal Consta	Risperidone	Microsphere	IM	Schizophrenia & bipolar I disorder (2003)
Ozurdex	Dexamethasone	Implant	Intravitreal Injection	Macular edema, non-infectious uveitis, diabetic macular edema (2009)
Bydureon	Exenatide	Microsphere	SQ	Improve glycemic control in adults with type 2 diabetes
Signifor LAR	Pasireotide pamoate	Microsphere	SQ	Cushing's disease/ Acromegaly (2014)

There are several potential reasons for the slow development and approval of PLGA delivery systems, including challenging encapsulation and manufacturing methods, undesirable release kinetics, and maintaining drug stability within the polymer for the product duration [6]. Nutropin Depot® demonstrates some of the manufacturing difficulties associated with LAR PLGA formulations. Nutropin Depot®, approved by the FDA in 1999, was a PLGA microsphere formulation encapsulating recombinant human growth hormone (hGH) and was administered either once or twice monthly for the treatment of growth hormone deficiency [53]. Nutropin Depot® performed well in preclinical studies and clinical trials but was discontinued in 2004 due to manufacturing challenges, such as low microsphere yield within the size range necessary for injection via a reasonable gauge syringe [4]. As a result, there was a high associated cost of manufacturing, which was a two-week process to manufacture one batch, and was not competitive when compared to daily injections of hGH, due to the reported 2-3 adverse reactions at the injection site per injection and the 21-gauge needle used for administration compared to the more pain-free 30-gauge needle used in the daily injections [4, 54-56].

1.4 Remote Loading

1.4.1 Aqueous Remote Loading Paradigm

A technique discovered in the Schwendeman lab called “self-healing” microencapsulation or aqueous remote loading of PLGA microspheres can help to overcome some of the problems of w/o/w double emulsion preparation by solvent evaporation. Including the harsh conditions such as exposure to the organic

solvent/aqueous interface, shear forces (e.g vortex and homogenization), residual solvent levels in microspheres and temperature fluctuations which can result in peptide/protein instability [57]. In the remote loading encapsulation method, drug free ("blank") w/o/w double emulsion PLGA microspheres are prepared by the solvent evaporation method with the leachable porosigen, trehalose, as the encapsulate instead of a drug/protein. During the evaporation, the porosigen leaches out creating microspheres with an interconnected porous network [41]. The dried microspheres are then incubated in a concentrated drug solution at a temperature below the glass transition temperature (T_g) to allow drug loading into the pores of the microspheres [41]. Once loading is complete, the microspheres can be heated to a temperature above the T_g to allow for spontaneous polymer chain rearrangement and thus close or "heal" the pores [41, 57]. Illustrations of conventional w/o/w microspheres prepared by solvent evaporation and remote loading are shown in (Figure 1.6).

This encapsulation paradigm minimizes the protein/peptide instability as there is little exposure to shear forces (e.g. homogenization), organic solvents, excess heating or freezing, or micronization of the drug [57, 59]. This technique also allows for terminal sterilization of the blank microspheres prior to protein/peptide encapsulation allowing a variety of sterile protein solutions to be mixed with the microspheres later. Therefore, microsphere formation does not require aseptic processing of organic solvents, so the cost of manufacturing can also be reduced [41].

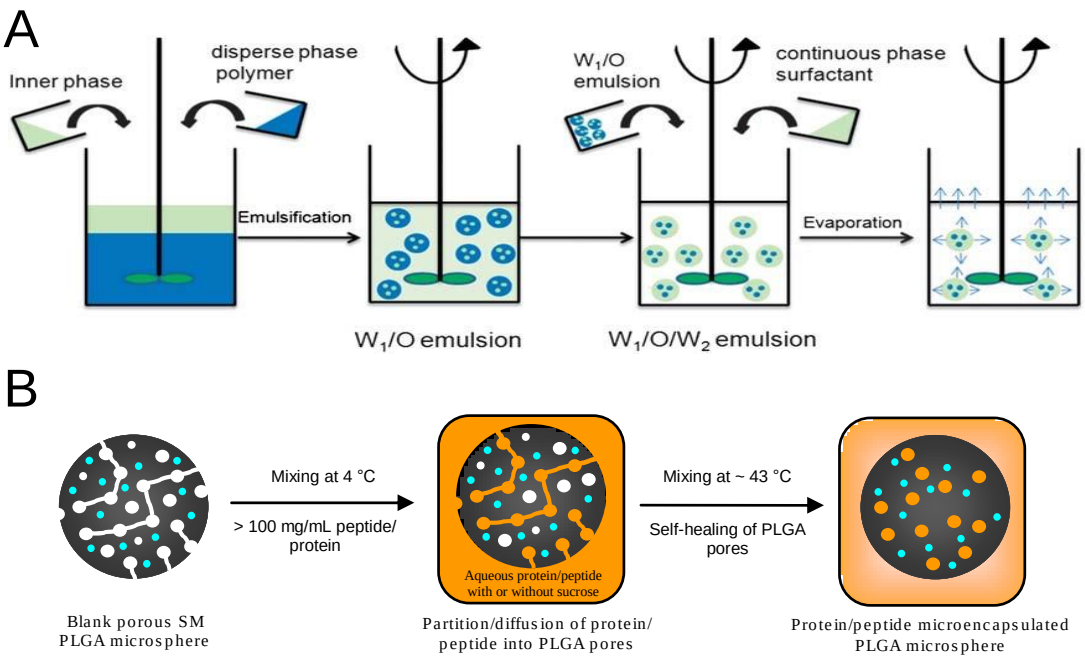

Figure 1.6 Illustrations of traditional w/o/w double emulsion solvent evaporation technique (A). Illustration of passive loading from a high concentration drug solution by aqueous remote loading (B). Adapted from [41, 58].

1.4.2 Active and Passive Remote Loading

The remote loading paradigm has been developed to both passively and actively load drugs. Initial studies using this “self-healing” microencapsulation paradigm to passively encapsulate the model proteins, lysozyme and bovine serum albumin, via diffusion resulted in 1-10 % w/w loading of the drug but this process had low encapsulation efficiencies as high initial protein concentrations (>100 mg/ml) were used [41, 57]. Encapsulation efficiency was improved by switching to an “active” loading method. Active remote loading can be achieved by adding a “trapping agent,” such as aluminum hydroxide gel, which can effectively bind and entrap drug from the external solution into the microspheres to achieve increased encapsulation efficiency [57]. The trapping agent was incorporated into the inner water phase during microsphere formation and was able to help increase protein encapsulation efficiencies to >85 %

while maintaining high w/w loading as demonstrated using ovalbumin and tetanus toxoid as models [57, 60].

Active drug encapsulation by remote loading has also been accomplished using various trapping agents such as biopolymers, including dextran sulfate, hyaluronic acid, and chitosan, to improve encapsulation and stability. A similar biomimetic approach was also successfully used in the commercial Nutropin Depot®: human growth hormone was complexed with zinc to maintain protein stability during encapsulation and release [61]. Like previously with aluminum hydroxide, the biopolymers were added to the inner water phase during microsphere formation and were present in the microsphere pores. Using this biomimetic approach, remote loading of lysozyme, VEGF and FgF-20 were achieved with encapsulation efficiencies >70% [61]. These examples for active and passive remote loading indicate the broad applicability for this technique. Additionally, drug stability is maintained during encapsulation and in the case of active loading, high encapsulation efficiencies can also be achieved [61].

1.5 Research Scope

The preceding introduction discusses controlled release from polymeric systems specifically PLGA microspheres, for drug delivery. Lupron Depot®, one of the first US FDA approved PLGA microparticle delivery systems, has experienced commercial success due to its ability to release therapeutic drug levels over several weeks from a single injection. However, there exists a gap in the development of PLGA LAR formulations as the commercial success of Lupron Depot® has not translated widely to the development of either innovator or generic PLGA microsphere drug delivery

systems. The work presented here will demonstrate the potential for the aqueous remote loading technique as a viable alternative to solvent evaporation as a mechanism for drug encapsulation in PLGA microspheres.

1.6 Thesis Overview

The overall goal of this thesis is to bridge the gap and use a novel aqueous remote loading technique to efficiently encapsulate the model peptide, leuprolide, at high drug loading and to achieve continuous release kinetics *in vitro* and *in vivo* and to ultimately enable future PLGA formulation development. In order to do so, the underlying mechanisms of interaction between the model peptide, leuprolide, and polymer are examined. Ultimately, this research should fundamentally help to further the development of new LAR PLGA depot formulations. This thesis consists of 6 chapters arranged around key concepts used to develop this research.

Chapter 2 of this thesis focuses on the development of a mechanistic understanding of the interaction between the model cationic peptide, leuprolide, and acid terminated PLGA microspheres. Specifically, this section seeks to understand the binding kinetics and thermodynamics of leuprolide absorption to PLGA. A visual understanding of how a cationic peptide interacts with the microspheres during loading is established using a fluorescently tagged peptide (octreotide).

Chapter 3 studies the aqueous remote loading technique as a function of various formulation parameters, e.g., microsphere concentration, porosity, and incubation time, in order to achieve elevated leuprolide loading and encapsulation efficiency comparable to that of commercial LAR products. Mathematical models to predict loading and

encapsulation efficiency are derived using binding isotherms. These models can be used to further refine the formulation parameters to maximize loading and EE.

Chapter 4 considers the next steps towards developing an alternate LD formulation and applies the remote loading technique to the PLGA composition, 75/25 LA/GA and 11-13 kDa - similar to that used in the commercial Lupron Depot®. Using the loading conditions optimized from Chapter 3, leuprolide is encapsulated in PLGA microspheres. The effects of various processing parameters, including gamma irradiation, on microsphere integrity and release *in vitro* are studied. Some of these formulations were then tested in rats to determine plasma testosterone levels and efficacy compared to the commercial 1-month Lupron Depot®.

Chapter 5 describes the applicability of the remote loading paradigm to other peptides. Octreotide, exenatide, salmon calcitonin, vasopressin, and protirelin are examined for their potential to remotely load based upon previous findings in earlier chapters. *In vitro* release of octreotide and exenatide is also monitored under similar conditions as previously described.

The conclusions of this research and potential future applications are discussed in chapter 6.

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Chapter 2: Mechanisms of Peptide Adsorption to PLGA

2.1 Abstract

Cationic peptides have been shown to interact with poly(lactic-*co*-glycolic) acid (PLGA) microspheres by an absorptive process using sorption isotherms, measuring peptide remaining in solution and peptide loading as a function of thickness [1, 2]. However, there has been little done to understand the interaction on the small scale/non-bulk case. This small-scale understanding can be achieved through thermal analysis (isothermal titration calorimetry (ITC), differential scanning calorimetry (DSC)) and confocal microscopy. In this work, these techniques are used to further the mechanistic understanding by which cationic peptides interact with PLGA. ITC experiments with a low molecular weight 50/50 PLGA demonstrated that leuprolide binds to 50/50 PLGA with high affinity (0.73 μM^{-1}) in agreement with previous results that fit peptide binding to a modified Langmuir equation [3]. The apparent enthalpy was determined by DSC as ~0.012 kJ/g and the Gibbs free energy was -0.097 J/g indicating that leuprolide binding to PLGA-COOH is spontaneous and may be entropically driven. Lastly, octreotide was tagged with bodipy in order to observe peptide loading over time in PLGA microspheres. An apparent increase in brightness is seen under confocal microscopy between 1 h and 8h of loading but there is minimal apparent increase in brightness during the last 16 h of loading which is supported by mass loss analysis of

octreotide loading in which microspheres achieves a quasi-equilibrium after 8h of incubation in an octreotide solution.

2.2 Introduction

Poly(lactic-*co*-glycolic) acid (PLGA) has been widely studied for use in long term (days to months) controlled release drug delivery due to its biocompatibility and biodegradability [4, 5]. The most common method for peptide encapsulation in PLGA microspheres is the double emulsion solvent evaporation, however, this method can cause peptide denaturation due to exposure to the water/oil interface [6]. Aqueous remote loading of microspheres allows for peptide loading by simple incubation of drug-free microspheres in an aqueous peptide solution, thus limiting peptide exposure to organic solvents that can cause degradation [5].

Previous studies have looked at the interaction between PLGA and several peptides, including salmon calcitonin and the model peptides leuprolide and octreotide. Tsai et al. found that salmon calcitonin exhibited a constant rate of adsorption ("transient equilibrium") to 50/50 PLGA followed by continuous adsorption [7]. In subsequent experiments by Na et al., the interaction between octreotide and hydrophobic and hydrophilic PLGA was studied. In the presence of acid end-capped PLGA (502H) octreotide was rapidly adsorbed to the PLGA (95%). However, in the presence of ester-terminated PLGA the initial adsorption of octreotide was much lower (20%) until day 21, indicating the importance of carboxyl end groups of PLGA for octreotide interaction [1].

The work of Tsai and Na suggested that peptides could interact with PLGA through surface adsorption. However, Sophocleous et al. studied this interaction between cationic peptides and acid terminated PLGA further. Through two key experiments this interaction was found to be caused by peptide absorption rather than a simple surface interaction. The first experiment looked at octreotide and leuprolide sorption as a function of PLGA film thickness and temperature. The sorption of both peptides increased linearly with thickness at 37°C indicating the peptides were absorbed into the polymer. The hypothesis of peptide absorption was further confirmed by first sorbing octreotide to a PLGA then removing sections of the film and determining the peptide remaining, which steadily decreased as polymer was removed. In the second experiment, leuprolide penetration into a PLGA film was observed by stimulated Raman scattering (SRS) and the characteristic peak of leuprolide was detected at all film depths with similar intensity shown for both leuprolide and acid terminated PLGA [2].

These three experiments confirmed that cationic peptides such as leuprolide and octreotide are able to interact with acid terminated PLGA (PLGA-COOH) and that this interaction occurs by absorption. However, there has been minimal work to understand the mechanisms behind this interaction beyond the necessity for salt formation between peptide and polymer. The following discussion will explore additional interactions that occur during peptide absorption to acid terminated PLGA such as calorimetric determination of binding constants and the thermodynamics associated with the unique physical interaction.

2.3 Materials and Methods

2.3.1 Materials

Free acid terminated PLGA Resomer® 502H (i.v.= 0.16-0.24 g/dL), Resomer ® 503H (i.v.= 0.32-0.44 g/dL) and PVA (80% hydrolyzed) were purchased from Sigma Aldrich (St. Louis, MO). PLGA 75/25 was purchased from Wako Chemicals (Osaka, Japan. Octreotide acetate and leuprolide acetate were purchased from SHNJH Pharmaceuticals (Shanghai, China). Bodipy FL dye and Hydroxyethyl-piperazineethanesulfonic acid (HEPES) were purchased from Thermo Fisher (Waltham, MA). All other materials were of analytical grade and purchased from commercial suppliers.

2.3.2 Microsphere Preparation

PLGA microspheres were prepared using a water-in-oil-in-water (w/o/w) double-emulsion solvent evaporation method. Free acid terminated 50/50 PLGA of two different molecular weights were used for these formulations; 503H PLGA (24-38 kDa) and 502H (7-17 kDa). For both polymers, PLGA was dissolved in methylene chloride at a concentration of 800 mg/mL. Once dissolved, 200 µL of the inner water phase containing trehalose (500 mg/mL) was added to the dissolved polymer solution and homogenized for 1 minute at 10,000 rpm to form a water-in-oil (w/o) emulsion. Next, 4 mL of 5% PVA was added and vortexed for 1 minute at high speed to form the w/o/w double emulsion. This emulsion was then transferred to a stirring bath of 0.5% PVA (100 mL) and stirred for 3 h, allowing for methylene chloride evaporation and microsphere hardening. Hardened microspheres were sieved (20-63 µm) and washed

with 1 L ddH_2O. Porous drug-free microspheres were then lyophilized and stored at -20 °C until use.

2.3.3 Isothermal Titration Calorimetry (ITC)

PLGA microspheres (502H) were suspended in a 0.1 M HEPES solution (pH 7.4) at a concentration of 6 mg/mL. Microspheres were incubated in the HEPES solution for 1 h at 37 °C. After incubation microspheres were centrifuged (5 minutes, 8000 rpm) and HEPES removed and replaced with fresh HEPES. A leuprolide solution was prepared by dissolving 5 mg of leuprolide in 1 mL of 0.1 M HEPES solution (pH 7.4). The leuprolide solution, microsphere suspension and HEPES were degassed for 10 minutes at 45 °C prior to titration. Isothermal titration calorimetry experiments were performed using a nanoITC (TA Instruments, DE, USA). The background heat of mixing for leuprolide and HEPES was first determined by 10 injections of 1.6 μL of leuprolide into 300 μL HEPES every 600 s with constant stirring (350 rpm) at 45 °C. Titrations of the 5 mg/mL leuprolide into 502H/HEPES suspension (1.8 mg/300 μL) was run using the same conditions for injection volume, injection interval and temperature. Binding of leuprolide to microspheres was modeled using an Independent model which is optimal when there are one or more identical binding sites for the same ligand with the same enthalpy and association constant (K_a), independent of each other (NanoAnalyze software, TA Instruments).

2.3.4 Differential Scanning Calorimetry (DSC)

PLGA microspheres (503H) were incubated in 1 mL 0.1M HEPES pH 7.4 at a microsphere concentration of 90 mg/ml for 1 h then centrifuged for 5 minutes at 8000 rpm and 800 μl quickly removed to create a slurry of microspheres of known polymer

concentration. A 20 mg/ml solution of leuprolide in HEPES was prepared by weighing out 40 mg of leuprolide and 47.66 mg of HEPES then dissolving in 1 mL ddH_2O. The leuprolide solution was titrated to pH 7.4 with 1 N NaOH and transferred to a 2-mL volumetric flask. Next, the remaining volume of ddH_2O was added to achieve a final volume of 2 mL. The leuprolide solution and microsphere slurry were stored on ice until running. DSC samples of leuprolide solution only, 503H microsphere slurry only, and leuprolide + microspheres were run using a modulated DSC (TA instruments, DE, USA) method from 5 °C – 60 °C. All DSC samples were compared to a reference pan of ddH_2O.

2.3.5 Bodipy Tagging of Octreotide

Octreotide was tagged with bodipy at a 1:1 molar ratio by adding 1.4 mg octreotide to 0.4 mg bodipy in 0.25 mL of DMSO and 40 μL triethylamine (TEA). The solution was stirred in darkness for 4 h. After stirring, PD MiniTrap G-10 columns (GE Healthcare Lifesciences, PA, USA), conditioned with 0.1 M HEPES, were used to separate and collect bodipy tagged octreotide (B-octreotide). Collected fractions were lyophilized and stored at -20 °C until use. Lyophilized fractions were reconstituted in 0.5 mL of HEPES buffer and the UV absorbance (280 nm) and fluorescence (excitation= 500 nm, emission= 530 nm) determined by spectrophotometry to determine fractions with tagged bodipy.

2.3.6 Encapsulation of Bodipy-Octreotide in PLGA Microspheres

Porous, drug-free microspheres were prepared as in Section 2.3.2. Briefly, 28 tubes each containing 90 mg of drug free microspheres were weighted out. Four 20 mg/mL solution of un-tagged octreotide and B-octreotide solutions were prepared.

Briefly, for un-tagged octreotide solutions 40 mg of octreotide and 47.66 mg of HEPES then dissolving in 1 mL ddH_2O. The octreotide solutions were titrated to pH 7.4 with 1N NaOH and transferred to a 2-mL volumetric flask. Next, the remaining volume of ddH_2O was added to achieve a final volume of 2 mL. B-octreotide solutions were prepared by combining 5 mg of B-octreotide with 35 mg of octreotide and 47.66 mg HEPES, then dissolving this in 1 mL of ddH_2O for each solution. The B-octreotide solution was titrated to pH 7.4 with 1 N NaOH then transferred to a 2-mL volumetric flask and the remaining water added.

Using the loading solution containing B-octreotide 0.5 mL was added to 7 tubes of microspheres and 0.375 mL to the next 7 tubes for final microsphere concentrations of 180 mg/mL and 240 mg/mL respectively. This process was repeated for the remaining 14 tubes of microspheres using un-tagged octreotide loading solution. All tubes were gently agitated to ensure microspheres were fully dispersed in loading solution and then incubated at 37 °C on a rotator.

At hours 1, 2, 4, 8, 12, 16 and 24 of incubation one tube of microspheres in the octreotide and B-octreotide solution mixture at each concentration were removed and centrifuged for 10 minutes at 8000 rpm and the supernatant collected. Microspheres were then washed 3 times with ddH_2O and lyophilized and stored at -20 °C until needed. This process was repeated at all time points.

2.3.7 Determination of Octreotide Loading and Encapsulation Efficiency

Octreotide and B-octreotide encapsulation in PLGA microspheres at both concentrations was determined for all time points by UPLC analysis. Octreotide loading solution supernatant collected after loading and the first wash supernatant was diluted

40x prior to analysis. Loading solution and washes were analyzed by UPLC to determine octreotide concentration remaining in solution. Octreotide loading was calculated as the mass ratio of drug in microspheres to mass of microspheres loaded. Encapsulation efficiency was calculated by the measured drug loaded divided by the theoretical loading.

2.3.8 Confocal Microscopy of Bodipy-Octreotide Loaded Microspheres

B-octreotide loaded microspheres for each hour time point at both microsphere concentrations (180 mg/mL and 240 mg/mL) were re-suspended in ddH_2O (~5 mg/0.2 mL) to create a slurry. Microspheres were examined using confocal microscopy (Nikon A1). Fluorescence of bodipy was detected using the FITC setting with excitation= 503 nm, and emission= 512 nm.

2.4 Results & Discussion

2.4.1 Leuprolide Has a Strong Affinity for 502H PLGA Microspheres

As we have used 503H for controlled release purposes the first ITC experiments were run with that polymer, although the results had less than desirable resolution. In order to increase the signal, we used 502H which is of lower MW and has more binding sites for the peptide. 502H PLGA (50/50 acid terminated) microspheres were incubated for 1 h in 0.1M HEPES buffer at 37 °C, with mild agitation, prior to isothermal titration calorimetry (ITC) experiments. This pre-incubation allows for microsphere equilibration with HEPES prior to running and therefore ensure that all heats of binding observed are due to leuprolide binding rather than HEPES binding, which was shown to have a binding of an equivalent of ~ 0.5 % w/w HEPES loading to blank acid terminated 503H

PLGA microspheres, as determined by nitrogen content (*data not shown*). The leuprolide solution was also incubated at 37 °C prior to running to minimize thermal effects. ITC experiments in which leuprolide was titrated into a suspension of 502H microspheres resulted in an association constant (Ka) = 0.73 μM^{-1} and a dissociation constant (Kd) = 1.37 μM (Figure 2.1). These results indicate that leuprolide has a strong affinity for acid terminated PLGA microspheres. This strong affinity is important in achieving a high encapsulation efficiency and loading.

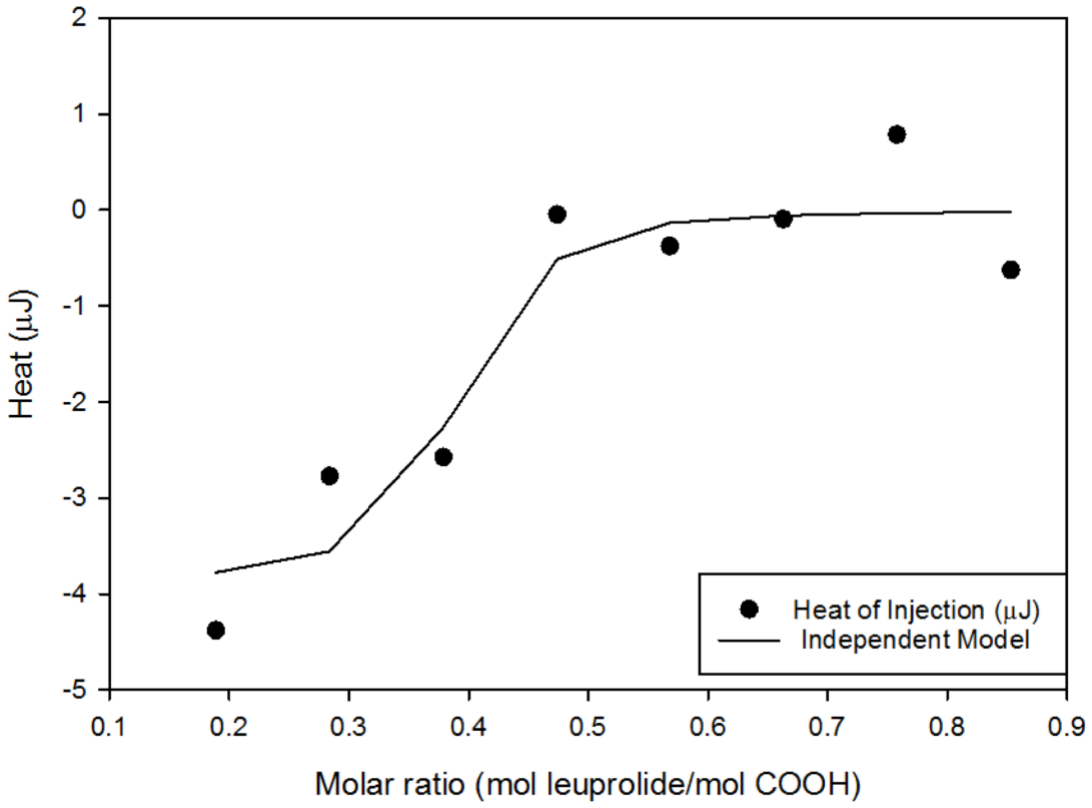

Figure 2.1 Titration of leuprolide binding to 502H PLGA microspheres at 45 °C.

Using an Independent model, described in Section 2.3.3 (discussed further in supplemental work) to fit the heats produced for leuprolide binding to 502H microspheres the enthalpy, entropy and Gibbs free energy were determined at 45 °C (Table 2.1). Based on the results at 37 °C the reaction is spontaneous and is

entropically driven based on the calculated value thermodynamic variables. The entropic effect can be rationalized according to the “hydrophobic effect” as peptide bound water molecules are likely released from the peptide surface upon polymer uptake.

Table 2.1 Thermodynamic variable determined by independent modeling of ITC titration of leuprolide into 502H microspheres.

Variable	Value
Kd (μM)	1.37
Ka (μM^{-1})	0.73
ΔH (kJ/mol)	-0.582
ΔS ($kJmol^{-1}K^{-1}$)	110.4
ΔG (kJ/mol)	-35.7

2.4.2 Determination of the Thermodynamics of Leuprolide Absorption

The apparent thermodynamics of leuprolide absorption to 503H microspheres was determined by modulated differential scanning calorimetry (DSC). From these experiments, we were able to determine the entropy, enthalpy, Gibbs free energy and heat capacity of leuprolide binding to PLGA as the temperature was increased past the polymer Tg, which has been shown critical for peptide absorption [2]. Using modulated DSC (mDSC) and ramping the temperature from 5 °C - 60 °C the interaction between PLGA microspheres and leuprolide was determined. From the DSC thermogram (Figure 2.2) the maximum heat capacity (transition midpoint, T_m) can be determined as the temperature at the peak maximum [8]. Integrating the non-reversing heat flow (Q_{nrev}) vs temperature curve gives the enthalpy (ΔH) of leuprolide absorption to 503H microspheres [8, 9].

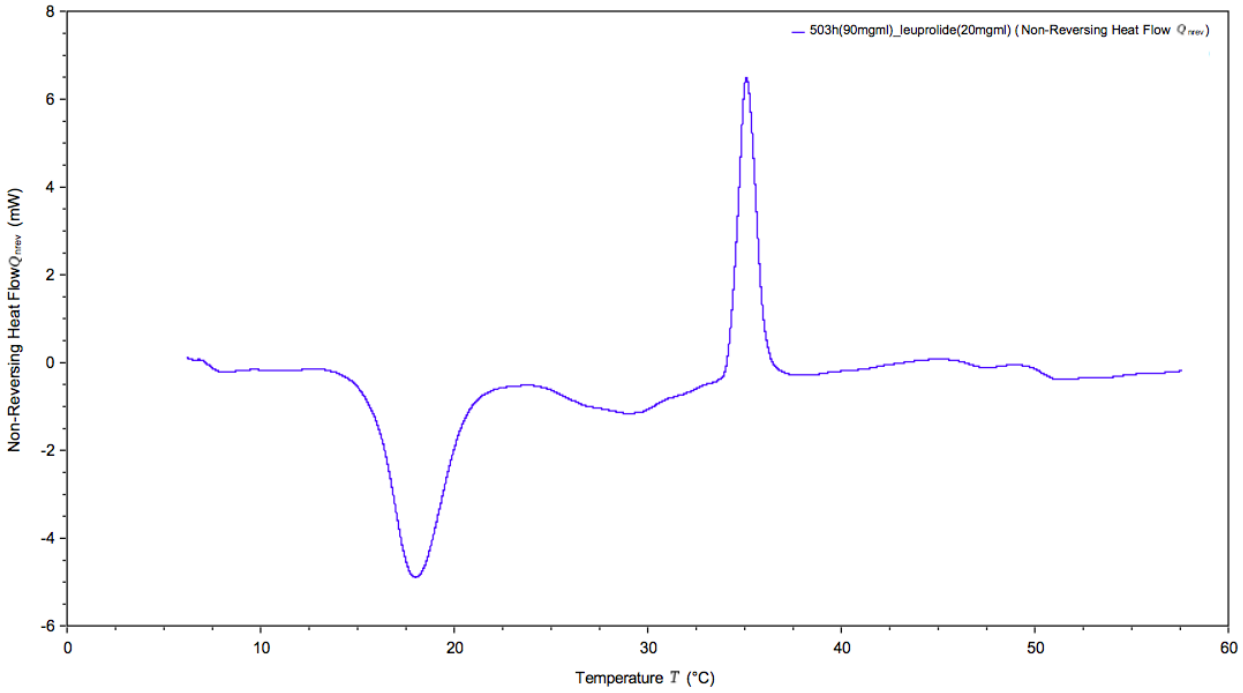

Figure 2.2 DSC thermogram of leuprolide interacting with 503H PLGA microspheres.

Using these three values determined from the thermogram the entropy (ΔS) and Gibbs free energy (ΔG) at 37°C can be determined. The calculated thermodynamic values are given in Table 2.2 below. As the DSC was run on a mixture of microspheres and leuprolide the overall thermodynamics are of the entire system not the individual components. However, it is expected that we would see a peak around the grass transition temperature of the polymer as previous work has shown that at 37 °C there is optimal interaction between the peptide and polymer [2]. The DSC thermograms of the individual components are shown in supplemental work.

Table 2.2 Thermodynamic parameters derived from experimentally determined values by DSC. Note all values are normalized to total sample mass

Parameter	Value
T_m	308.25 K
ΔH	0.012 kJ/g
ΔS	0.038 J/gK
ΔG (@ 37 °C)	-0.097 kJ/g

Based on the calculated values, leuprolide absorption to acid terminated PLGA (503H) is endothermic due to the positive enthalpy and is spontaneous/favorable since it has a negative Gibbs free energy. However, it is important to note the importance of temperature in determining the favorability of the reaction since we have a relatively small entropy as the temperature decreases the reaction would become less favorable. Therefore, suggesting that there is a critical minimum temperature at which leuprolide absorption to acid terminated PLGA will spontaneously occur. These results are similar to those observed by ITC as the reaction is still favorable and entropically driven. Future work will look at better comparing thermodynamic parameters on a molar basis and with 50/50 PLGA of the same molecular weight.

2.4.3 Bodipy Tagged Octreotide Loads Similar to Un-tagged

In order to visualize octreotide loading in PLGA microspheres over time octreotide was tagged with the fluorescent dye bodipy. Bodipy was chosen for fluorescent tagging as it is a stable, non-pH sensitive dye and can be conjugated to the primary amino groups of octreotide [2]. Octreotide was used for these experiments because of its two primary amino groups for labeling. However, to ensure that bodipy tagging did not affect octreotide absorption, as bodipy is conjugated to the amino groups which are the same residues that allows for peptide interaction with PLGA-COOH, un-tagged octreotide was also loaded. Microspheres were loaded at both 180 mg/mL and 240 mg/mL with both concentrations rapidly absorbing bodipy tagged octreotide (B-octreotide) in the first 4 h of incubation then achieving a quasi-equilibrium of 6.25% and 5.4% w/w load respectively (Figure 2.3 (A)). Both concentrations also

achieved over 60 % encapsulation efficiency (Figure 2.3(B)). Bodipy conjugation did not have an effect on octreotide absorption (Figure S 2.3).

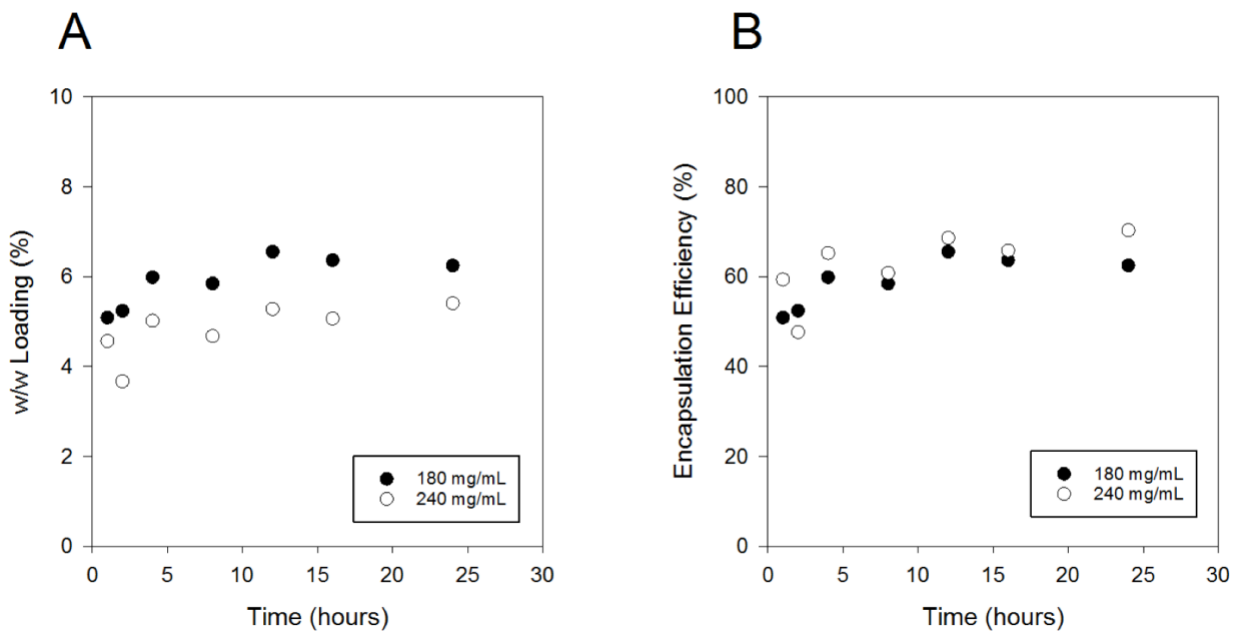

Figure 2.3 Loading (A) and encapsulation efficiency (B) of bodipy-octreotide loaded at 180 mg/mL and 240 mg/mL microsphere concentration over 24 h.

2.4.4 Confocal Imaging of Octreotide Loading

After establishing no impact for fluorescent tagging, microspheres loaded with B-octreotide were observed with confocal microscopy for a visualization of peptide absorption during 24 h loading. Microsphere fluorescence became brighter as the microspheres were allowed to load for longer periods of time, up to a certain point; this is particularly evident when comparing 1 h and 8 h loaded microspheres at 180 mg/mL and 240 mg/mL microsphere concentration. However, when comparing 8h loaded microspheres and 24 h loaded microspheres there is minimal apparent increase in brightness. The increasing brightness over the first 8 h confirms that there is more octreotide absorption occurring compared to the last 16 h of loading in which there is a

minimal increase in brightness, indicating a minimal increase in octreotide absorbed (Figure 2.4) and an achieved equilibrium after 8h.

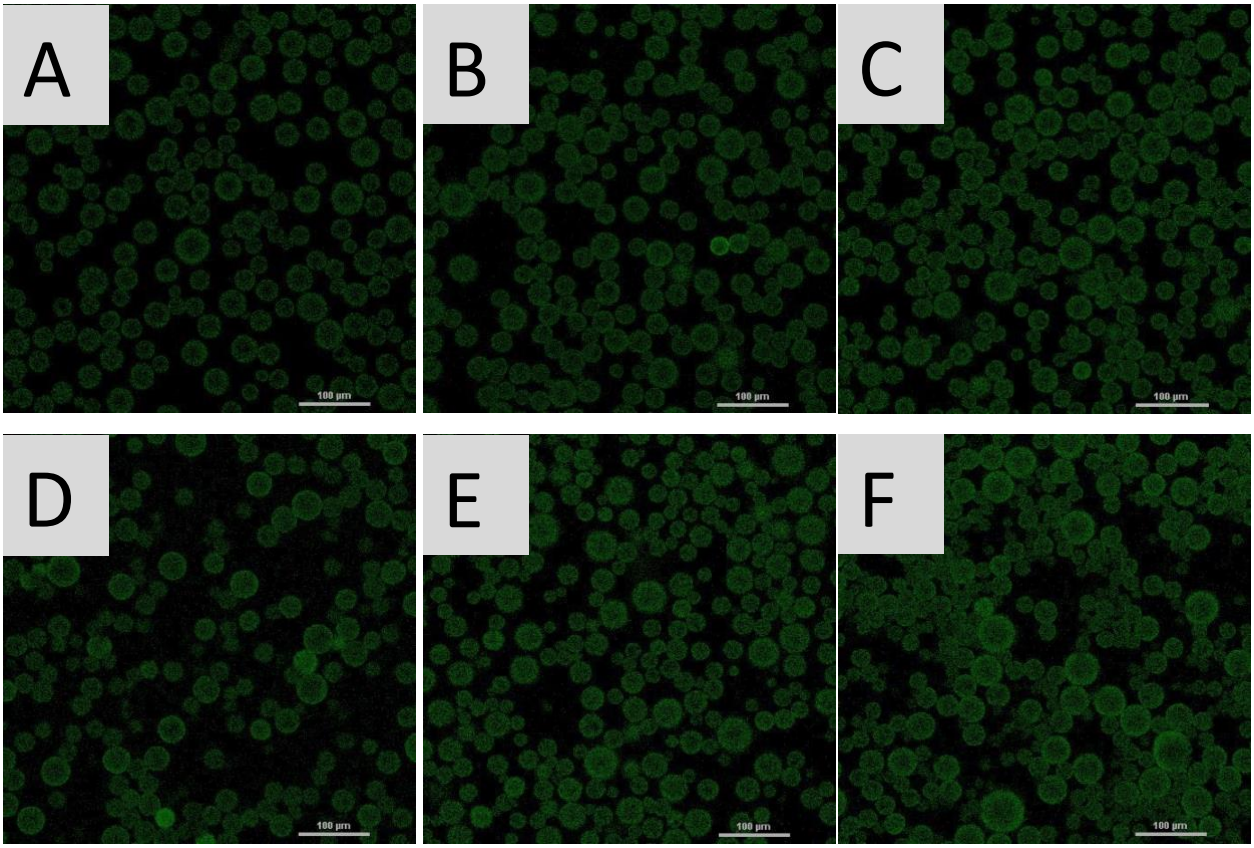

Figure 2.4 Confocal microscopy of bodipy loaded microspheres at 1h (A, D), 8h (B, E) and 24h (C,F) of incubation at 180 mg/mL (A-C) and 240 mg/mL (D-F) microsphere concentration.

While we see fluorescence throughout the microspheres, fluorescence appears to be stronger at the periphery of the microspheres and/or concentrated in specific regions. This could be due to two different distributions of peptide both in the polymer phase and in the polymer pores. Distribution of tagged peptide in the pores would be expected to be heterogeneous in the polymer matrix owing to a distribution of percolating networks (i.e., those that percolate would not contain peptide whereas those that do not percolate would likely contain peptide). It is unknown the level of response from peptide tagged in pores vs. polymer.

2.5 Conclusion

An improved understanding of the mechanisms behind cationic peptide absorption to acid terminated PLGA is important in helping to understand how these model peptides, leuprolide and octreotide, are able to rapidly absorb in PLGA. These experiments support that the cationic peptides interact with acid terminated PLGA via absorption in a spontaneous manner with a high binding affinity, which is primarily entropy driven. Confocal imaging of octreotide support that the loading occurs rapidly in the first 8 hours of loading as it achieves a quasi-equilibrium after that time.

The potential to use these techniques to better understand peptide interaction with the polymer can be important in determining the potential for aqueous remote loading with other peptides as well as helping to determine the duration of incubation time necessary to ensure high encapsulation efficiency.

2.6 References

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2.7 Supplemental

For ITC analysis, the heat produced for each injection was analyzed by an Independent model, which is optimal when there are one or more identical binding sites for the same ligand with the same enthalpy and association constant (K_a), independent of each other, to derive thermodynamic parameters. Heats produced from leuprolide injection into HEPES buffer was subtracted from heats produced from leuprolide injection into 502H microspheres. An independent model was used, as peptide binding to PLGA-COOH is non-specific.

For thermodynamic analysis of 503H PLGA microspheres and leuprolide controls of microspheres only and leuprolide only were also run on DSC. Individual thermographs are shown below (Figure S 2.1, Figure S 2.2). As with samples of microspheres + leuprolide, hydrated samples of microspheres or leuprolide were ramped from 5 °C - 60 °C.

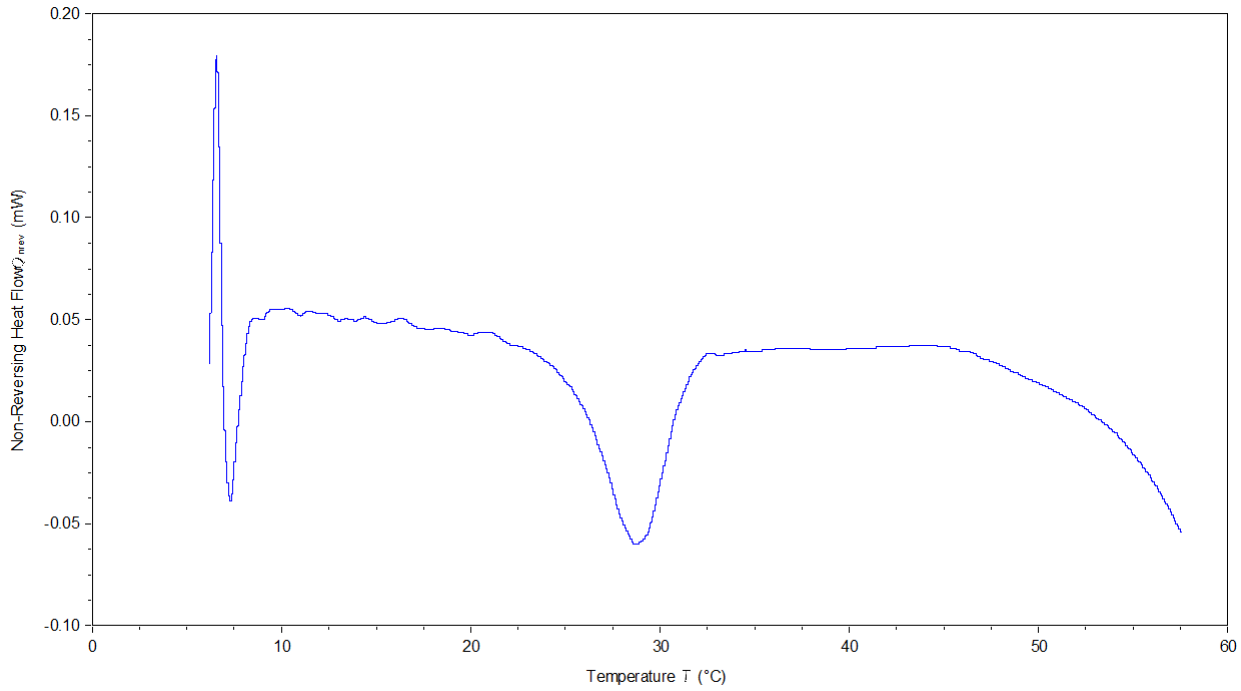

Figure S 2.1 DSC thermograph of 503H microspheres at a concentration of 90 mg/mL.

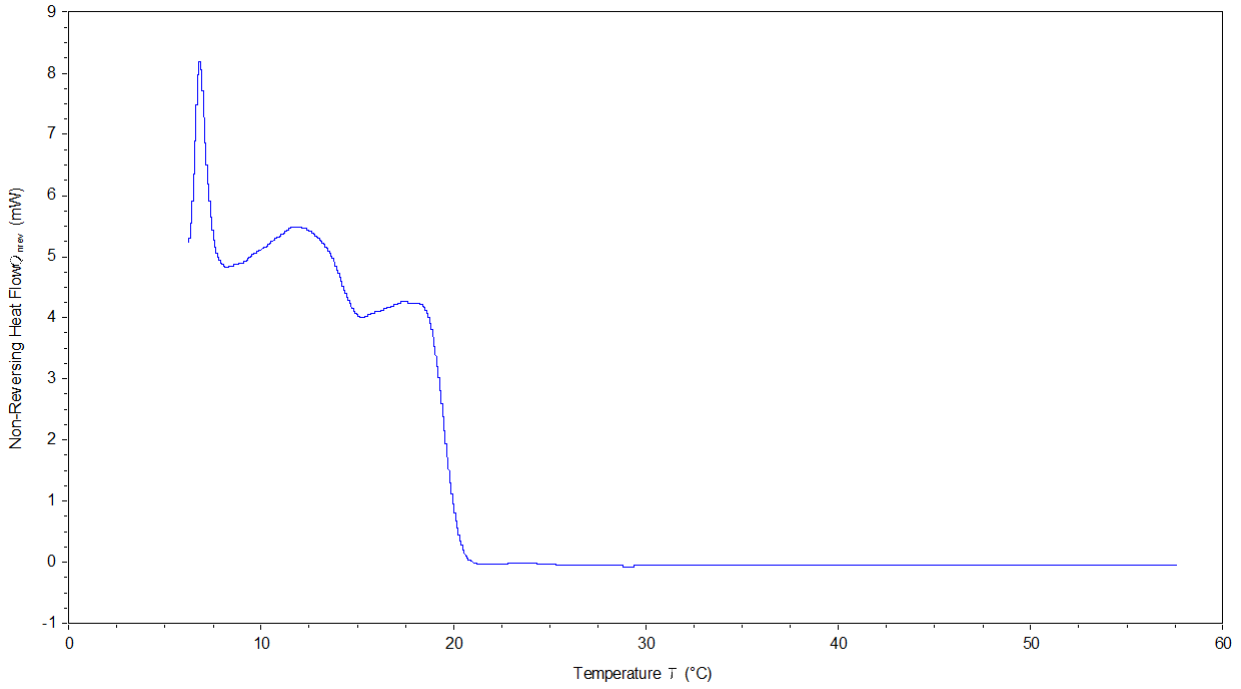

Figure S 2.2 DSC thermograph of 20 mg/mL leuprolide.

In control experiments for B-octreotide absorption to 503H microspheres over 24 h, untagged octreotide also showed rapid absorption to PLGA microspheres an attained a quasi-equilibrium by after 8 h of incubation. For 180 mg/mL microsphere concentration octreotide was loaded at 5.47% with an encapsulation efficiency of 54.6%. For 240 mg/mL microsphere concentration loading of 4.81% and encapsulation efficiency of 62.5% was achieved (Figure S 2.3)

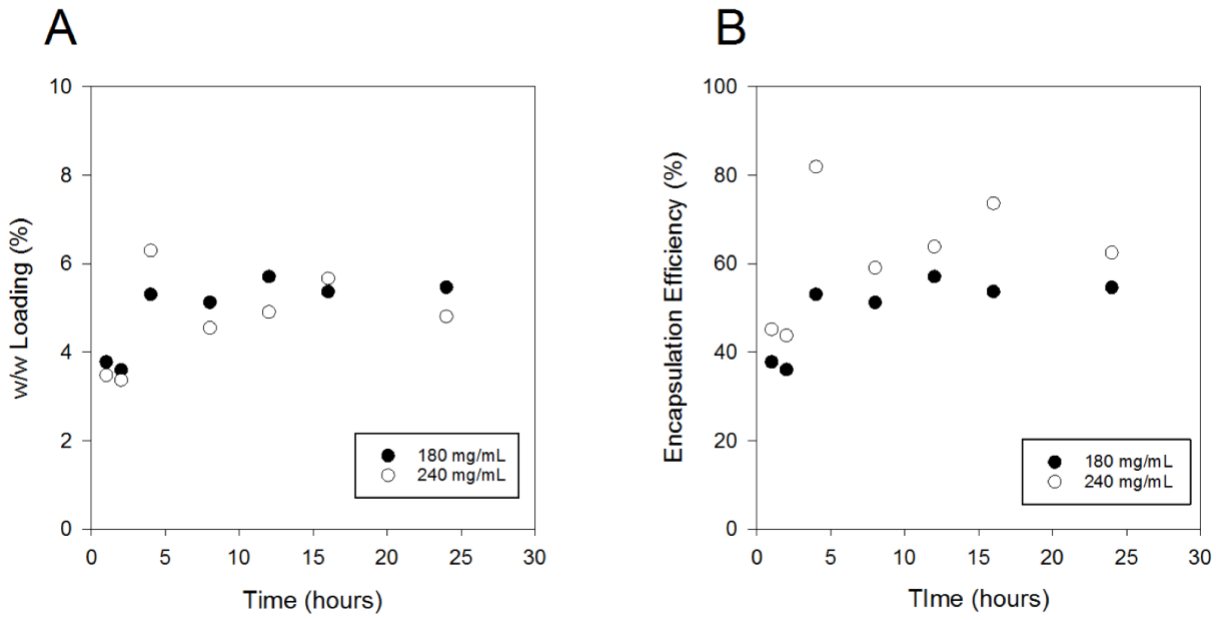

Figure S 2.3 Octreotide loading (A) and encapsulation efficiency (B) in 503H microspheres over 24 h at 180 mg/mL and 240 mg/mL microsphere concentrations.

Chapter 3: Aqueous Remote Loading of Leuprolide in PLGA Microspheres

3.1 Abstract

Moderately sized cationic peptides, roughly 1 kDa molecular weight, such as leuprolide and octreotide, have been shown to strongly interact with free acid terminated poly(lactic-*co*-glycolic acid) (PLGA-COOH). Above the polymer glass transition temperature, this interaction involves peptide absorption in the polymer rather than adsorption at the polymer surface. An aqueous remote loading paradigm was used to load pre-formed 50/50 PLGA-COOH microspheres with leuprolide acetate in a 0.1 M HEPES buffer (pH 7.4) at elevated encapsulation efficiencies and loading. Assuming the quasi-equilibrium absorption of leuprolide, a model for encapsulation efficiency (EE) and loading (l) was derived with *EE* found dependent on the binding strength, capacity, the polymer water content, and initial polymer concentration, while l depended on the EE and the peptide to polymer mass ratio. Initial studies with microspheres were able to achieve loading of ~ 9.8 % but had a low EE (~ 38 %). However, these microspheres continuously released *in vitro* for over 1 month with a low initial burst. In order to increase EE and loading several parameters were studied and optimized including: microsphere concentration in the loading solution, duration of absorption, inner water phase volume, and porosity. We found that loading from microsphere concentrations of 180 mg/ml and 240 mg/ml improved EE (> 55 %) with encapsulation rapidly occurring in

the first 8 h to achieve a quasi-equilibrium. By applying the theoretical analysis, drug loading can be manipulated to optimal levels by first controlling the pre-formed microsphere concentration and then adjusting the peptide concentration, maximizing the potential for future applications for peptides with limited aqueous solubility and encapsulation performed on a very small scale.

3.2 Introduction

Peptides and proteins often suffer from poor *in vivo* half-lives and poor bioavailability when delivered via the oral, intramuscular, or subcutaneous routes, and therefore require frequent injections to maintain therapeutic efficacy. The high frequency of injections necessary for peptide and protein therapies often leads to poor patient compliance, discomfort, and psychological stress [1, 2], thus making peptides ideal candidates for controlled release delivery systems. Controlled release or long-acting release (LARs) depots present many benefits to patients to overcome challenges resulting from frequent injections, and can lead to improved compliance and efficacy relative to immediate release injections [3]. Poly(lactic-*co*-glycolic acid) (PLGA), a biodegradable and biocompatible polymer, has been used in several FDA approved products [2] to deliver peptides for the duration of weeks to months. Although the current products on the market using PLGA have often shown success, the broader development of LARs has been limited in part due to several challenges including: peptide and protein instability, poor polymer release kinetics, the presence of residual organic solvents in formulation, and elevated cost of manufacturing [2, 4, 5]. For example, Nutropin Depot® was approved in 1999 for the treatment of growth hormone

deficiency [6] but was removed from the market in 2004 due to the reported high cost of manufacturing and marketing, in addition to poor efficacy compared to daily injections of the solution form of growth hormone [5]. Moreover, despite several PLGA dosage forms being marketed for more than 25 years, and well beyond patent expiration, there are currently only 15 PLGA products approved in the US, and there are currently no PLGA-based generics approved by the US FDA. This lack of PLGA generics also suggests that there may be formulation/manufacturing difficulties associated with current PLGA dosage forms that have impeded progress toward novel and “generic” development.

Traditionally, emulsion, spray drying, and coacervation methods have been used to encapsulate peptides into PLGA [7]. However, these techniques expose the peptide to harsh conditions such as organic solvents, micronization (e.g., large shear forces of homogenization), and drying [4], and have proven difficult at times to manufacture at a desirable cost of goods. Remote loading is an alternative encapsulation method that can be used to ameliorate many of these issues. In this technique, peptides are loaded into pre-formed microspheres in an aqueous solution, which avoids drug exposure to harsh conditions e.g. organic solvents and drug micronization. The water-based remote loading also provides other advantages to product performance compared to traditional methods. For example, this technique in theory provides better control over the amount of residual organic solvent present during encapsulation and allows for simpler production as microspheres can be terminally sterilized prior to sterile aqueous peptide addition, eliminating the need to handle organic solvents in a sterile environment. Using pre-formed, drug-free microspheres is also advantageous because there is greater

control over the final microsphere attributes (e.g. size, surface morphology) used in the final formulation. Microspheres having desired characteristics, from the same or different batches, can be theoretically selected for use during encapsulation steps thus reducing the risk of large batch failure and potential waste/loss of expensive peptide.

One of the most commercially successful LARs is the Lupron Depot® (LD) which delivers the gonadotropin releasing hormone (GnRH) agonist leuprolide. LD was approved in the US in 1989 for the treatment of prostate cancer and was among the first group of LARs to reach the market. LD consists of PLGA microspheres encapsulating the cationic peptide, leuprolide acetate, and can have a dose frequency between 1-6 months. Cationic peptides such as leuprolide or octreotide, the latter of which is used in Sandostatin® LAR, have also been shown to strongly interact with low molecular weight free acid end group PLGA (PLGA-COOH) at neutral pH, via the peptide alpha amino group (when unprotected) and cationic side chains of arginine, histidine and lysine [8-10]. In conventional organic-solvent based formulations, these interactions can be exploited to enable slow continuous drug release over several weeks, improve encapsulation efficiency, and reduce initial burst release [9, 10]. Our group recently demonstrated that the interaction between PLGA-COOH and these cationic peptides is due to an absorption process, in which peptides rapidly form a salt with the acid end groups of mobilized polymer chains, providing a potential to be remotely loaded in the PLGA for later controlled release [11]. We first demonstrated the 1-day peptide absorption to ground 50/50 PLGA-COOH particulates in a neutral pH zwitterionic buffer solution could form an injectable LAR depot at high peptide loading capable of chemical castration of rats for 2 months when dosed every 2 weeks [11]. However, the proof-of-

principle absorption to ground PLGA-COOH suffered from low encapsulation efficiency and had a high initial burst, and clearly was not designed to compete with existing peptide products such as the LD [11].

Here, we combine the two principles of the aqueous remote loading paradigm and principle of peptide absorption in acid-capped PLGA to encapsulate the model cationic peptide, leuprolide acetate, in pre-formed 50/50 PLGA-COOH microspheres. Our objective was to determine if the remote loading strategy when applied to leuprolide could provide high encapsulation efficiency, low initial burst, and continuous peptide release for 1 month *in vitro*. To accomplish our goals, we utilized a higher molecular weight (24 - 38 kDa) 50/50 PLGA-COOH, compared to what was previously used [11], for preparation of pre-formed microspheres and subsequently optimized the loading conditions by both developing a theory for predicting encapsulation and using this theory to help optimize the formulation experimentally.

3.3 Predicting Encapsulation Efficiency and Loading from Sorption Isotherms

Absorption of leuprolide in PLGA-COOH has been shown to reach a quasi-equilibrium absorption [11], so we sought to determine the relationship of encapsulation efficiency (*EE*) and drug loading (l) on starting system variables, such as initial peptide and microsphere mass and polymer water content, to understand how to optimize encapsulation.

EE is determined from the quotient of polymer bound peptide to total peptide in the loading solution as follows:

$$EE = \frac{n_b}{n_b+n_f} = \frac{m_b}{m_b+m_f} \quad (1)$$

where n_b is the moles of bound peptide after sorption, n_f the moles of free peptide after sorption, m_b the mass of bound peptide after sorption, and m_f the mass of free peptide after sorption. Similarly, l is

$$l = \frac{m_b}{m_b + M_p} \quad (2)$$

where M_p is the mass of polymer microspheres in the loading solution.

As the peptide is expected to bind as a salt with the polymer at roughly a 1:1 mole ratio with the polymer end-groups [11], the fractional bound peptide (f_b) can be defined as

$$f_b = \frac{n_b}{M_p \kappa} \quad (3)$$

where κ is the mole of –COOH end-group per unit mass of the polymer equivalent to the maximal peptide binding or binding capacity of the polymer.

When analyzing equilibrium sorption data, sorption isotherms typically follow a number of different sorption models and the fractional bound peptide is a function, *f*, of the concentration of free peptide after sorption as follows noting (3):

$$f_b = \frac{n_b}{M_p \kappa} = f(n_f / V_f) \quad (4)$$

where V_f is the volume of loading solution after sorption, which can be different significantly from the initial volume because of polymer water uptake during sorption. Rearranging, and inserting (4) into (1) for n_b, *EE* can be determined from the sorption model as follows:

$$EE = \frac{M_p \kappa f(n_f/V_f)}{M_p \kappa f(n_f/V_f) + n_f} \quad (5)$$

From our data, we observed at low free peptide concentrations the binding of peptide was proportional to free peptide (see below) and therefore:

$$f_b = \frac{n_b}{M_p \kappa} = f(n_f/V_f) = \alpha\ n_f/V_f \quad (6)$$

where α is a binding constant (M^{-1}). Inserting (6) into (5):

$$EE = \frac{M_p \kappa \alpha\ n_f/V_f}{M_p \kappa \alpha\ n_f/V_f + n_f} = \frac{M_p \alpha \kappa}{M_p \alpha \kappa + V_f} \quad (7)$$

We see that the *EE* becomes independent of the final concentration of free peptide and dependent on the final volume of the loading solution as *EE* approaches the theoretical maximum, at steady state. As the loading solution will decrease as the initial concentration of polymer increases, *EE* can reasonably be maximized (as one would expect) by maximizing the sorbent (polymer) concentration in the loading solution.

The V_f can be written in terms of the initial volume of the loading solution (V_i) and the polymer water (φ) uptake per mass of microspheres as follows:

$$V_f = V_i - \varphi M_p = \frac{M_p}{C_{p,i}} - \varphi M_p = M_p\left(\frac{1}{C_{p,i}} - \varphi\right) \quad (8)$$

where $C_{p,i}$ is the initial mass concentration of microspheres in the loading solution. Inserting (8) into (7) gives

$$EE = \frac{M_p \alpha \kappa}{M_p \alpha \kappa + M_p\left(\frac{1}{C_{p,i}} - \varphi\right)} \quad (9)$$

Rearranging (9) gives an expression for *EE* in terms of $C_{p,i}$, α, κ and φ as follows:

$$EE = \frac{\alpha \kappa C_{p,i}}{(\alpha \kappa - \varphi) C_{p,i} + 1} \quad (10)$$

or in dimensionless form:

$$EE = \frac{Bi}{(Bi - V^*) + 1} \quad (11)$$

where a binding number (*Bi*) and volume ratio (*V**) are two dimensionless variables that can be defined as follows:

$$Bi \equiv \alpha \kappa C_{p,i}$$

$$= binding\ capacity\ x\ initial\ concentration\ of\ absorptive\ sites\ in\ loading\ solution \quad (12)$$

and

$$V^* \equiv \varphi C_{p,i}$$

$$= final\ volume\ of\ water\ in\ polymer\ matrix / initial\ volume\ of\ loading\ solution \quad (13)$$

To determine the value for loading, l, m_b from (1) is inserted into (2) as follows:

$$l = \frac{m_b}{m_b+M_p} = \frac{EE\ (m_b+m_f)}{EE\ (m_b+m_f)+M_p} = \frac{EE\ m_{pep,i}}{EE\ m_{pep,i}+M_p} = \frac{EE\ C_{pep,i}}{EE\ C_{pep,i}+C_{p,i}} \quad (14)$$

where $m_{pep,i}$ and $C_{pep,i}$ are the initial peptide mass (total mass of free and bound peptide) and initial peptide concentration in the loading solution, respectively. Rearranging and inserting (10) into (14) gives:

$$l = \frac{1}{1+\frac{(\alpha\kappa-\varphi)C_{p,i}+1}{\alpha\kappa C_{pep,i}}} \quad (15)$$

and in dimensionless form:

$$l = \frac{1}{1+\frac{(Bi-V^*)+1}{Bi\,C^*}} \quad (16)$$

where one more dimensionless group, an initial concentration ratio appears, and is defined as follows:

$$C^* \equiv \frac{C_{pep,i}}{C_{p,i}}$$

$$= initial\ peptide\ concentration / initial\ polymer\ concentration \quad (17)$$

Together the equations for *EE* and l, (10) and (15), tell us that while l depends on initial peptide concentration, *EE* does not. Therefore, again, to maximize *EE,* $C_{p,i}$ should be maximized, and then to achieve a desired loading, $C_{pep,i}$ should be adjusted according to (15).

3.4 Materials and Methods

3.4.1 Materials

Leuprolide acetate was purchased from SHNJH Pharmaceuticals (Shanghai, China). 50/50 poly(lactic-*co*-glycolic acid) (Resomer® RG 503H) was purchased from Evonik (Essen, Germany). Polyvinylalcohol was purchased from Sigma Aldrich Chemical Co (St. Louis, MO). Hydroxyethyl-piperazineethanesulfonic acid (HEPES) was purchased from Thermo Fisher Scientific (Waltham, MA). All other materials were of analytical grade and purchased from commercial suppliers.

3.4.2 PLGA Microsphere Formation

Porous PLGA drug-free microspheres were prepared by using a double emulsion solvent evaporation method. Porosity was introduced to increase the rate of peptide absorption. PLGA, 800 mg, was dissolved in 1 mL methylene chloride. Once dissolved, 200 μl of a 500 mg/mL trehalose in ddH_2O solution was added to the polymer solution and then homogenized (VirTis Tempest I.Q.^{2}) for 1 min at 10,000 rpm to create a water-in-oil (w/o) emulsion. Next, 4 mL of a 5% poly vinyl alcohol (PVA) solution was added to the emulsion, which was then vortexed (Scientific Industries Vortex Genie 2) for 1 min at maximum speed. The solution was then added to a 100-mL stirring bath of 0.5 % PVA and stirred for 3 hours for solvent evaporation. After hardening, microspheres were washed extensively with ddH_2O and sieved for size in the range of 20-63 μm. Microspheres were lyophilized (Labconco FreeZone 2.5) and stored frozen (-20 °C) until further use.

3.4.3 Leuprolide Loading Solution

A low concentration leuprolide loading solution of 3.6 mg/mL leuprolide acetate was made by dissolving the peptide in a 0.1 M HEPES (pH 7.4) solution. High concentration leuprolide loading solution was made by dissolving 40 mg of peptide and 47.66 mg HEPES in 1 mL ddH_2O, titrated to pH 7.4 using 0.1 N sodium hydroxide (NaOH). A final loading solution volume of 2 mL was attained by adding ddH_2O at a volume necessary to achieve 2 mL after titration.

3.4.4 Remote Loading of Microspheres

Pre-formed blank PLGA microspheres were loaded with a solution of leuprolide in the aforementioned HEPES buffer solutions. Low concentration remote loading was done by incubating 14 mg of microspheres with 1 mL of 3.6 mg/mL leuprolide loading solution at 37 °C with mixing for 24 h. High concentration loading was achieved by dispersing 90 mg of microspheres in either 0.5, 0.375, or 0.25 mL of high concentration leuprolide loading solution (20 mg/mL) for final initial polymer concentrations of 180, 240, and 360 mg/mL, respectively. Dispersed microspheres were incubated at 37 °C and mixed for 24 h. After incubation, microspheres were centrifuged (Eppendorf 5424R) for 10 min at 5,000 rpm and the supernatant was collected. Microspheres were next washed three times with 1 mL ddH_2O and the supernatant was saved; centrifuging at 5,000 rpm for 10 min between each wash. Loaded and washed microspheres were then lyophilized to remove excess water and stored at -20 °C until future use.

3.4.5 Microsphere Morphology

The surface morphology of PLGA microspheres (loaded and unloaded) were analyzed by gold coating (Polaron Sputter Coater) for 90 s followed by imaging with a

scanning electron microscope (AMRAY 1910 Field Emission Scanning Electron Microscope), using a gun voltage of 5 kV.

3.4.6 Peptide Loading Analysis

Total peptide loading in microspheres was estimated by peptide loss from loading solution after correction for water uptake, and determined directly by two-phase extraction and elemental analysis. The amount of leuprolide remaining in the loading solution supernatant and three ddH_2O washes were analyzed by reverse phase UPLC (Acquity UPLC, Waters, USA). The mobile phase was composed of 25:75 v/v gradient (acetonitrile + 0.1 % TFA: ddH_2O + 0.1 % TFA) over 3.5 min with a flow rate of 0.5 mL/min. Standards in PBST and samples were injected onto a C18 (Acquity BEH C18, .7 μm, 2.1 x 100 mm) column. Leuprolide was detected by UV at 215 nm. The determined concentrations were added to calculate the amount of leuprolide that was loaded into microspheres based on the leuprolide that was lost from solution.

For two-phase extraction, 5 mg of loaded particles were dissolved in 1 mL methylene chloride by brief vortexing. Once dissolved, 2 mL sodium acetate buffer (50 mM, pH 4) was added, followed by vortexing for 1 min. After vortexing, microspheres were centrifuged for 4 min at 4,000 rpm, which generated the two phases. 1.5 mL of the aqueous phase was collected, saved and replaced with a fresh 1.5 mL of sodium acetate buffer. This procedure was repeated a total of 5 times. After the 5^{th} extraction, 1.5 mL of 50 mM sodium acetate + 1 M sodium chloride (pH 4) was added to dissolved microspheres. Extractions were repeated with sodium acetate + NaCl 6 more times for a total of 11 extractions, saving the supernatant in a separate tube from the first 5 iterations. The extracted supernatants of 50 mM sodium acetate and 50 mM sodium

acetate + 1 M NaCl were analyzed for peptide concentration using the UPLC method described previously.

Total peptide content was extrapolated from total nitrogen content using a modified automated Dumas technique [12]. Briefly, 1-4 mg of leuprolide loaded microspheres were aliquoted into tin pans, in triplicate, then crimped to remove excess air prior to analysis. Samples were run on a Leco TrueSpec® Micro CHN (LECO, USA). The instrument was first blanked without samples or tins to establish atmospheric baselines. Using USP grade EDTA the percent of carbon, hydrogen, and nitrogen were verified to be within anticipated range and these values were set as standards. Lyophilized leuprolide acetate standards were run to verify the percent nitrogen in the peptide and set a peptide factor. Microsphere samples were then dropped into a combustion chamber at 1050 °C, which converts all nitrogen to nitrogen gas, which is then quantified by a thermal conductivity cell. Peptide content was determined by multiplying the nitrogen mass by the leuprolide peptide factor after first subtracting the nitrogen mass from negative controls (unloaded microspheres).

3.4.7 Analysis of In Vitro Leuprolide Release

In vitro leuprolide release from loaded microspheres was performed under sink conditions as follows; approximately 10 mg of leuprolide loaded microsphere were weighed out into 2 mL tubes, in triplicate, and incubated with 1 mL of phosphate buffered saline (PBS) containing 0.02% Tween 80 (PBST) with mild agitation at 37 °C. At pre-determined time points samples were centrifuged for 5 min at 8,000 rpm and 1 mL of the release buffer was completely removed and replaced with fresh release buffer. The amount of leuprolide in release solution was analyzed using UPLC.

3.4.8 Kinetics of Leuprolide Sorption to PLGA microspheres

Leuprolide sorption to PLGA microspheres at elevated polymer concentrations of 180 mg/mL and 240 mg/mL were observed over 24 hours and sorption isotherms were determined at 1, 2, 4, 8, 12, 16 and 24 h of incubation. Briefly, leuprolide loading solutions of 20 mg/mL in 0.1 M HEPES, 2 mL total volume (titrated to pH 7.4 with 1 N NaOH). For both polymer concentrations 90 mg of blank microspheres were weighed out into tubes in duplicate for each time point. Microspheres were dispersed in the HEPES loading solution as described in Section 3.4.4, by light mixing, at a volume of 0.5 mL and 0.375 mL, resulting in polymer concentrations of 180 mg/mL and 240 mg/mL respectively, and incubated at 37 °C with rotation. At hours 1, 2, 4, 8, 12, 16 and 24 microspheres were removed from the incubator, centrifuged 10 min at 8,000 rpm and the supernatant removed. After washing 3 times with ddH_2O, the leuprolide loading solutions were analyzed by UPLC to determine the loss of leuprolide used for loading relative to the initial concentration. Mass loss of peptide, corrected for water uptake, was used to indirectly estimate how much leuprolide was loaded at each time point.

The amount of leuprolide sorbed to 50/50 PLGA microspheres over time was also determined directly by two-phase extraction and nitrogen analysis, as previously described.

3.4.9 Hydrated Polymer Glass Transition Temperature

The hydrated glass transition temperature of PLGA microspheres was determined by incubating 10-12 mg of blank (unloaded) microspheres in 200 μl of 0.1 M HEPES solution pH 7.4 at 37 °C for 24 hours on a rotator. After incubation microspheres were centrifuged 5 min at 8,000 rpm and 100 μl of supernatant removed

to create a slurry of microspheres in HEPES. Fifty microliters (50 μl) of the microsphere slurry was then added to a hermetic pan for differential scanning calorimetry (DSC) analysis (Discovery, TA Instruments). Samples underwent a heat/cool/heat cycle from 5 °C to 90 °C to determine the hydrated glass transition temperature. The hydrated glass transition temperature from the second heat cycle was used for analysis. Based on the hydrated glass transition temperature, the ideal temperature for polymer chain mobility and leuprolide loading was determined.

3.4.10 Microsphere Porosity

The intrusion volume and porosity of PLGA microspheres was determined by mercury intrusion porosimetry (AutoPore V Series, Micromeritics). PLGA microspheres were prepared as in Section 3.4.2 with various amounts of the porosigen, trehalose, controlled by the inner water phase volumes of 500 mg/mL disaccharide (i.e., 0 µl, 50 µl, 100 µl, 200 µl and 350 µl), and lyophilized. Between 0.25 mg- 0.35 mg of microspheres were weighted out into the penetrometer. Analysis was performed over low and high pressure ranging from 0.5 psia – 61,000 psia with a fill rate of 0.5 s and equilibration of 10 s at each pressure.

3.4.11 Determination of Moles of Binding Capacity

The binding capacity was determined by the acid number (*AN*) of 503H microspheres. To determine *AN* 300 mg of microspheres were dissolved in 20 mL of 1:1 v/v dehydrated acetone: dehydrated tetrahydrofuran. A 0.5 weight % phenolphthalein in dehydrated methanol solution was prepared and 2-3 drops added to the microsphere solution. The microsphere solution was then titrated with 0.1 M methanolic potassium

(KOH) solution, with constant stirring, until a pink color was observed in the entirety of the solution for a few seconds. The *AN* and binding capacity, κ, are calculated as:

$$(AN) = \frac{(V_t\, C_t\, MW_t)}{M_p} \quad (18)$$

$$\kappa = \frac{V_t\, C_t}{M_p} \quad (19)$$

The acid number is the milligrams of KOH needed to neutralize 1 g of PLGA, where V_t is the volume of titrant (L), C_t is the concentration of titrant (M), MW_t is the molecular weight of the titrant (g/mol) and M_p is the mass of polymer microspheres (g). The *AN* was used to determine the binding capacity (κ) in micromole of –COOH end group per grams of polymer microspheres.

3.4.12 Estimation of Water Uptake

Microsphere water uptake (φ) during loading was estimated as described previously accounting for inter-particle water [13]. Briefly, 90 mg of dry PLGA microspheres were suspended in 0.5 mL of HEPES buffer (0.1 M, pH 7.4) at 4 °C, where negligible water is taken up in the PLGA, and rapidly filtered and dried for 48 h. Inter-particle water (W_i) was determined as the weight differences between wet and dry particles as follows:

$$W_i = \frac{W_1' - W_2'}{W_2'} \quad (20)$$

where W_1' and W_2' are the wet and dry microsphere weights, respectively.

Using the determined inter-particle water, the water uptake of microspheres during loading was determined by loading microspheres with leuprolide at 180 mg/mL. After loading, microspheres were washed 3 times with water, rapidly filtered and dried for 48 h. Water uptake (φ) was determined as

$$\varphi = \frac{W_1 - W_2 - W_2 W_i}{W_2}. \quad (21)$$

3.5 Results and Discussion

3.5.1 Leuprolide Loading is Possible in PLGA Microspheres

Sophocleous et al. [11] previously showed that ground particles of low molecular weight PLGA 50/50 could absorb leuprolide, suggesting feasibility of remote loading of cationic peptides in PLGA-COOH by polymer absorption. However, the proof-of-principle experiment resulted in a low encapsulation efficiency and high initial burst. We sought to improve this by using microspheres, instead of ground particles, of a higher molecular weight polymer for better leuprolide loading and longer release. Blank microspheres of 50/50 PLGA were incubated with low concentration (3.6 mg/mL) of leuprolide loading solution. Low concentration (14 mg/mL) of blank 50/50 PLGA microspheres achieved loading of ~9.8 % w/w leuprolide but with an encapsulation efficiency of just ~38 %. *In vitro* leuprolide release from these microspheres was, however, continuous over 1 month and demonstrated a low initial burst with near zero-order release kinetics (Figure 3.1B). Therefore, remote loading into blank PLGA microspheres could provide improved *in vitro* release kinetics at high drug loading, but the low loading efficiency indicated that the loading conditions could be further improved.

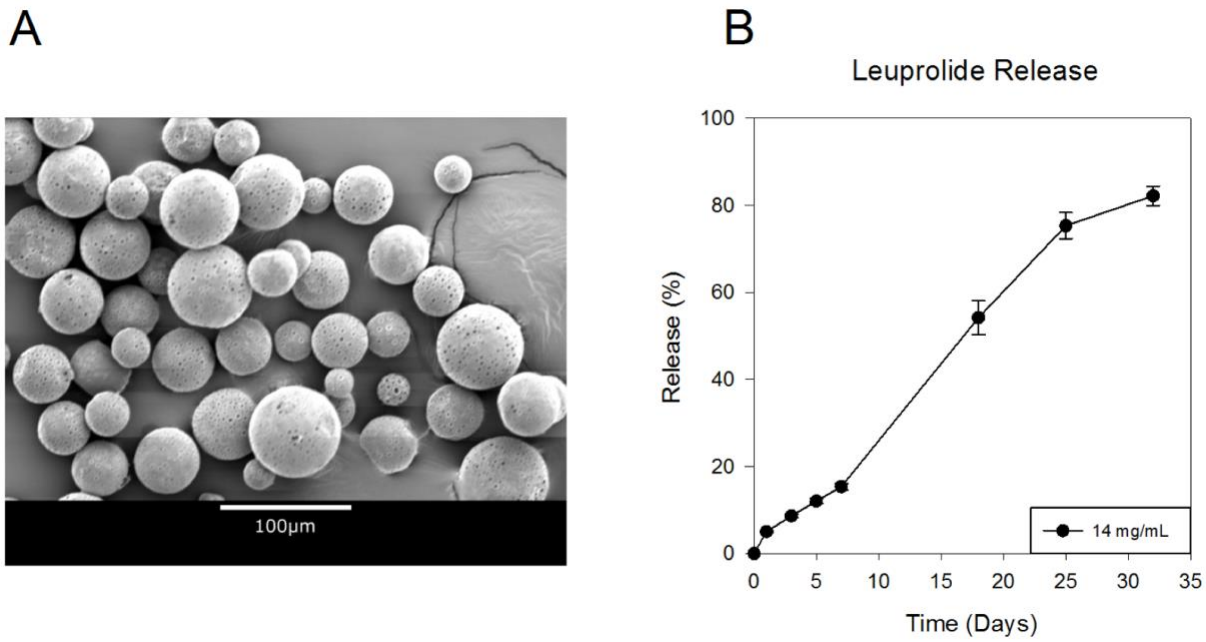

Figure 3.1 Evaluation of leuprolide loaded PLGA microspheres (14 mg/mL) formed from low concentration peptide solution. Scanning electron microscopy (SEM) image (A) and in vitro release kinetics (B) of the microspheres, Data represent Mean ± SEM (n=3).

3.5.2 Increased Efficiency of Encapsulation in PLGA with Increased Microsphere content

To understand the quasi-equilibrium sorption behavior sorption isotherms were determined by varying initial peptide concentration in the loading solution ($C_{pep,i}$) at two different initial microsphere concentrations ($C_{p,i}$) and applying the model developed above. As shown in Figure 3.2A, the sorption isotherm (f_b vs. (n_f/V_f)) for the data set followed a proportional behavior (see equation (6)) between fraction bound and free peptide concentration in solution ($r^2 = 0.96$). From the least squares regression, a binding constant, α, was determined as 0.22 mM^{-1}, which is of similar magnitude as those determined previously for 502H particles [11].

In order to predict the encapsulation efficiency (*EE*) from the sorption data, the equilibrium microsphere water uptake was also estimated by the inter-particle water (equation (23)) and water uptake during loading (equation (24)). Using the fraction of inter-particle water (0.61 ± 0.05 mL/mg, n=3), the 37 ºC intra-particle water uptake (φ) was determined as ~ 0.9 mL/mg (0.88 ± 0.15, n=3) and the binding capacity (κ), from titrating the end groups, as ~ 107 μmol COOH/g PLGA (107 ± 3 μmol COOH/g PLGA, n=3). These parameters were plugged into the theoretical equation for *EE*. As shown in Figure 3.2B, the *EE* was experimentally increased either by increasing the mass of microspheres (M_p) or decreasing the initial volume (V_i) of the solution, and thereby increasing $C_{p,i}$ in both cases (Figure S 3.1). As expected, also shown in Figure 3.2B, the theoretical *EE* calculated by equation ((10)) and the aforementioned determined parameters described these trends too. Note that the experimentally determined sorption data was used to predict *EE* during the same experiment. Therefore, while not an independent and full test of the theory, the quasi-equilibrium sorption isotherm and the theoretical analysis provide an explanation as to why the formulation variables such as mass of particles and volume of the loading solution have a highly variable effect on *EE* (and thus loading, l). These data also indicate it is possible to attain very high (~ 80% or higher) encapsulation efficiencies by the remote loading method. However, we see that as we increase the *EE*, this occurs at the expense of l (Figure 3.2C). This is due to loading being inversely related to polymer concentration (C_p) (equation ((14)). In order to improve loading (l) we would have to alter the peptide/polymer mass ratio at a constant C_p.

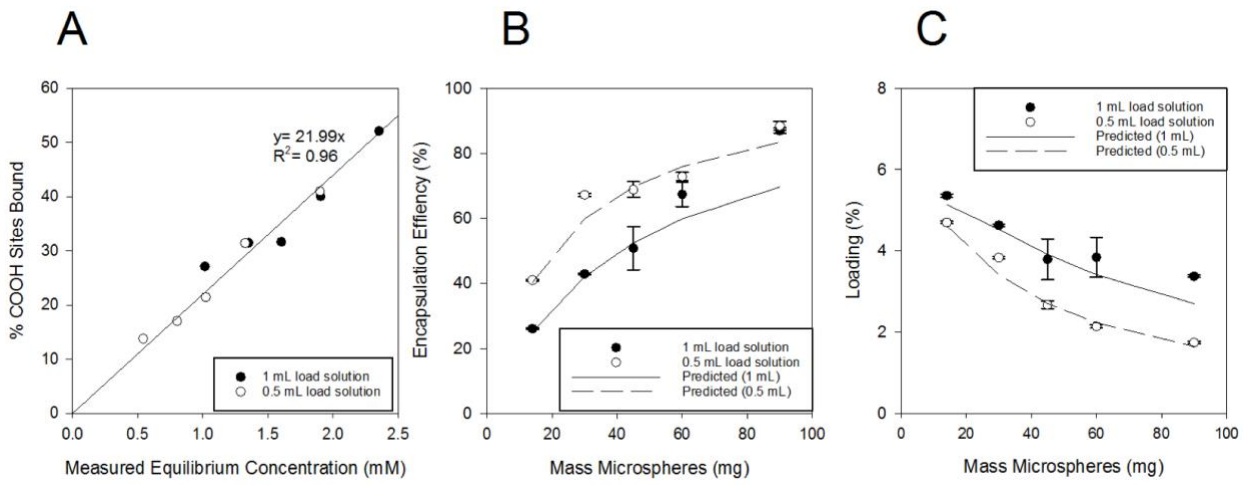

Figure 3.2 Encapsulation efficiencies were used to determine the % COOH sites bound at equilibrium (A). Encapsulation efficiency increases with increasing mass of microspheres similarly to the predicted curves determined using equation 3 (B). However, leuprolide loading decreases as the microsphere concentration increases (C), Data represent Mean ± SEM.

This binding data fit suggested the potential to use this data to theoretically predict the encapsulation efficiency (*EE*) and loading (l) for given variables of initial peptide mass ($M_{pep,i}$), polymer mass (M_p), initial polymer concentration ($C_{p,i}$), binding strength (α), and binding capacity (κ). Using equations (3) and (5) we were able to predict the *EE* and fraction loading (l) as a function of initial polymer concentration (Figure 3.3). This theory implies that encapsulation efficiency depends on the peptide binding strength (α), peptide binding capacity (κ), polymer water content (φ), and initial polymer concentration ($C_{p,i}$), but not on the peptide mass ($M_{pep,i}$), concentration ($C_{pep,i}$), or loading solution volume (V_i). Since loading is dependent on the encapsulation efficiency it also depends on the previously specified parameters. Loading is also dependent on the ratio of peptide mass to polymer mass ($M_{pep,i}/M_p$). However, it does not depend on the loading solution volume. This theory suggests optimizing encapsulation by first optimizing the initial polymer concentration to obtain a desirable

encapsulation efficiency, then keeping the polymer concentration and *EE* constant, a desired fraction loading can be achieved by adjusting the peptide/polymer mass ratio. Based on these experimental and theoretical results we selected initial polymer concentrations of 180 mg/mL and 240 mg/mL for studies going forward as they should result in *EE* > 80 %, which would mean minimal peptide wasted (see sample calculations in supplemental section).

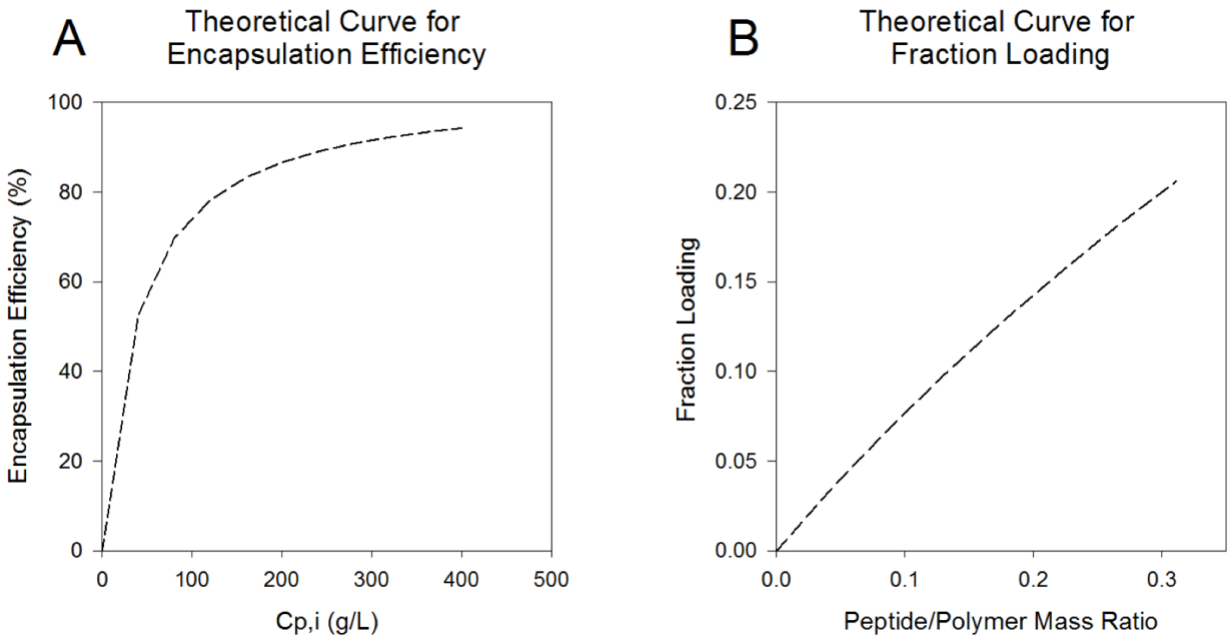

Figure 3.3 Theory for the prediction of encapsulation efficiency (A), and fraction loading (B) based on initial polymer concentration and peptide/polymer mass ratio. Theoretical curves determined using $f_b = 0.25$, $\kappa = .000107$ mmol COOH/mg PLGA, and $\varphi = 3.3x10^{-6}$ L/mg PLGA.

3.5.3 Kinetics of Leuprolide Binding to PLGA Microspheres

The binding equilibria of leuprolide to microspheres is not only important to optimize encapsulation but also to ultimately understand the binding mechanism. Most of the leuprolide encapsulation in PLGA by remote loading occurs in the first hour of incubation. Leuprolide binding to 50/50 PLGA microspheres was followed over 24 h for

the two different polymer concentrations, 180 mg/mL and 240 mg/mL, (Figure 3.4). For both concentrations, leuprolide rapidly bound to the polymer microspheres in the first hour (~40 - 50 %), which is consistent with previous results for leuprolide binding 50/50 PLGA particles [11]. After 8 hours, a quasi-equilibrium was reached as little increase in leuprolide binding was observed. Microspheres also became slightly less porous during loading (Figure 3.5). This could be due to polymer chain rearrangement during incubation leading to pore closure over time [14, 15]. It is noted that microspheres could also be loaded at 360 mg/mL initial polymer concentration, although at this concentration the microspheres imbibed the entire loading solution (i.e., V_f = 0 mL).

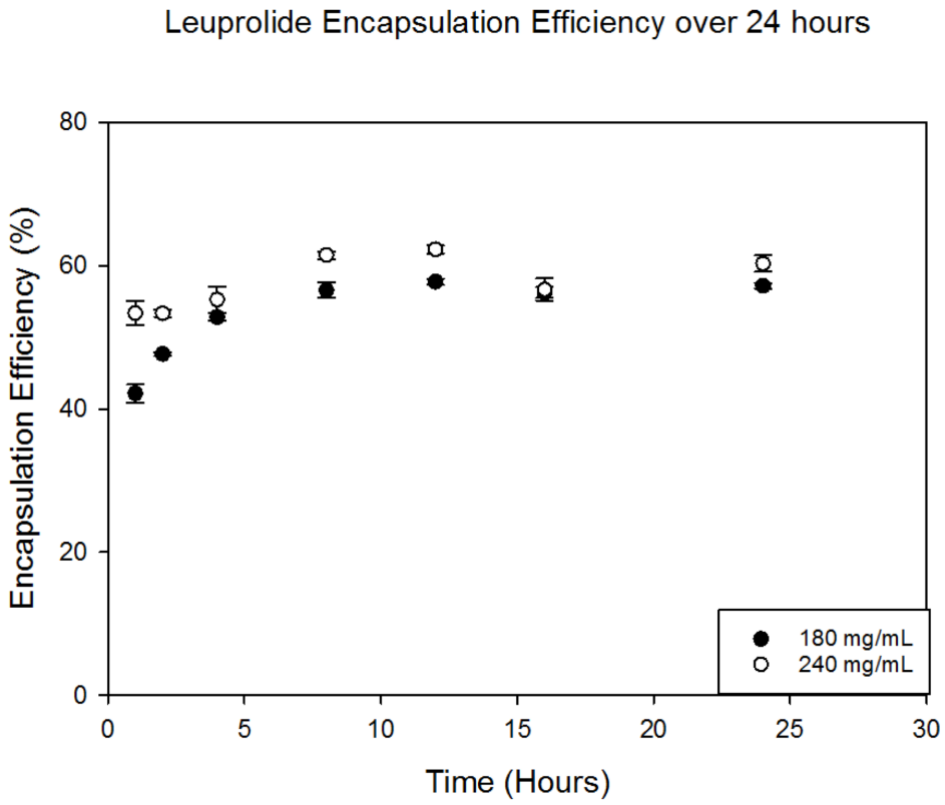

Figure 3.4 Kinetics of remote loading and encapsulation efficiency at 180 mg/mL, and 240 mg/mL microsphere concentrations were recorded by extraction/UPLC. Data represent Mean ± SEM.

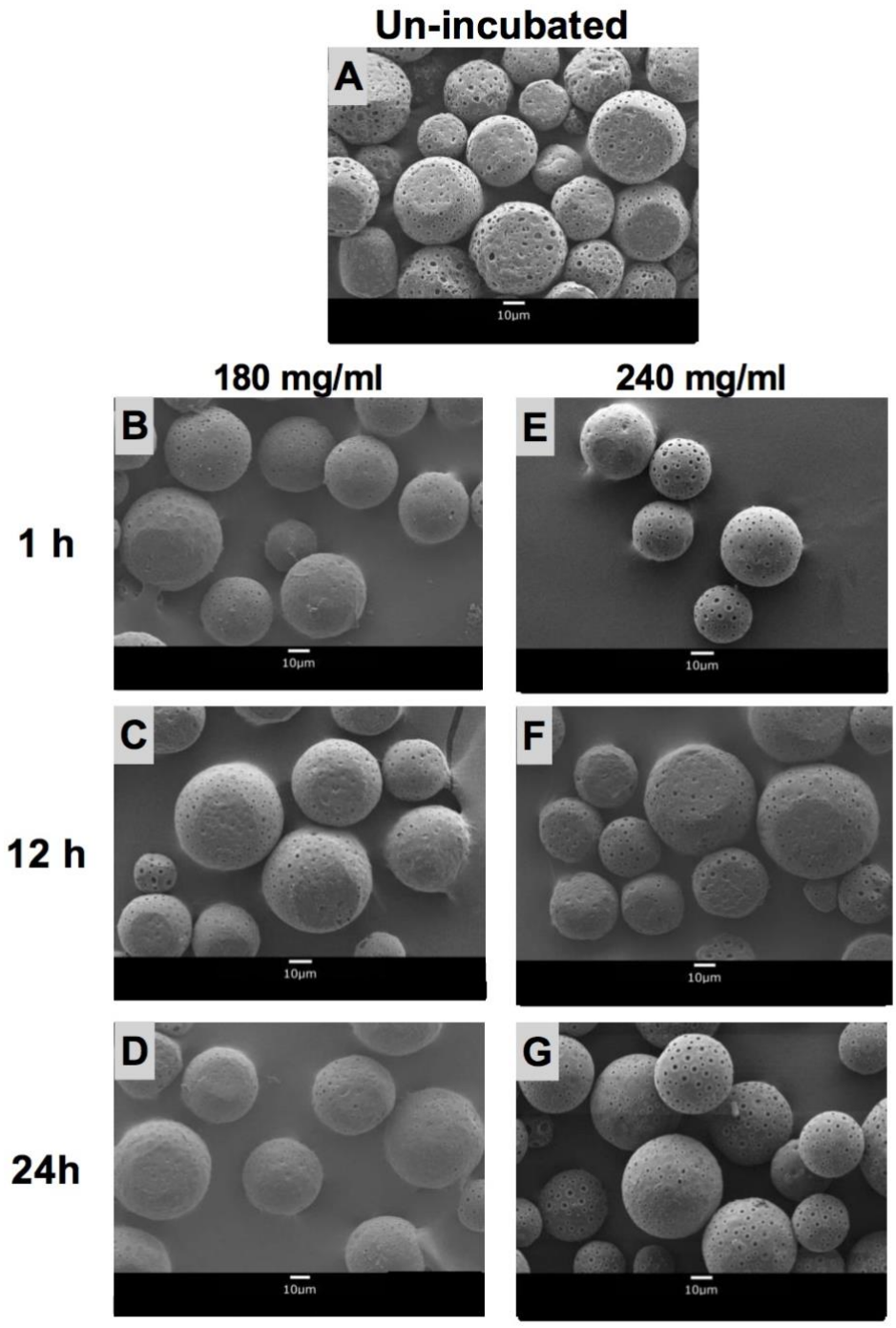

Figure 3.5 Surface morphology of microspheres by SEM during leuprolide loading from 20 mg/mL peptide solution. Images of drug-free (A), 1 hour 180 mg/mL (B), 12 hour 180 mg/mL (C), 24 hour 180 mg/mL (D), 1 hour 240 mg/mL (E), 12 hour 240 mg/mL (F), and 24 hour 240 mg/mL (G).

3.5.4 Microspheres with High Encapsulation Efficiency and Loading Slowly Release Leuprolide

Optimized microspheres with both high loading (180 mg/mL, 5.72 ± 0.04 %; 240 mg/mL, 4.64 ± 0.09 %) and encapsulation efficiency (180 mg/mL, 57.2 ± 0.4 %; 240 mg/mL, 60.3 ± 1.1 %) were evaluated for *in vitro* for their release kinetics. Initial burst release and *in vitro* release were determined for the two high-polymer concentration formulations (180 mg/mL and 240 mg/mL) in Figure 3.6. Microspheres loaded from 180 mg/mL exhibited a higher initial burst release (~30 %) relative to those loaded at 240 mg/mL (~20 %). This result is interesting given that the surface of the 240 mg/mL microspheres appear as porous as the 180 mg/mL loaded ones (Figure 3.7). This difference in initial burst release could be due to difference in particle size distribution as smaller particles can release faster than larger particles [16]. Therefore, if more particles are closer to 20 µm rather than 63 µm a faster release rate would be expected. Also, the different drug loading for the two microsphere concentrations can affect release rate as microspheres with higher loading had a higher initial burst release due to a smaller polymer to drug ratio [17].

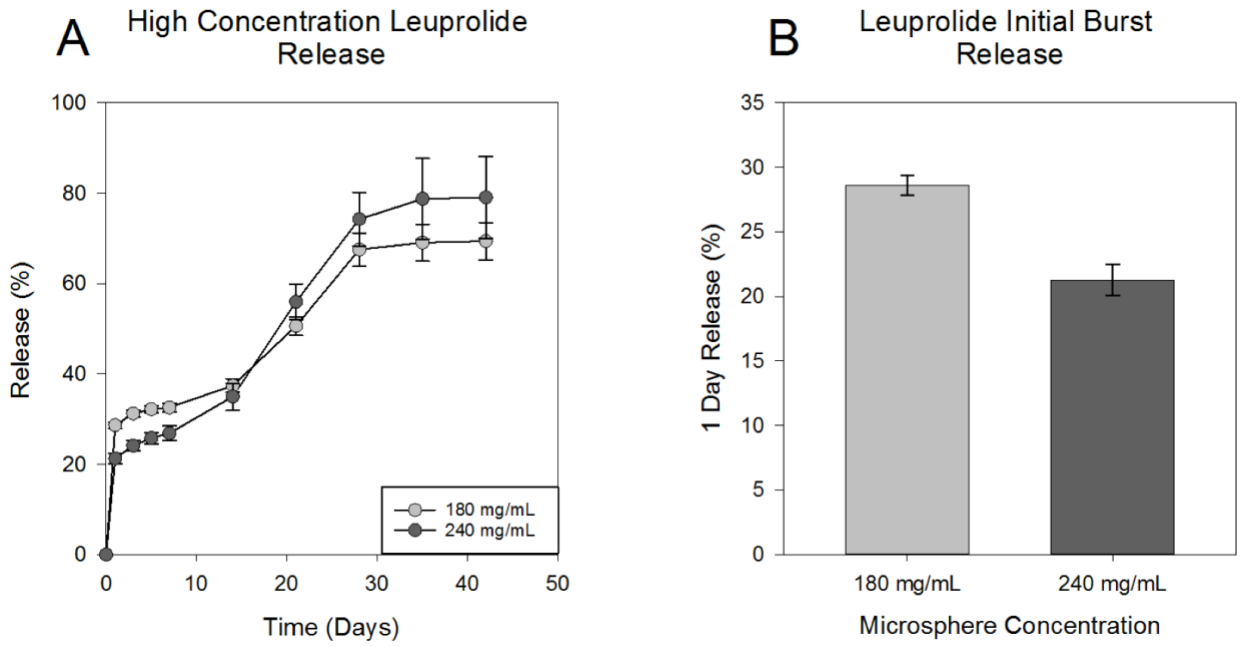

Figure 3.6 Leuprolide release kinetics from PLGA microspheres loaded at 180 mg/mL, and 240 mg/mL microsphere concentration (A). Initial burst after 1 day release from the same microspheres (B). Symbol/bars represent Mean ± SEM (n=6).

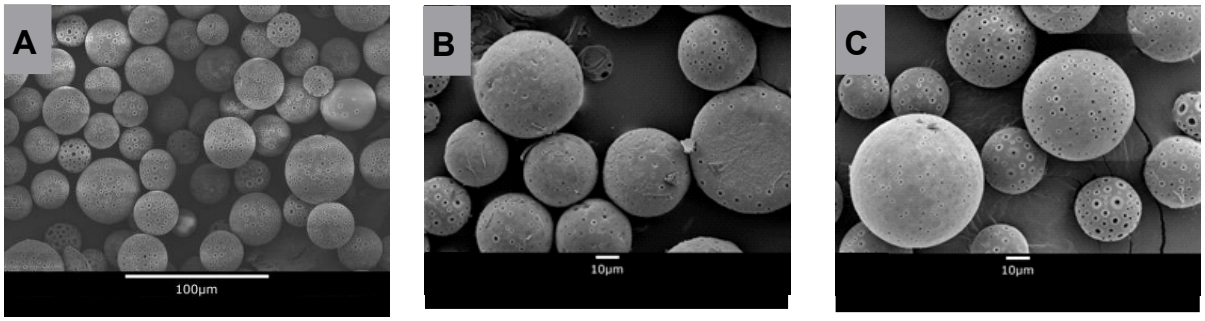

Figure 3.7 Scanning electron micrographs of PLGA microspheres loaded from high microsphere concentrations; un-incubated (A), 180 mg/mL (B), and 240 mg/mL (C). Initial leuprolide concentration was 20 mg/mL.

3.5.5 Microsphere Porosity Does Not Strongly Affect Encapsulation Efficiency or Loading

Porosity is a key parameter of controlled release microspheres as it can affect both drug loading and drug release rate [18, 19]. Microspheres with increasing volumes of the inner water phase trehalose (500mg/mL) showed an increase in porosity from

38% - 60% (Figure 3.8A), with the greatest increase in porosity occurring between 0 μl to 50 μl added trehalose solution. Microspheres of increasing porosigen content were loaded with leuprolide at a polymer concentration of 240 mg/mL; the encapsulation efficiency (Figure 3.8B) and loading (Figure 3.8C) increased slightly with increasing porosigen volume from 0 μl - 100 μl. After 100 μl trehalose the encapsulation and loading was similar for the microspheres containing 100 μl – 350 μl at ~ 70 % encapsulation and ~5.8 % loading. These results further support that the peptide is being absorbed into the polymer phase as increasing the porosity did not affect the encapsulation particularly for the 100 μl- 350 μl microspheres. Increasing the porosity did, however, increase the percent leuprolide released in 1 day from 0 μl - 100 μl added trehalose (Figure 3.8D).

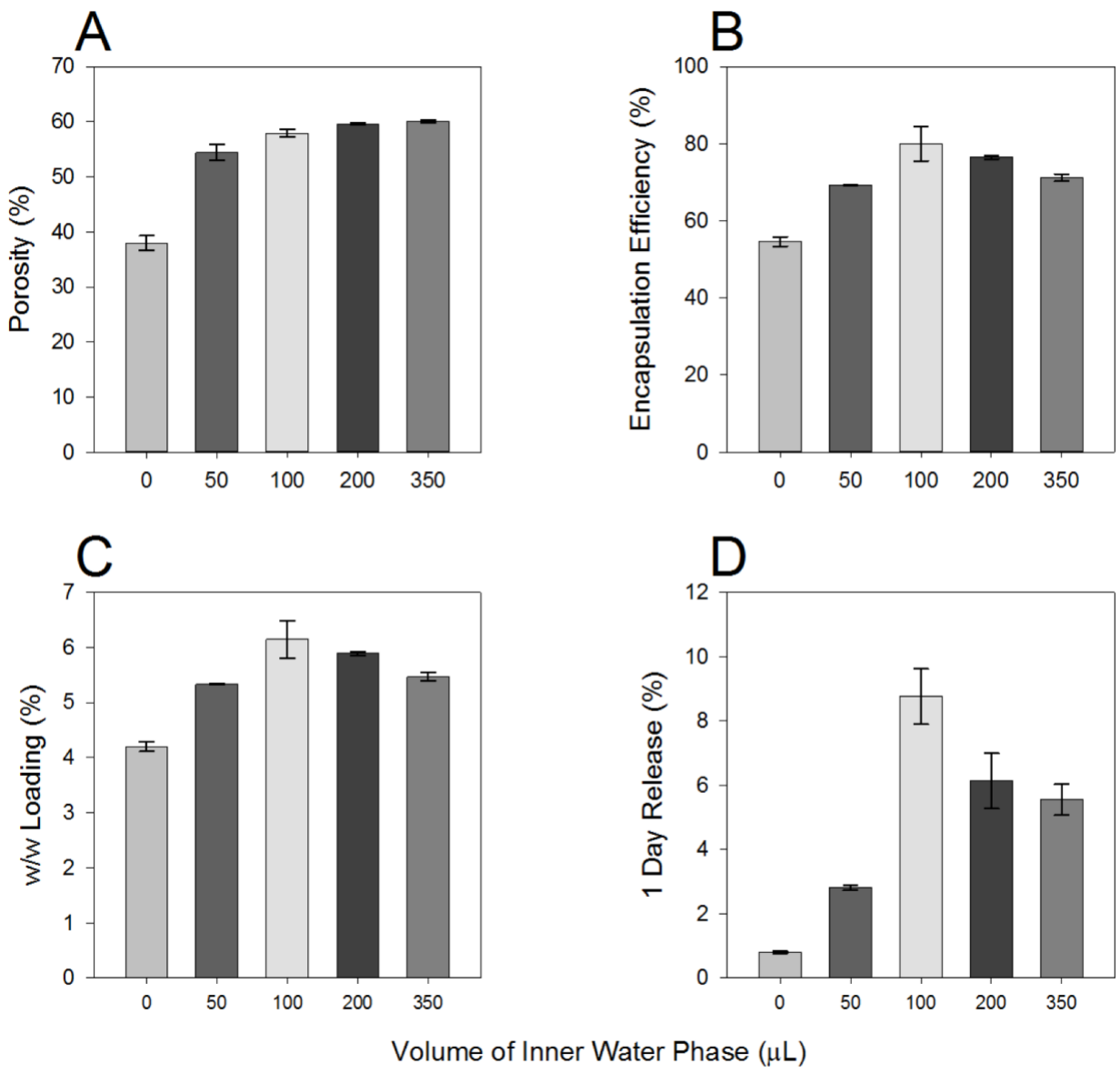

Figure 3.8 Effect of trehalose on 50/50 PLGA microsphere porosity (A), encapsulation efficiency (B), loading (C), and leuprolide initial burst release (D). Data represent Mean ± SEM (n=3).

The lower porosity microsphere may have had more difficulty attaining quasi-equilibrium absorption, based on their lower *EE* and loading after 24 hours on incubation. To test this, microspheres prepared with 0, 50, and 100 uL inner-water phase volumes were loaded longer, i.e., for 48 and 72 hours. However, as seen in Figure 3.9 leuprolide loading did not improve with longer loading period but rather

started to decrease in the case of 50 μL and 100 μL. The small difference in peptide loading after 72 h compared to 24 h loading may be related to the amount of peptide loaded into the pore volume versus the polymer volume of the microspheres, which can be up to ~1 % difference in some cases (*data not shown*). We note the contrast in remote loading by absorption with that of passive self-healing into percolating pores (e.g., for lysozyme [20, 21]). In the former, loading is mostly indifferent to polymer porosity because the mass of sorbent is kept constant, whereas the latter depends strongly on the porosity as more pores provide additional space for encapsulation on a constant pre-formed microsphere weight basis.

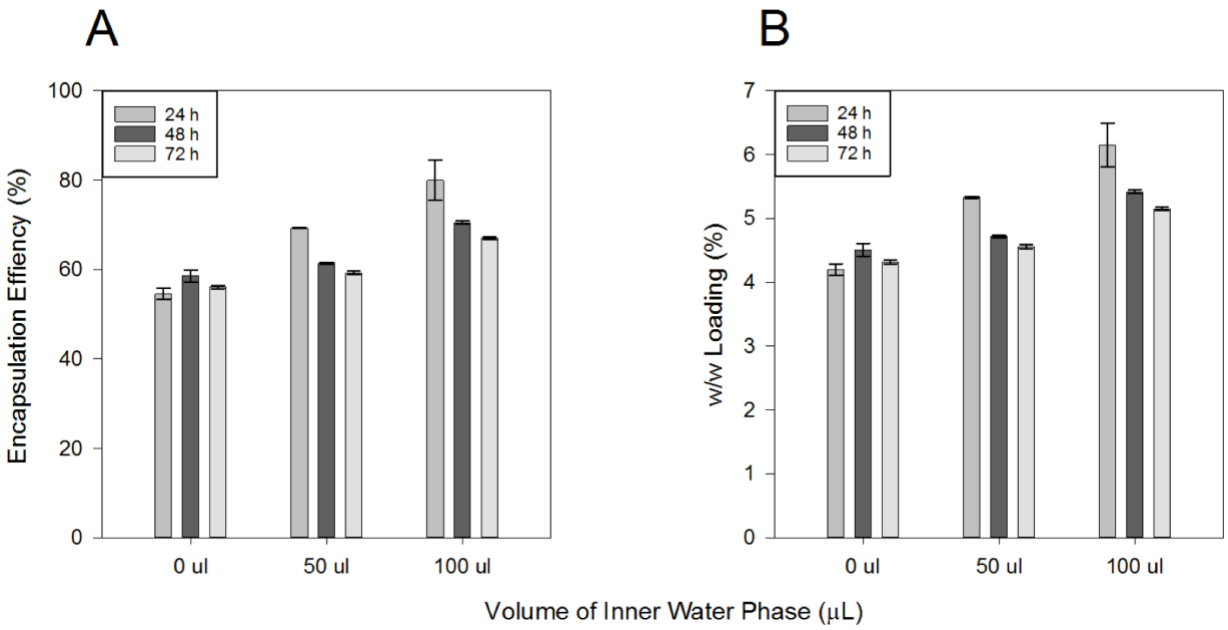

Figure 3.9 Effect of loading time on encapsulation efficiency (A), and loading (B) of microspheres prepared with 0 μl – 100 μl inner water phase. Data represent Mean ± SEM (n=3).

The resulting release kinetics of the different porous microspheres was also interesting. All samples had a low initial burst release of less than 10%, with microspheres having 100 μL inner-water phase showing the highest 1 day release

(Figure 3.8D). Microspheres of 50 μL- 350 μL inner-water phase displayed similar continuous release kinetics over 35 days. However, microspheres with 0 μL inner water phase showed minimal release over the first 2 weeks and then showed a faster release rate between day 14-35 (Figure 3.10). This slight change could be due a higher resistant of peptide to mass transfer owing to the more nonporous PLGA polymer matrix.

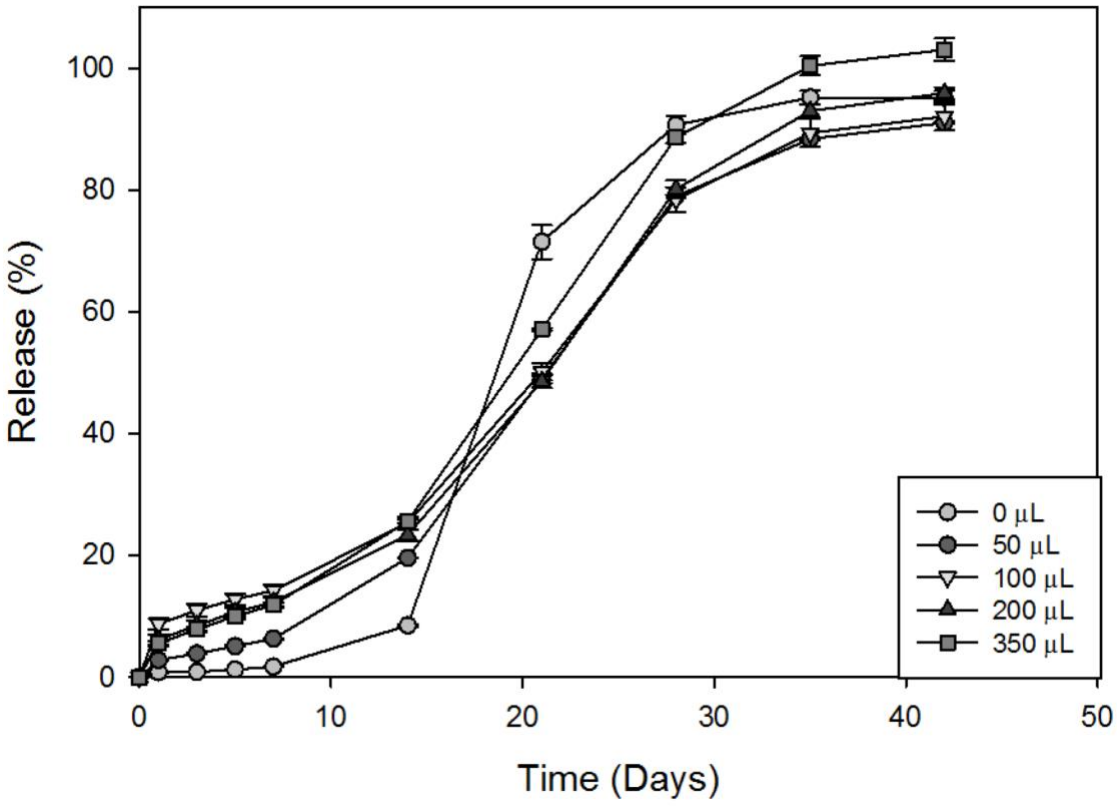

Figure 3.10 Leuprolide release from 50/50 PLGA microspheres of increasing inner water phase volume. Data represent Mean± SEM (n=3).

3.6 Conclusions

This work demonstrates that it is possible to use aqueous remote loading to encapsulate the cationic peptide leuprolide in acid terminated PLGA microspheres with high efficiency (>80%) and loading (>4%). Theoretical analysis indicates the importance of first controlling the concentration of the pre-formed microsphere sorbent in the loading solution to control *EE* before adjusting peptide concentration to manipulate drug loading. Utilizing this theoretical approach of relating the equilibrium absorption isotherms to the loading and encapsulation efficiency helped to optimize of loading conditions. Remote loaded-microspheres display a low initial burst (<30%) and continuous release of peptide over 1 month *in vitro*. The slow release indicates that peptide desorption kinetics of leuprolide is extremely slow compared to the fast absorption to the polymer during loading. The remote loading technique presents many potential advantages in lowering manufacturing costs of peptides with limited aqueous solubility, and for encapsulation of peptides on the small scale during early phase drug development, as one could imagine needing only a small amount (μg) of peptide as desired based on the amount of microsphere absorbent required during loading on the small scale. Typical microencapsulation by solvent evaporation requires significant levels of peptide if high loading is desired. In the future, this technique can be tested on additional peptides and biodegradable polymers of different lactic acid content to be utilized in long-term controlled release formulations, and validate the *in vivo* efficacy of such formulations.

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3.8 Supplemental

3.8.1 Increased Encapsulation with Increasing Microsphere Content

In order to improve encapsulation efficiency, the mass of microspheres loaded was increased from 14 mg to 90 mg at 1 mL and 0.5mL, effectively altering the initial polymer concentration ($C_{p,i}$). We see that as the mass of microspheres increases and the volume of loading solution decreases we are able to increase encapsulation efficiency (Figure S 3.1).

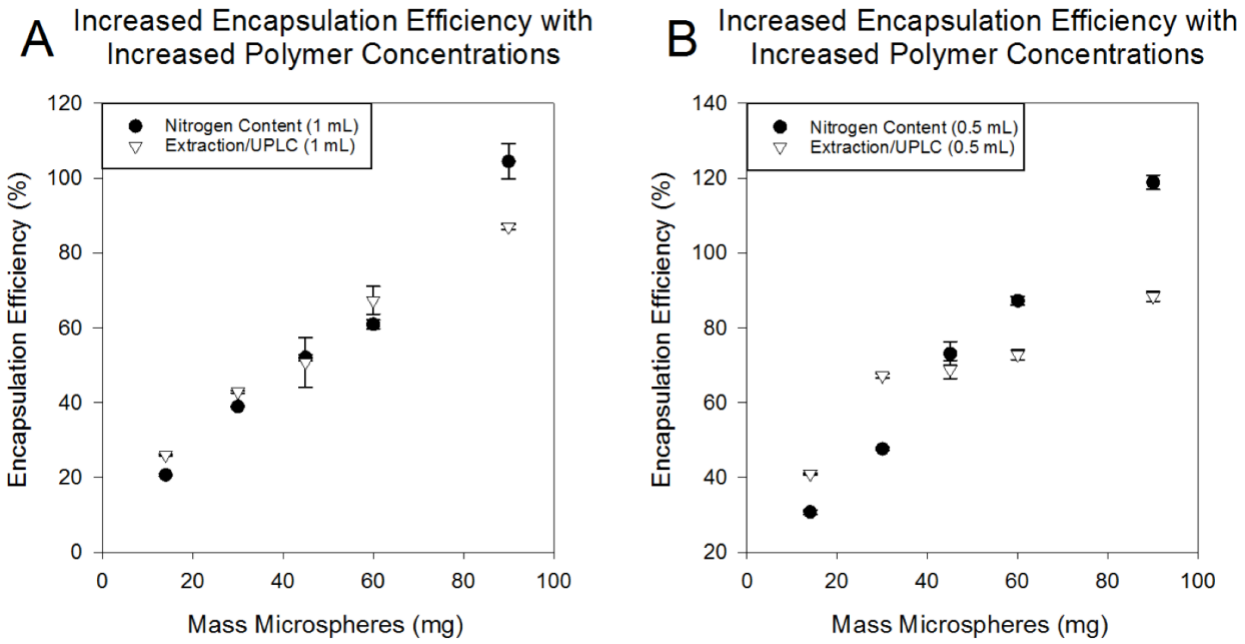

Figure S 3.1 Encapsulation efficiency of leuprolide increases with increasing mass of polymer microspheres with loading solution as determined by nitrogen content and extraction/UPLC. Microspheres were loaded in 1 mL (A), and 0.5 mL (B) of 3.6 mg/mL leuprolide in 0.1 M HEPES, pH 7.4.

3.8.2 Sample Calculations for Encapsulation Efficiency

Sample calculations for encapsulation efficiency (equation (10)) and loading (equation (14)), at a microsphere concentration of 180 mg/mL, based on the model discussed in section 2 are shown below. Given the constants for binding strength (α) = 0.25 M^{-1}, binding capacity (κ) = 107 μmol COOH/g PLGA and water uptake (φ) = $8.85\text{x}10^{-7}$ L/mg;

$$EE = \frac{\alpha\kappa C_{p,i}}{(\alpha\kappa - \varphi)C_{p,i} + 1}$$

$$EE = \frac{(.25) * (107\text{x}10^{-6})(180000)}{\big((.25)(107\text{x}10^{-7}) - (8.85\text{x}10^{-7})\big)(180000\big) + 1}$$

$$EE = \frac{4.815}{(.00002675 - (8.85\text{x}10^{-7}))(180000) + 1}$$

$$EE = \frac{4.815}{(2.59\text{x}10^{-5})(180000) + 1}$$

$$EE = \frac{4.815}{4.656 + 1}$$

$$EE = \frac{4.815}{5.646}$$

$$EE = 0.853$$

$$EE = 85.3\ \%$$

Keeping the polymer concentration constant, and by extension the *EE* constant, the loading, given a polymer mass (M_p) of 90 mg and peptide mass ($M_{pep,i}$) of 10 mg, can be determined as follows:

$$l = \frac{EE(M_{pep,i})}{EE\left(M_{pep,i}\right) + M_p}$$

$$l = \frac{EE(^{M_{pep,i}}/_{M_p})}{EE\left(^{M_{pep,i}}/_{M_p}\right) + 1}$$

$$l = \frac{(0.853)(^{10}/_{90})}{(0.853)(^{10}/_{90}) + 1}$$

$$l = \frac{(.853)(.111)}{(.853)(.111) + 1}$$

$$l = \frac{.095}{.095 + 1}$$

$$l = \frac{.095}{1.095}$$

$$l = .087$$

$$l = 8.7\ \%$$

Chapter 4: Development of clinically relevant PLGA microspheres for 1-month Leuprolide Delivery

4.1 Abstract

Poly (lactic-*co*-glycolic) acid (PLGA) is commonly used in long-acting release (LAR) drug delivery due to its biocompatibility and biodegradability. PLGA products have been on the US market for decades. However, no generics have reached approval in the US, likely due to the complex characterization required, difficulties in *in vitro* testing, discrepancies between *in vitro* and *in vivo* release, and manufacturing challenges such as maintaining activity of the drug and reducing batch-to-batch variation [1-3]. From the previous chapter, it has been shown that model cationic peptides, i.e. leuprolide and octreotide, can interact with acid terminated 24 kDa, 50/50 PLGA, allowing for elevated encapsulation efficiency (improved to ~60% from 38% in initial studies), due to peptide absorption. Here we test the potential to remotely load leuprolide in the same polycondensation PLGA 75/25 (11-13 kDa) as used in the commercial product, Lupron Depot® (LD) in an effort to create a clinically useful formulation. Consistent with the high level of end-groups available for leuprolide binding, over 80% leuprolide encapsulation was achieved for 24 h loading at a leuprolide concentration of 20 mg/mL and microsphere concentrations of 180 mg/mL, 240 mg/mL and 360 mg/mL. All three microsphere concentrations continuously released leuprolide,

in vitro, for over 49 days and had a low initial burst of <20% on day 1, comparable to LD. Drug-free microspheres were exposed to gamma radiation in order to explore the potential for terminal sterilization prior to drug loading and further test the commercial feasibility of this loading paradigm. Gamma irradiation did not significantly affect the glass transition temperature, molecular weight (MW) or surface morphology of microspheres. Microspheres loaded after irradiation exhibited continuous release *in vitro* comparable to non-irradiated microspheres. Leuprolide release from irradiated (240 mg/mL) and non-irradiated microspheres (180 mg/mL and 240 mg/mL) were additionally compared to 1 month LD *in vivo*. Chemical castration was achieved and maintained for 3 months when dosed monthly for all three formulations in the same way as the LD positive control. This work demonstrates that aqueous remote loading can be applied to 75/25 PLGA and achieve loading, loading efficiency, and *in vitro* and *in vivo* release comparable to a commercial product, LD.

4.2 Introduction

Lupron Depot® (LD) was first approved in the United States in 1989 as a monthly injection for the treatment of prostate cancer. LD is considered the gold standard for polymeric LAR depots as it was one of the first commercially successful microparticle drug delivery systems [4]. However, a generic form of Lupron Depot® does not exist in the US likely due to the difficulty of manufacturing this dosage form [5]. Takeda Pharmaceuticals, the makers of LD, tested several types of PLGA with varying molecular weights, lactic acid/glycolic acid ratios, and also tested different particle sizes, additives, and inner water phase viscosities [6, 7] to determine the optimal formulation

to achieve both maximal encapsulation and zero-order release for over one month. Changes to any of the stated parameters could adversely affect a previously optimized parameter and subsequently the overall product quality, demonstrating the difficulty in producing LD [5].

There are currently 15 FDA approved PLGA or PLA based products on the market, none of which have generics available. This lack of generics can be burdensome on patients as they have little option but to pay more for the brand name drug or to seek other, less desirable treatment options. For example, the use of GnRH agonists, such as leuprolide, in general has overcome the undesired treatment of surgical castration for prostate cancer as patients are instead chemically castrated when given these agonists. In addition, the loss of testosterone level is reversible once dosing is discontinued. One dose of 1-month LD costs ~$1,400 [8]. There are two approved products for controlled release of leuprolide, Lupron Depot® and Eligard®. There are several differences between the two products. Eligard® is a subcutaneous *in-situ* forming gel injection which comes as two separate syringes containing separate solutions of polymer and peptide dissolved in a biocompatible organic solvent, and the formulation must be refrigerated prior to use [9]. LD is an intramuscular injection of leuprolide in PLGA microspheres and diluent separated in a pre-filled syringe, and can also be stored at room temperature prior to administration [10]. Therefore, while the two deliver the same peptide the ease of use in terms of comfort, storage conditions, and preparation time makes the LD more favorable [8].

In the development of LAR generic products, when developed according to the simpler regulatory route, they must show no significant difference in drug release rate

among other criteria by being both qualitatively (Q1) and quantitatively (Q2) the same as the innovator product [1]. However, this is difficult to control when working with PLGA as small changes in the polymer can have significant effects on mechanism and rate of release [1]. Nutropin Depot®, which was approved in 1999 and subsequently discontinued in 2004 due to the high cost of manufacturing illustrates how difficult it is to make a cost-effective, effective, and competitive PLGA microsphere product [4].

Manufacture of the LD involves a 14-step process [11], which is not only complex but also costly. A remote loading method, in which PLGA microspheres are incubated in a drug solution, developed by Schwendeman et.al. may provide a simpler, cheaper alternative [12-14]. The interaction between leuprolide, and acid terminated PLGA suggests aqueous efficient remote loading is possible in a commercial context. Previous studies have shown that simply incubating acid terminated 50/50 PLGA microspheres with leuprolide results in elevated leuprolide loading and the particles are able to release leuprolide, *in vitro,* over 1 month [14]. If leuprolide can be similarly encapsulated in 11-13 kDa 75/25 PLGA microspheres by remote loading, then the cost and steps needed to make leuprolide microspheres could be decreased. Drug-free, porous 75/25 PLGA microspheres were prepared by a w/o/w double emulsion solvent evaporation method and then incubated in an aqueous peptide solution for peptide absorption. Aqueous remote loaded, low molecular weight microspheres were prepared as in Chapter 3 and were compared to the commercial LD formulation in terms of loading efficiency and drug loading, initial burst, long-term release kinetics and *in vivo* efficacy. As we wanted to determine the applicability of the entire process for its potential

commercial viability for long-term controlled release formulations, the effect of terminal sterilization of microspheres prior to peptide absorption was also studied.

4.3 Materials & Methods

4.3.1 Materials

Leuprolide acetate was purchased from SHNJH Pharmaceuticals (Shanghai, China). Low molecular weight 75/25 poly(lactic-*co*-glycolic acid) was purchased from Wako Chemicals (Osaka, Japan). Poly vinyl alcohol, carboxymethylcellulose, aprotinin from bovine lung, D-mannitol and Tween 80 were purchased from Sigma Aldrich Chemical Co (St. Louis, MO). Hydroxyethyl-piperazineethanesulfonic acid (HEPES) was purchased from Thermo Fisher Scientific (Waltham, MA). Lupron Depot® (lot # 1064067) was purchased from the University of Michigan Hospital Pharmacy. All other materials were of analytical grade and purchased from commercial suppliers.

4.3.2 PLGA Microsphere Formulation

Low molecular weight PLGA microspheres were prepared using a double emulsion solvent evaporation method. PLGA, 1 g was dissolved in 1 mL methylene chloride. Once dissolved, 100 µl of a 500 mg/mL trehalose in ddH_2O solution was added to the polymer solution and then homogenized (VirTis Tempest I.Q.^{2}) for 1 min at 10,000 rpm to create a water-in-oil (w/o) emulsion. A 5% poly vinyl alcohol (PVA) solution was added to the emulsion, which was then vortexed (Scientific Industries Vortex Genie 2) for 1 min at maximum speed. The solution was then added to a stirring bath of 0.5 % PVA and stirred for 3 hours to allow for solvent evaporation. After

hardening, microspheres were washed with 1 L ddH_2O and sieved for size, 20-63 μm. Microspheres were lyophilized (Labconco FreeZone 2.5) and stored until further use.

4.3.3 Leuprolide Loading Solution

High concentration leuprolide loading solutions of 20 mg/mL were made by dissolving 40 mg of peptide and 47.7 mg of HEPES in 1 mL ddH_2O. The solution was titrated to pH 7.4 using 0.1 N sodium hydroxide (NaOH). The solution was then transferred to a 2-mL volumetric flask and the remaining ddH_2O added to achieve a final loading solution volume of 2 mL.

4.3.4 Irradiation of Microspheres

Preformed, unloaded microspheres were weighed out into glass ampules, ~ 100 mg per ampule, and sealed under vacuum. Sealed ampules were exposed to gamma irradiation at a dose of 1.8 Mrad and dose rate of 0.64 Mrad/hr. Gamma irradiation was done by the Oregon State University Radiation Center (Corvallis, OR).

4.3.5 Remote Loading of Microspheres

Irradiated and non-irradiated microspheres were loaded by incubating 90 mg of microspheres in an aqueous peptide solution of either 0.5 mL or 0.375 mL of leuprolide loading solution for 24 h at 37 °C with rotation. The final microsphere concentrations loaded were 180 mg/mL and 240 mg/mL. After incubation, microspheres were centrifuged for 5 min at 8,000 rpm and the supernatant removed. Loaded microspheres were washed three times with 1 mL of ddH_2O. Loaded microspheres were then freeze dried and stored at -20 °C.

4.3.6 Leuprolide Encapsulation Efficiency & Loading Analysis in Low Molecular Weight PLGA Microspheres

Leuprolide encapsulation in PLGA microspheres was determined by mass loss from solution after loading, two-phase extraction, and total nitrogen analysis. The supernatant was removed after loading and wash solutions were analyzed by UPLC (Acquity H-Class, Waters). Briefly, for extractions, ~5 mg of loaded microspheres were dissolved in 1 mL methylene chloride and 2 mL of 1M sodium acetate (pH 4) was added to create the two phases. The 3 mL solution was vortexed for 1 minute then centrifuged for 4 min at 4,000 rpm and 1.5 mL of the supernatant was removed. The removed 1.5 mL was replaced with a fresh 1.5 mL of sodium acetate and the process repeated 4 more times for a total of 5 extractions. After the 5th extraction, 1.5 mL of 1 M sodium acetate + 1 M sodium chloride (pH 4) was added. This process of vortexing then centrifugation was repeated 6 times; resulting in 11 total extractions. For total nitrogen analysis ~ 2 mg of microspheres were weighed into tin pans for nitrogen analysis. Samples of EDTA ranging in mass from 0.5 mg – 3 mg were used to calibrate the nitrogen analyzer (TruSpec Micro, Leco).

4.3.7 Evaluation of PLGA Glass Transition Temperature

The dry and hydrated glass transition temperature (T_g) of 75/25 PLGA microspheres were measured by differential scanning calorimetry (DSC) analysis (Discovery DSC, TA Instruments). The dry T_g was determined by weighing 4-5 mg of unloaded irradiated and non-irradiated microspheres into non-hermetic pans. Samples were analyzed by ramping the temperature from 5 °C to 90 °C. The hydrated glass transition temperature was determined by first weighing out 10 -12 mg of unloaded

irradiated and non-irradiated microspheres in 200 µl of 0.1 M HEPES solution (pH 7.4) at 37 °C for 24 h on a rotator. After incubation, microspheres were centrifuged and the supernatant removed to create a slurry, which was then analyzed over the same temperature range as done with non-hydrated microspheres.

4.3.8 Analysis of In Vitro Release

Leuprolide release, *in vitro*, from irradiated and non-irradiated microspheres loaded at 180 mg/mL and 240 mg/mL was measured by determining the percent remaining in microspheres. Briefly, 10 mg of loaded microspheres were weighted out, in duplicate, for each time point to be tested (Day 1, 3, 5, 7, 14, 21, 28) and incubated with 1 mL of phosphate buffered saline + 0.02% Tween 80 (PBST) with mild agitation at 37 °C. At each time point the samples were centrifuged for 5 min at 8,000 rpm and 900 µl of the release buffer was removed to ensure that no particles were collected. Samples for Day 1 were then dried for 24- 48 h at 25 °C. For all other samples not stopped on that day, 900 µl of the removed release media was replaced with fresh PBST and incubated until the next time point. From the sample that was stopped and dried, approximately 5 mg of microspheres were weighed out into an Eppendorf tube for analysis of percent peptide remaining by two-phase extraction.

4.3.9 In Vivo analysis of Microspheres Compared to Lupron Depot®

Leuprolide release from irradiated and non-irradiated microspheres was tested *in vivo* and compared to the commercial 1-month Lupron Depot®. Non-irradiated microspheres loaded at 180 mg/mL and 240 mg/mL and irradiated microspheres loaded at 240 mg/mL formulations were tested in Male Sprague Dawley rats (n=6 rats/group).

4.3.10 In Vivo Evaluation of Plasma Testosterone Levels in Sprague-Dawley Rats Over 3 Months

The efficacy of leuprolide loaded microspheres at testosterone suppression were tested in male Sprague-Dawley rats and compared to commercial 1-month Lupron Depot®. Both non-irradiated and irradiated microspheres were injected into rats at a dose of 100 µg/kg/day. Microspheres were suspended in an injection vehicle of 0.5 % low viscosity carboxy methyl cellulose (CMC), 0.1 % w/v Tween 80 and 5 % D-mannitol and were administered subcutaneously. Lupron Depot® was reconstituted according to the package insert and added to 4 mL of injection vehicle. Blood was sampled over the first 24 h to determine leuprolide levels in plasma. To ensure leuprolide stability, 25 µl of 1 mg/mL aprotinin was added to each blood collection tube. Blood was also sampled on days 1, 3, 7 and weekly thereafter to determine plasma testosterone levels. Whole blood samples were centrifuged for 10 minutes at 4000 rpm (Eppendorf 5810R) to separate the plasma. The plasma was then removed and stored at -80 °C until analysis. Plasma testosterone samples were analyzed, in duplicate, by RIA analysis at the University of Pennsylvania (RIA Biomarkers Core, Penn Diabetes).

4.4 Results

4.4.1 Remote Loading in Low Molecular Weight PLGA Microspheres

Low molecular weight PLGA microspheres, at three different microsphere concentrations, 180 mg/mL, 240 mg/mL and 360 mg/mL, were loaded by aqueous remote loading using 20 mg/mL of leuprolide. All microsphere concentrations achieved greater than 4% w/w loading (Figure 4.1A) and over 80% encapsulation efficiency

(Figure 4.1B). Polymer concentration had an inverse relationship with loading as lower polymer concentrations had higher loading. Encapsulation efficiencies, however, did not seem to vary with polymer concentration.

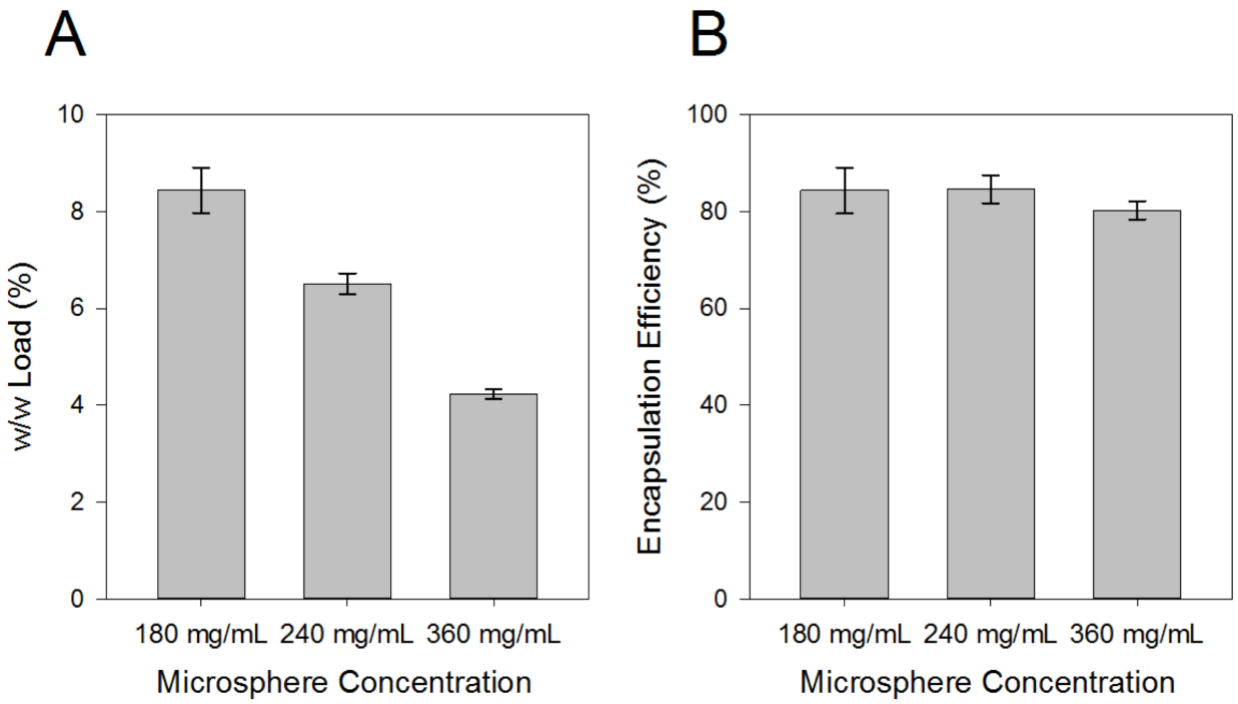

Figure 4.1 Leuprolide loading (A) and encapsulation efficiency (B) in low molecular weight 75/25 PLGA microspheres at 180 mg/mL, 240 mg/mL, 360 mg/mL microsphere concentration. Mean ± SEM (n=3).

Leuprolide release *in vitro,* measured by concentration in release medium, from all three formulations was continuous over 42 days (Figure 4.2A) with a low initial burst release of less than 20% on day 1 (Figure 4.2B). The release curves show near zero order release for week 2-6 and the 180 mg/mL and 240 mg/mL formulations both achieved 83 ± 3% and 87.5 ± 0.4% release, respectively.

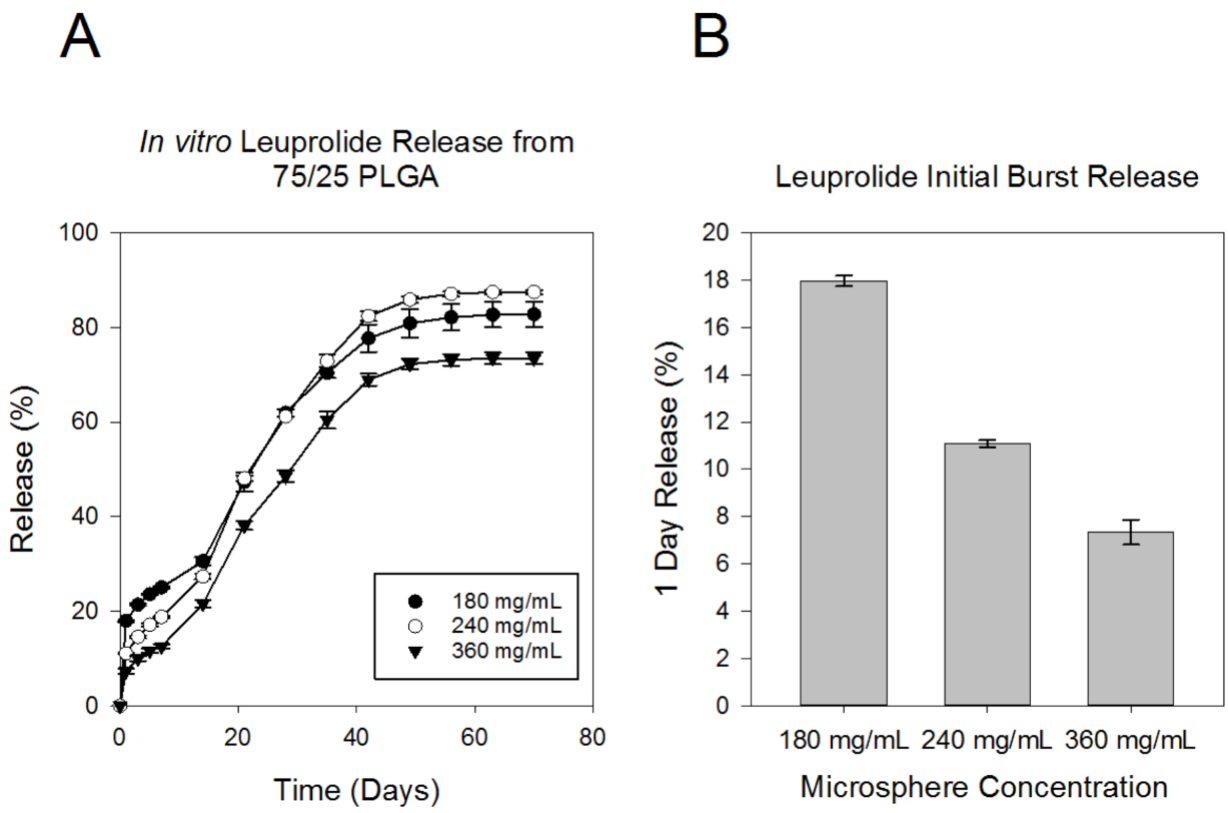

Figure 4.2 In vitro release (A) and initial burst release (B) of leuprolide from low molecular weight 75/25 PLGA loaded at 180 mg/mL, 240 mg/mL, and 360 mg/mL microsphere concentration. Mean ± SEM.

4.4.2 Effect of Gamma Radiation Exposure on PLGA Microsphere Properties

Drug-free, low molecular weight microspheres were exposed to gamma irradiation and then analyzed to ensure that there were no adverse effects due to radiation exposure. Four sample vials from the same initial batch of microspheres were characterized by their molecular weight, glass transition temperature and surface morphology following gamma irradiation. There was minimal change in molecular weight of irradiated microspheres compared to the molecular weight of non-irradiated microspheres, as all test vials had average molecular weights ranging from 11.3 kDa – 11.4 kDa, compared to 11.3 kDa for non-irradiated microspheres (Figure 4.3A). The glass transition temperature of the microspheres also showed no change as the glass transition temperature was ~45 °C before and after gamma irradiation exposure (Figure

4.3B). Scanning electron microscopy images of the microspheres before gamma irradiation exposure (Figure 4.4A) and from four different vials after gamma irradiation (Figure 4.4B-E) appeared similar. Microspheres showed a smooth surface with a few surface pores and were still within the desired size range of 20-63 microns post radiation.

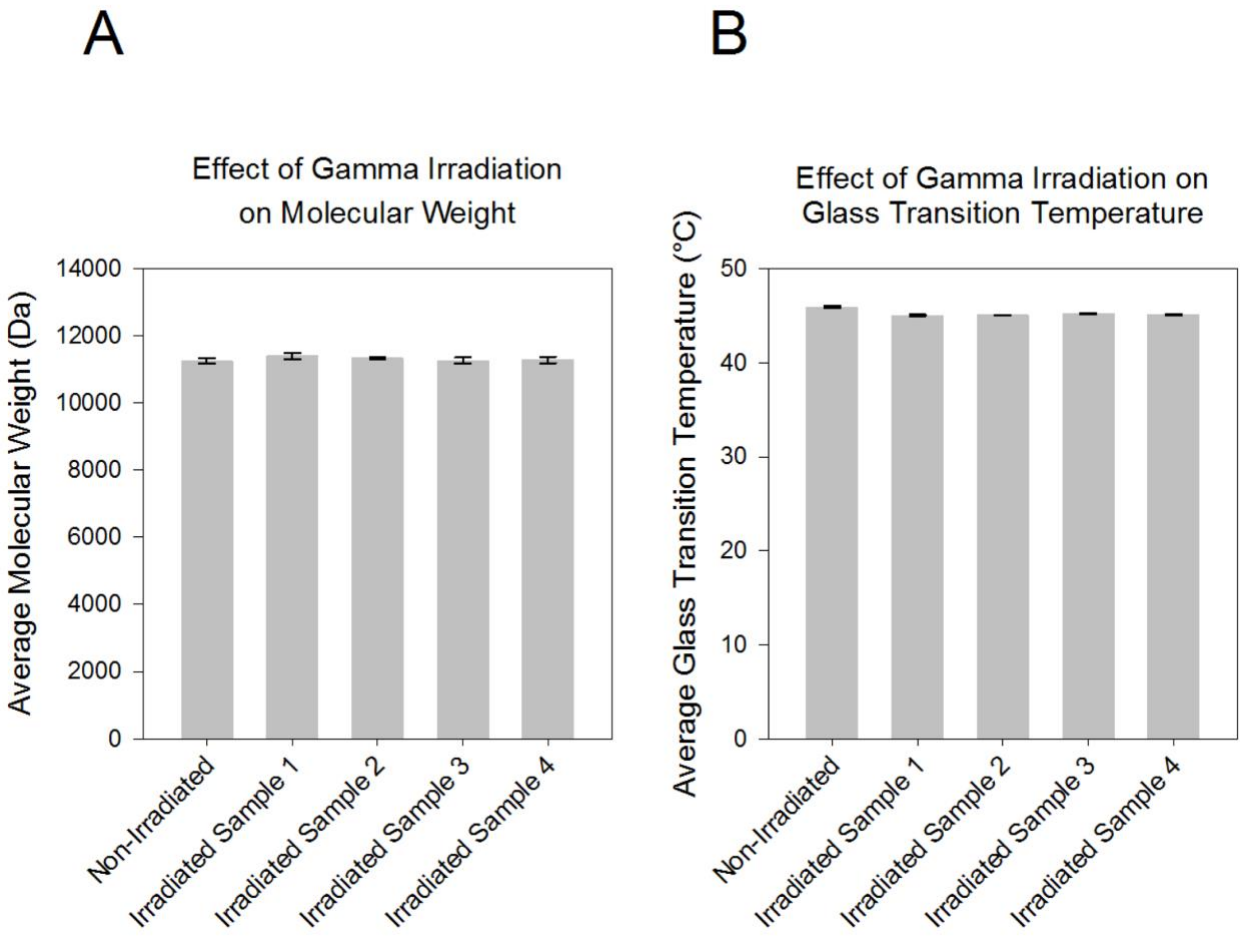

Figure 4.3 Effect of gamma irradiation on low molecular weight 75/25 PLGA microsphere molecular weight (A), and glass transition temperature (B). Microspheres were exposed to 1.8 Mrad dose of gamma irradiation and 4 different sample vials were randomly selected and tested. Mean ± SEM (n=3).

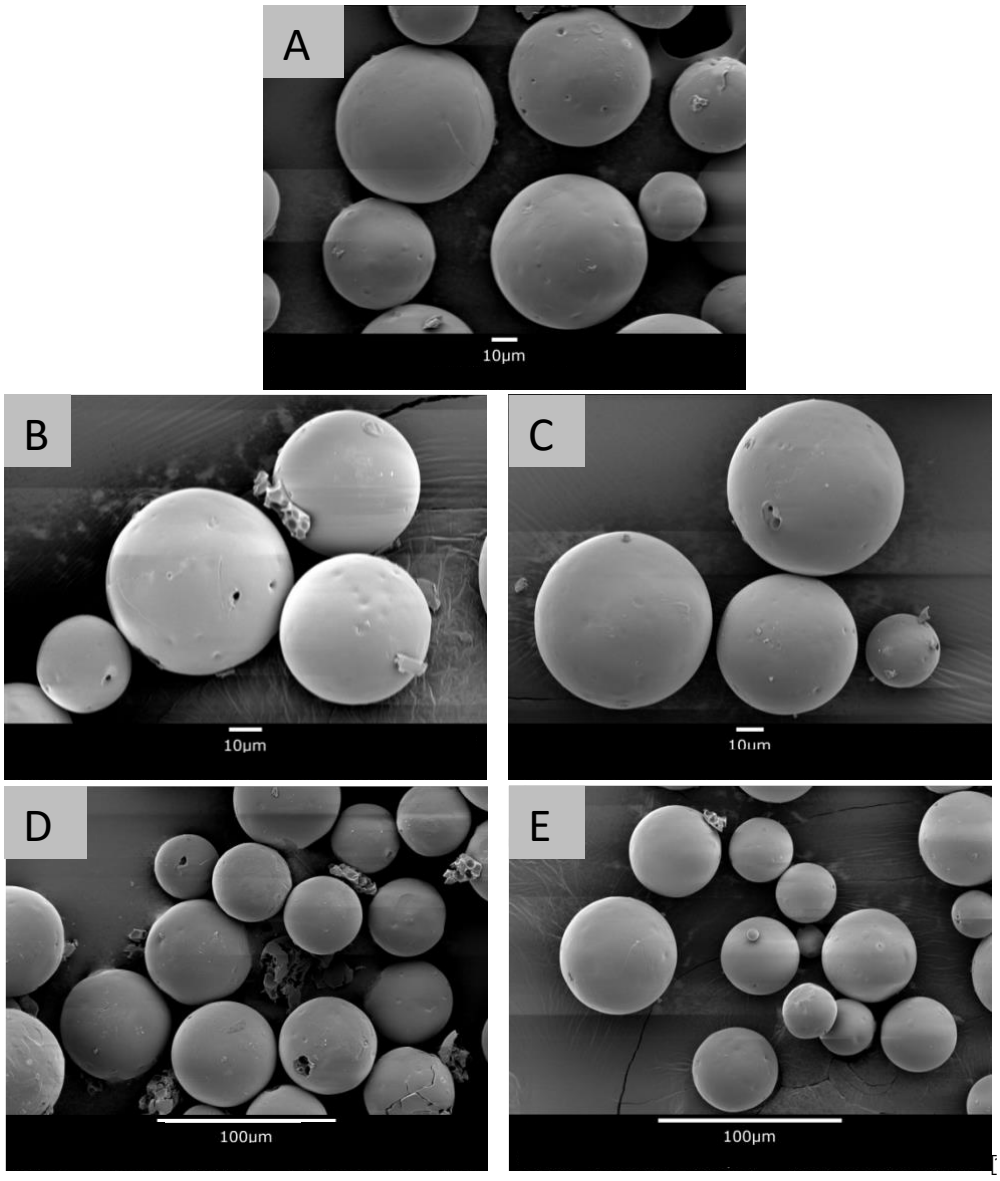

Figure 4.4 Scanning electron microscopy (SEM) images of low molecular weight 75/25 PLGA microspheres before gamma irradiation (A), and after gamma irradiation exposure (B-E). Four vials were tested; irradiated sample 1 (B), irradiated sample 2 (C), irradiated sample 3 (D), irradiated sample 4 (E).

4.4.3 Irradiated Microspheres Load Similar to Non-irradiated Microspheres

Irradiated microspheres were loaded with leuprolide at 180 mg/mL and 240 mg/mL microsphere concentration similar to non-irradiated microspheres. A new batch of microspheres from those previously loaded were used. Irradiated microspheres showed a negligible decrease in leuprolide encapsulation efficiency and loading

compared to non-irradiated microspheres; 9.5 ± 0.013 % compared to 9.65 ± 0.001 % w/w loading (95.07 ± 0.13 % EE to 96.37 ± 0.05 % EE) for 180 mg/mL and 7.24 ± 0.01 % compared to 7.42 ± 0.01 % w/w loading (94.1 ± 0.16 % EE to 96.51 ± 0.11 %EE) for 240 mg/mL (Figure 4.5). These results show that gamma irradiation does not affect the loading or encapsulation efficiency of leuprolide, further confirming gamma irradiation as a viable method for terminal sterilization of microspheres prior to remote loading.

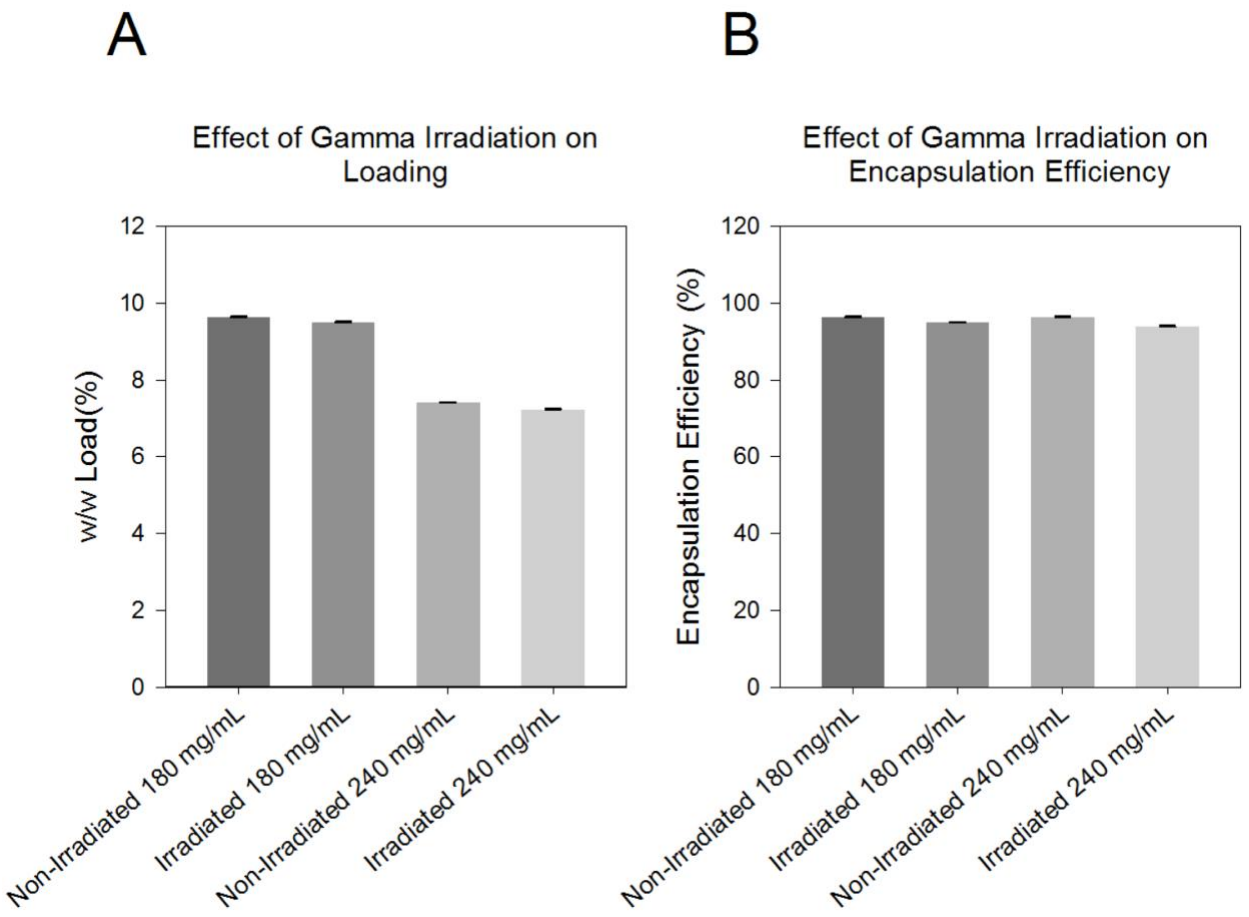

Figure 4.5 Comparison of leuprolide loading (A) and encapsulation efficiency (B) in low molecular weight 75/25 PLGA microspheres that were either exposed or not to gamma irradiation prior to loading at 180 mg/mL, and 240 mg/mL microsphere concentration. Mean ± SEM (n=2).

4.4.4 In Vitro Leuprolide Release from Irradiated and Non-irradiated Microspheres

Leuprolide release from irradiated and non-irradiated microspheres were measured for 1 month *in vitro* (Figure 4.6A). The release profiles are similar for

irradiated and non-irradiated microspheres for both polymer concentrations (180 mg/mL and 240 mg/mL). All formulations show near zero order release after 1 week. The initial burst (Figure 4.6B) was higher than desirable (> 20 %) although the total leuprolide release (76 % - 84 %) in 1 month is acceptable. From Figure 4.6A, it appears the microspheres may still be releasing after a month but the experiment was stopped after 28 days.

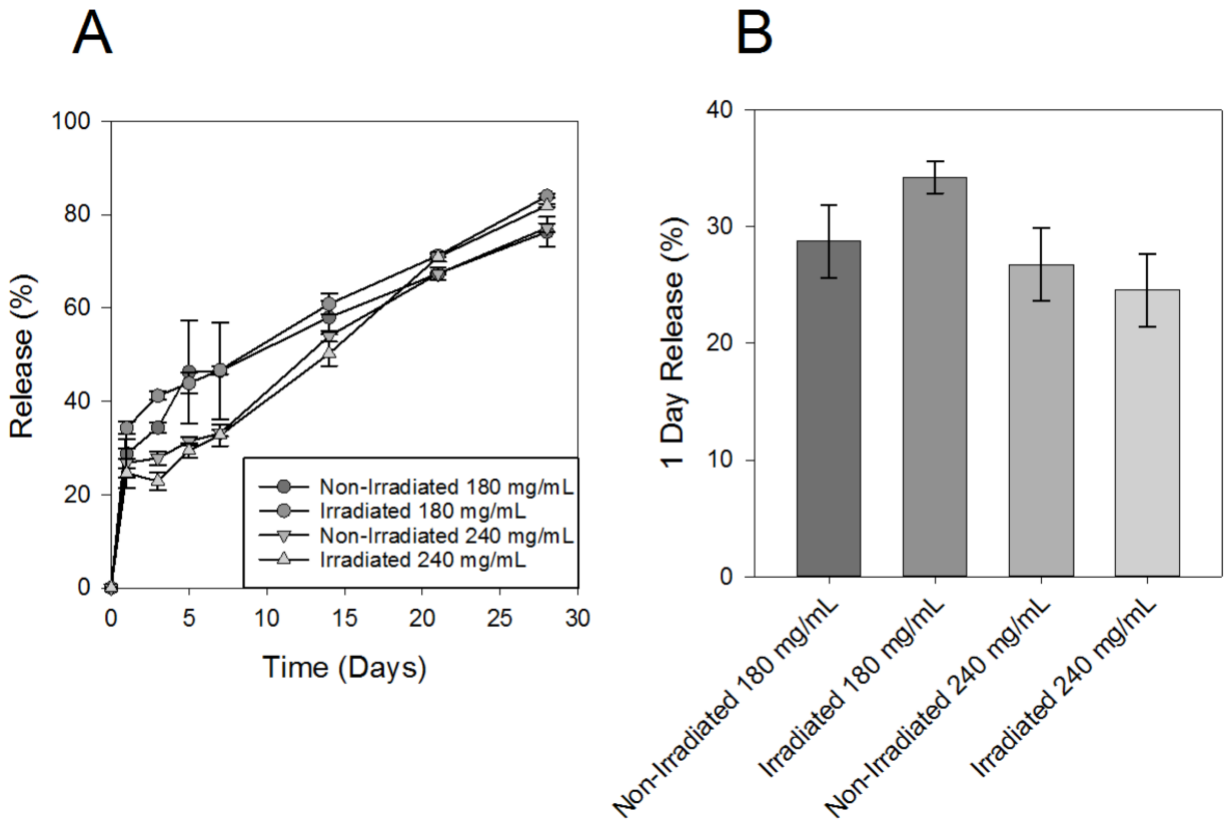

Figure 4.6 In vitro release of leuprolide from irradiated and non-irradiated microspheres at 180 mg/mL, and 240 mg/mL concentration (A) and initial burst release (B). Mean ± SEM (n=2).

4.4.5 Chemical Castration Achieved over 3 Months with Monthly Dosing

The *in vivo* efficacy of non-irradiated (180 mg/mL and 240 mg/mL) and irradiated (240 mg/mL) microspheres were tested in rats over 3 months receiving monthly dosing of different leuprolide LAR formulations. *In vivo* leuprolide release is evident with an

initial increase in plasma testosterone levels from baseline and one day post injection followed by a decrease in testosterone levels as the gonadotropin releasing hormone receptor is downregulated resulting in a decrease in plasma testosterone levels [15, 16]. In all formulations castration is achieved by day 7 with the irradiated and non-irradiated 240 mg/mL microspheres achieving castration by day 3 (Figure 4.7). All formulations achieved and maintained castration over the first month and continued to maintain castration levels after the 2nd and 3rd injections of the microspheres on days 28 and 56.

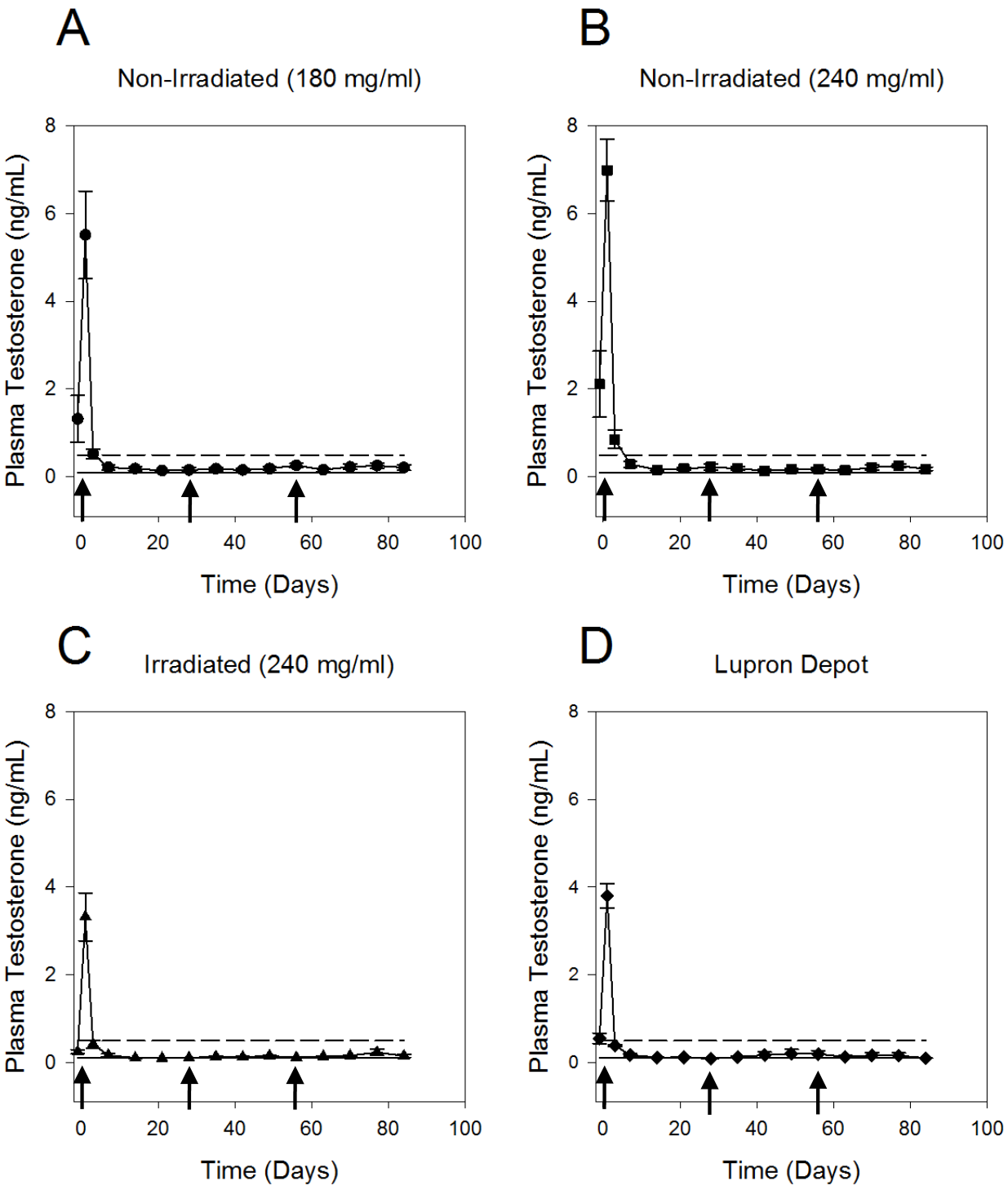

Figure 4.7 In vivo plasma testosterone levels of male Sprague-Dawley rats administered 3 monthly doses of leuprolide loaded microspheres; non-irradiated 180 mg/mL (A), non-irradiated 240 mg/mL (B), irradiated 240 mg/mL (C), and Lupron Depot® (D). Dashed and solid lines indicate castration level (0.5 ng/mL) and the analysis limit of detection (0.1 ng/mL). Mean ± SEM (n=6). Arrow show days of dosing.

4.5 Discussion

Aqueous remote loading of PLGA has been shown to be a viable method to achieve elevated loading and encapsulation of leuprolide [14]. Remote loading decreases the steps needed to encapsulate leuprolide from ~14 steps to ~9 steps and from ~14 aseptic steps to ~2 aseptic steps. Here we have applied this technique to PLGA with a similar lactic acid content and molecular weight as LD. In using 75/25 PLGA leuprolide should be released more slowly *in vivo*, compared to 50/50 PLGA which was tested previously, and ultimately achieved 1 month release *in vivo* [17]. Low molecular weight 75/25 PLGA microspheres loaded at 180 mg/mL microsphere concentration achieved 8.4 ± 0.5 % w/w loading of leuprolide which is comparable to the monthly, commercial, LD formulation which has a loading of 10 % w/w. This indicates that remote loading can achieve loading similar to the commercial product. When comparing *in vitro* release for these microspheres to LD we see similar near zero order release kinetics after the first week and a comparably low initial burst release particularly for microspheres loaded at 240 mg/mL (Figure S 4.1)

Ensuring sterility is a key criterion for a commercialized injectable product. There are several acceptable methods for sterilization of pharmaceutical products according to The International Pharmacopoeia, including, saturated steam, filtration, gas, and ionizing radiation such as gamma irradiation [18]. Gamma irradiation is ideal for sterilizing PLGA microspheres because it reduces the potential for PLGA hydrolysis, as is the case with steam sterilization, and avoids the production of toxic residues and altering the polymer properties, which can occur via ethylene oxide sterilization [19-21]. To determine if gamma irradiation exposure had an effect on the potential to remotely

load leuprolide, we tested two properties which were intrinsic to the polymer and could impact product quality: molecular weight and glass transition temperature. Comparing these properties in microspheres that were and were not exposed to irradiation showed no significant changes, indicating that gamma irradiation can be used for terminal sterilization of microspheres without changing their physical attributes. Radiating before leuprolide encapsulation ensures that the peptide is not damaged during this sterilization processes; the two materials can be mixed sterilely subsequently to achieve loading. The microspheres also retained their surface morphology features suggesting that the gamma irradiation dose and dose rate did not cause physical damage. Microspheres that were not exposed to irradiation had a marginally higher encapsulation efficiency than irradiated microspheres but all had over 90 % encapsulation efficiency, indicating very little leuprolide from the loading solution was wasted. The slight different in encapsulation efficiency can be due to the fact that gamma ray exposure causes a decrease in polymer molecular weight and molecular number, which can decrease the number of available acid terminations in the microspheres available for leuprolide absorption [14, 20].

Microspheres that were exposed to gamma irradiation were further compared to non-irradiated microspheres' ability to release leuprolide *in vitro*. Similar release rates for irradiated and non-irradiated microspheres further supports that exposure to gamma irradiation at a dose of 1.8 Mrad did not affect the ability to release leuprolide. This is in agreement with previous indications suggesting no obvious physical changes were observed between irradiated and non-irradiated microspheres. In the case of the 180 mg/mL microspheres the irradiated microspheres have a slightly faster release rate

which can be explained by small differences in molecular weight of microspheres exposed to irradiation. It might be possible to mitigate this decrease in molecular weight due to gamma irradiation by using a lower dose of irradiation over a longer period of time to achieve desired sterility [21, 22]. However, as the current standard for gamma sterilization of PLGA is a dose of 2.5 Mrad we can expect that exposure at this level would result in even faster degradation rate of the polymer and subsequent drug release [20, 21]. The similar release kinetics seen over one month for irradiated microspheres compared to microspheres not exposed to irradiation indicates that we would expect similar release kinetics during *in vivo* studies.

In vivo studies were performed over 3 months in rats receiving monthly subcutaneous doses of 100 µg/kg/day, which has been shown to cause a decrease in prostate weight, seminal vesicle growth and testis growth of male Sprague-Dawley rats 2-weeks post microsphere injection [14, 23, 24], therefore, we would expect to see an effect on castration in rats dosed at this level. Irradiated or non-irradiated microspheres were able to achieve and maintain chemical castration, comparable to that of 1-month Lupron Depot® in terms of time to reach chemical castration and ability to maintain castration. Rats administered irradiated microspheres achieved castration faster than non-irradiated microspheres of the same concentration indicating that leuprolide release from irradiated microspheres was slightly faster than in non-irradiated microspheres. This could be due to the slight decrease in molecular weight after gamma irradiation and the fact that release rate of leuprolide has been shown to be faster *in vivo* than *in vitro* [25].

4.6 Conclusion

Low molecular weight 75/25 PLGA microspheres were successfully encapsulated with leuprolide using an aqueous remote loading process. This aqueous remote loading process was optimized for performance consistent with commercial monthly leuprolide formulations [6], determined by comparable loading and encapsulation efficiencies, low initial burst and long-term release *in vitro*, and *in vivo* efficacy [10, 26]. Effectiveness of gamma irradiation for producing sterile microspheres was also explored; microspheres exposed to gamma irradiation had similar molecular weight, glass transition temperature and surface morphology to microspheres that were not exposed. Irradiated microspheres also achieved comparable loading, encapsulation efficiency, and *in vitro* release rate as non-irradiated microspheres. The minimal effect of irradiation on microspheres indicates that microspheres can be terminally sterilized prior to aqueous remote loading of leuprolide which can help lower the cost of manufacturing. This entire process-microsphere formation, sterilization and leuprolide loading was combined to formulate a “generic” LD. Lastly, these formulations were administered to rats and demonstrated comparable efficacy through their ability to achieve and maintain chemical castration on the same time scale as commercial 1-month Lupron Depot®. These results demonstrate the potential for this aqueous remote loading paradigm for future development of commercialized peptide LAR formulations.

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4.8 Supplemental

In vitro leuprolide release from 75/25 PLGA microspheres, 180 mg/mL and 240 mg/mL, was compared to *in vitro* release of Lupron Depot® (Figure S 4.1) [7]. Leuprolide

microspheres had similar low initial burst release, especially for 240 mg/mL microspheres, and showed near zero order release kinetics after 1 week similar to that seen for the commercial product.

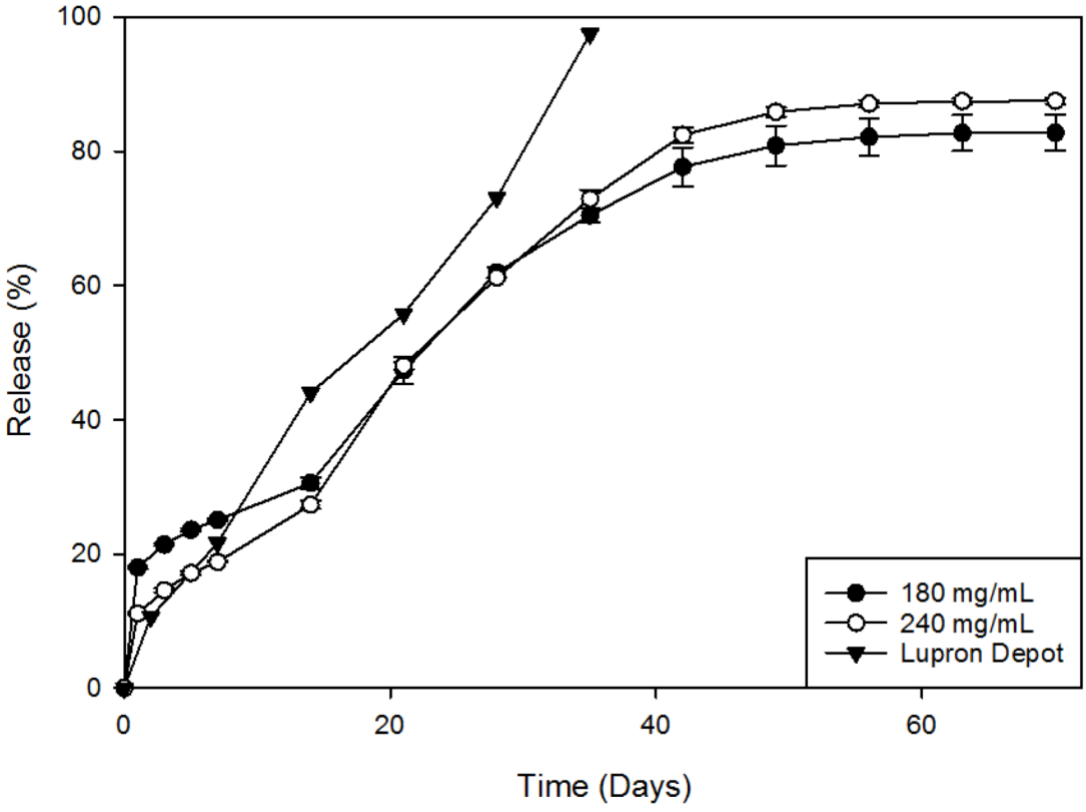

Figure S 4.1 In vitro release of 75/25 PLGA microspheres loaded by aqueous remote loading at 180 mg/mL and 240 mg/mL compared to Lupron Depot Release in vitro. Lupron Depot release adapted from [7].

Chapter 5: Generality of Aqueous Remote Loading

5.1 Abstract

Leuprolide has been shown to absorb to acid terminated PLGA microspheres by simply incubating drug-free microspheres in an aqueous peptide solution for 24 h above the polymer hydrated glass transition temperature. This aqueous remote loading technique has been optimized both theoretically and experimentally to achieve elevated loading and encapsulation efficiency of leuprolide consistent with the commercial product, Lupron Depot®. Using loading conditions optimized for leuprolide, the aqueous remote loading technique was applied to 5 different peptides of varying molecular weights and charges. The 5 peptides: exenatide, octreotide, protirelin (thyrotropin releasing hormone), salmon calcitonin, and vasopressin were loaded at 180 mg/mL microsphere concentration in low molecular weight acid terminated 75/25 PLGA and 20 mg/mL peptide concentration. Octreotide, vasopressin, and salmon calcitonin achieved high encapsulation efficiencies (> 65 %) and loading (> 6.5 %), comparable to values achieved for leuprolide. When applied to exenatide and protirelin, reduced levels of peptide encapsulation and loading were observed (< 30 % encapsulation efficiency and <3 % w/w load) for both peptides. Low loading of exenatide may be attributed to the low isoelectric point (pI = 4.9) compared to loading pH and the presence of amino acids with negative side chains. Protirelin has a partial positive charge due to histidine (pKa ~ 6),

which may affect the ability of protirelin to absorb to the microspheres and has lower molecular mass, creating higher equivalents in solution for salt formation with the polymer on a weight basis. *In vitro* release of octreotide over 2 months was monitored and compared to the commercial product Sandostatin® LAR. Formulations had a low initial burst release of octreotide (< 10 %) and exhibited near zero-order continuous release over the two months. *In vitro* release of exenatide from microspheres was analyzed over 1 month by percent remaining in microspheres. These microspheres displayed a low initial burst (< 10 % on day 1) and showed continuous release of exenatide. This work builds upon previously optimized parameters and supports the generality of aqueous remote loading of peptides in low molecular weight PLGA microspheres.

5.2 Introduction

It has been shown that model cationic peptides, leuprolide and octreotide, can interact with ground 50/50 acid terminated PLGA via peptide absorption [1]. This aqueous remote loading technique allows for peptide encapsulation without exposure to potential degradants (i.e. sheer stress, organic solvent exposure, temperature fluctuations) during microsphere formulation [2]. In addition to improving peptide stability, remote loading can help to lower the cost of manufacturing as microspheres can be prepared in a non-sterile environment prior to sterilization and incubated with sterile peptide, and microspheres can be tested on the small scale first to reduce the risk of batch failure during scale-up and reduce loss of expensive drug [2, 3]. Therefore,

remote loading could prove to be a viable alternative to traditional techniques for peptide encapsulation in PLGA.

In order to demonstrate the practical potential for remote loading it is important to test this loading strategy in several peptides. Two currently marketed products using PLGA are Sandostatin LAR® and Bydureon™. Sandostatin® LAR is a monthly intragluteal injection of the somatostatin analogue, octreotide, for treatment of acromegaly. Octreotide is encapsulated in glucose star polymer PLGA at 4.6 % octreotide w/w [4]. Bydureon™ is a weekly subcutaneous injection of the glucagon-like peptide-1 (GLP-1), exenatide, for glycemic control in type 2 diabetes mellitus. Bydureon encapsulates exenatide in 50/50 PLGA by a coacervation technique at 5 % w/w [5, 6]. However, the coacervation method in particular is complex with many different organic solvents and large scale manufacture and high product yields have been important issues [7, 8].

Vasopressin is a peptide hormone that causes vasoconstriction and is available clinically as Vasostrict® to increase blood pressure in patients with vasodilatory shock [9]. There have been a few *in vivo* studies on vasopressin encapsulation in polypropylene controlled release devices and on vasopressin release in Brattleboro rats [10, 11], however, encapsulation in PLGA has not been extensively studied as it is not administered for chronic therapy. The peptide hormone salmon calcitonin is used for the treatment of osteoporosis as a once daily nasal spray or as a sterile solution for intramuscular or subcutaneous administration [12]. Salmon calcitonin is ideal for management of osteoporosis due to its high potency and is less likely to aggregate compared to calcitonin [13]. However, when administered intranasally the peptide has a

lower therapeutic effect compared to dosing by parenteral administration [13]. *In vivo* salmon calcitonin has a short half-life ($t^{1/2}$ ~ 15 - 20 minutes) and is susceptible to degradation, and therefore, encapsulation in PLGA is a viable alternative that has been explored in acid terminated 50/50 PLGA microspheres using the solvent evaporation method [14]. Protirelin is a tripeptide hormone that stimulates thyrotropin and prolactin release from the pituitary. Due to its many roles in the central nervous system, protirelin has been commercially developed in Japan as a CNS-stimulating drug [15]. There are several studies on protirelin encapsulation in 75/25 PLGA, using a double emulsion solvent evaporation technique, and release *in vitro* and *in vivo* [15-17]. However, by encapsulating protirelin by double emulsion solvent evaporation the peptide is exposed to potential degradants, as mentioned earlier.

While several of these peptides have been studied for encapsulation in PLGA, with some being commercially available, drug stability and cost of goods hurdles remain that limit the number of microsphere products to become US FDA approved. Our long-term goal is to simplify drug encapsulation by aqueous remote loading to overcome these critical obstacles. This chapter further advances this goal by studying the general applicability of the remote loading encapsulation method via peptide absorption by testing the optimized PLGA microsphere formulation and loading conditions developed in the previous chapters. The drug loading, encapsulation efficiency, and drug release kinetics in certain cases are examined and rationalized based on the structure and properties of the peptides.

5.3 Materials and Methods

5.3.1 Materials

Octreotide was purchased from SHNJH Pharmaceuticals (Shanghai, China). Exenatide was purchased from Amneal Pharmaceutics (India). Salmon calcitonin, vasopressin, and protirelin were purchased from Bachem (Bubendorf, Switzerland). Low molecular weight 75/25 poly(lactic-co-glycolic acid) was purchased from Wako Chemicals (Osaka, Japan). Poly vinyl alcohol, and Tween 80 were purchased from Sigma Aldrich Chemical Co (St. Louis, MO). Hydroxyethyl-piperazineethanesulfonic acid (HEPES) was purchased from Thermo Fisher Scientific (Waltham, MA). All other materials were of analytical grade and purchased from commercial suppliers.

5.3.2 Microsphere Preparation

PLGA microspheres of 75/25 were prepared using a water-in-oil-in-water (w/o/w) double-emulsion solvent evaporation method. PLGA was dissolved in methylene chloride at a concentration of 1000 mg/mL. Once dissolved, 100 µL of the inner water phase containing trehalose (500 mg/mL) was added to the dissolved polymer solution and homogenized for 1 min at 10,000 rpm to form a water-in-oil (w/o) emulsion. Next, 4 mL of 5 % PVA was added and vortexed for 1 min at high speed to form the w/o/w double emulsion. This emulsion was then transferred to a stirring bath of 0.5 % PVA (100 mL) and stirred for 3 h, allowing for methylene chloride evaporation and microsphere hardening. Hardened microspheres were sieved (20 - 63 µm) and washed with 1 L ddH_2O. Porous drug-free microspheres were then lyophilized and stored at -20 °C until use.

5.3.3 Peptide Loading Solution

For all peptides, solutions of 20 mg/mL were used for loading. Briefly, 40 mg of peptide and 47.6 mg of HEPES were dissolved in 1 mL of ddH_2O and vortexed. With constant stirring the solutions for octreotide, exenatide, vasopressin, and salmon calcitonin were titrated to pH 7.4 with 1 N NaOH. For protirelin and exenatide loading solutions the pH was also titrated to pH 6 and 6.7 using 1 N NaOH or HCl. After titration solutions were transferred to a 2-mL volumetric flask and the remaining volume needed of ddH_2O added to reach 2 mL total volume.

5.3.4 Peptide Loading in 75/25 PLGA Microspheres

Pre-formed drug-free microspheres of low molecular weight 75/25 PLGA were loaded with each peptide by incubation. Briefly, 90 mg of microspheres were weighed out and 0.5 mL of peptide loading solution added. Microspheres were fully dispersed in the peptide solution then incubated for 24 h at 37 °C with rotation. After incubation, loaded microspheres were centrifuged for 10 min at 8,000 rpm and the remaining loading solution was removed. Microspheres were then washed 3 times with 1 mL ddH_2O centrifuging for 10 min at 8,000 rpm between each wash.

5.3.5 Analysis of Peptide Absorption in PLGA Microspheres

Peptide absorption in microspheres was determined by ultra-performance liquid chromatography (UPLC), analysis of mass loss from solution during loading, and total nitrogen analysis. For octreotide loaded microspheres, peptide absorption was also determined by two-phase extraction.

To determine peptide absorption by mass loss the concentration of peptide remaining in solution after 24 h incubation and in the three washes were analyzed by

UPLC. For octreotide samples a gradient of 25 % Acetonitrile (ACN) + 0.1 % trifluoroacetic acid (TFA):75 % ddH_2O + 0.1 % TFA to 35 % ACN + TFA:65 % ddH_2O + TFA over 4 minutes with a flow rate of 0.4 mL/min was run. For vasopressin samples, an isocratic method of 20 % ACN+TFA:80 % ddH_2O at 0.4 mL/min was run over 2 minutes. Salmon calcitonin samples were also run using an isocratic method of 37 % ACN + TFA:63 % ddH_2O at 0.5 mL/min over 1.8 minutes. Protirelin was run with a gradient of 5 % ACN: 95 % ddH_2O to 6.5 % ACN: 93.5 % ddH_2O over 2 minutes at 0.4mL/minute. Exenatide was run using a gradient method of 25 % ACN: 75 % ddH_2O to 90 % ACN:10 % ddH_2O over 4 minutes at a flow rate of 0.5 mL/min.

Peptide absorption was also determined by total nitrogen analysis of loaded microspheres. In triplicate, ~2 mg of microspheres were weighed into tin capsules for analysis. Calibration standards of EDTA (0.5 mg - 3 mg) were weighed and run prior to microspheres and peptides. A protein factor for each peptide was determined by running ~0.5 mg of peptide only and determining the protein factor to produce 100% protein. The determined protein factor for each peptide was used to convert the percent nitrogen determined for each corresponding sample of loaded microspheres run. As only the peptide contributes to the nitrogen content in the microspheres the percent nitrogen can be used to determine the amount of peptide absorbed.

5.3.6 Analysis of Peptide Release from Microspheres

Octreotide release from microspheres was measured in phosphate buffered saline + 0.02 % Tween 80 (PBST) at pH 7.4. In triplicate, 10 mg of loaded microspheres were incubated in 1 mL of PBST at 37 °C with mild agitation. At specific time points samples were centrifuged 5 minutes at 8,000 rpm and the supernatant removed and

replaced with fresh PBST. Release supernatants were analyzed by UPLC using the methods described in Section 5.3.5 to determine loading by mass loss.

Exenatide release was carried out in HEPES buffered saline (HBS) (pH 7.4), to ensure peptide stability, and determined by calculating percent peptide remaining in microspheres at each time point. Approximately 10 mg of microspheres were weighed out in duplicate for each time point (days 1, 3, 5, 7, 14, 21, 28) and incubated in 1 mL of HBS at 37 °C with mild agitation. At each time point all samples were centrifuged for 5 minutes at 8,000 rpm and the release supernatant removed. Samples corresponding to the day were dried at room temperature for 48 h and stored at -20 °C until analysis by total nitrogen analysis. Release media for all future time points was replaced with fresh HBS and incubated again until the next time point.

5.4 Results & Discussion

5.4.1 Comparison of Peptide Properties to Leuprolide

Using the previously optimized parameters for remote loading with leuprolide, remote loading was applied to 5 peptides (octreotide, vasopressin, salmon calcitonin, exenatide and protirelin) to determine the generality of remote loading. Before loading the peptides in 75/25 PLGA-COOH microspheres at a concentration of 180 mg/mL microspheres the size, isoelectric point, presence of negative side chains and primary amines were compared with leuprolide (

Table 5.1) in order to help better understand our loading results.

Table 5.1 Comparison of Peptide properties. Size cutoffs are, small = MW < 600 Da, medium = 600 < MW < 1500 Da, large = 1500 < MW < 5808 (Insulin) Da.

Peptide	Molecular Weight (Da)	Size	Isoelectric Point	Negative Side Chains	Primary Amine	Classification

Leuprolide	1209.4	Medium	8.0	No	Yes	Cationic
Octreotide	1019.2	Medium	9.1	No	Yes	Cationic
Vasopressin	1084.2	Medium	9.1	No	Yes	Cationic
Samlnon Calcitonin	3431.9	Large	9.7	Yes	Yes	Basic
Exenatide	4186.6	Large	4.9	Yes	Yes	Acidic
Protirelin	362.4	Small	10.5	No	No	Basic

5.4.2 Continuous Slow Release of Octreotide from 75/25 Microspheres In Vitro

Based on previous work with octreotide [1], it is known that octreotide interacts with acid terminated 50/50 PLGA via absorption. Here we looked at the ability to aqueously remote load low molecular weight 75/25 PLGA microspheres and continuously release octreotide *in vitro*. PLGA microspheres absorbed 6.8 ± 0.12 % octreotide with an encapsulation efficiency of 67.9 ± 1.2 %. The lower loading and encapsulation, compared to leuprolide, may be due to the lower binding affinity of octreotide to PLGA compared to leuprolide [1] or the lower molecular weight of octreotide which would result in lower loading compared to leuprolide at constant mole absorption. Another potential explanation is the difference in charged amino acids in the two peptides and how they interact with the PLGA-COOH. Octreotide has two reactive amines (N-terminal amine and lysine side chain) while arginine and histidine in leuprolide are non-reactive. Microspheres exhibited a low initial burst release of 5.5 ± 0.02 % in the first day. This low release is comparable to initial burst release seen from the commercial Sandostatin® LAR during *in vitro* tests in phosphate buffered saline + triethyl citrate (PBST, pH 7.4). Octreotide slowly and continuously released from

microspheres over 2 months, exhibiting near zero-order release kinetics after day 1 (Figure 5.1). This release profile is slower than that of leuprolide loaded 75/25 microspheres and differs from the commercial product which had a burst in octreotide release after day 21 (Figure 5.1). This difference in release profile may be due to the different polymer as Sandostatin® LAR uses a glucose star polymer (50/50 PLGA) with no carboxylic acid end-groups in the formulation compared to the 75/25 acid-capped PLGA used for octreotide remote loading. Also, the different release buffers used can affect release, as the presence of the plasticizer triethyl citrate in release buffer can increase polymer chain mobility as it is taken up by the microspheres into the polymer phase, accelerating hydrolysis [18]. This accelerated hydrolysis can result in faster polymer erosion and diffusion within the polymer microsphere, [18] thus accelerating octreotide release rate.

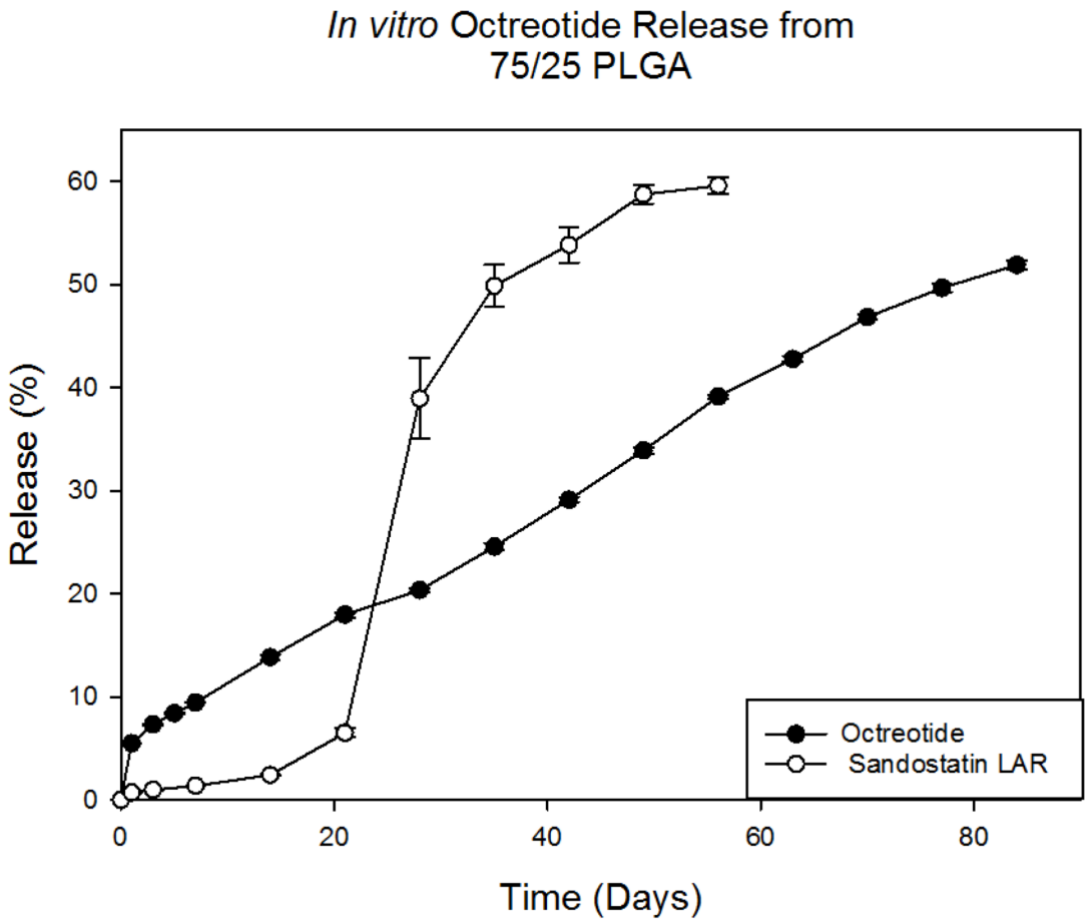

Figure 5.1 Octreotide release from 75/25 PLGA microspheres in vitro compared to the commercial product Sandostatin® LAR. Mean ± SEM (n=3).

5.4.3 Remote Loading of Vasopressin & Salmon Calcitonin

Vasopressin and salmon calcitonin were encapsulated in acid terminated 75/25 PLGA microspheres via remote loading of 180 mg/mL microsphere concentration. Microspheres incubated in 20 mg/mL vasopressin loaded 8.5 ± .2 % w/w vasopressin with 85 ± 1.5 % encapsulation efficiency. Vasopressin, a 10-amino acid peptide of molecular weight 1084.2 g/mol, is a cationic peptide with an isoelectric point (pI) of 9.1. Vasopressin can ionically interact with the acid terminated PLGA microspheres, similar to leuprolide and octreotide, through the primary amine of arginine and N-terminal

hydrogen allowing for peptide absorption. Vasopresin has a molecular weight similar to octreotide and the same pI but had peptide loading and encapsulation more similar to that seen with leuprolide (Figure 5.2), a more positive peptide with a slightly higher molecular weight. Comparing the positively charged amino acids for these three peptides, vasopressin has an N-terminal amino and an arginine, octreotide an N-terminal amine and lysine, and leuprolide an arginine and histidine. Looking at the isoelectic points of these three amino acids' side chains, arginine has the highest pI and therefore would lead to a stronger positive charge compared to histidine or lysine. A potential explanation for vasopressin loading compared to octreotide and leuprolide may be differences in peptide binding affinity to PLGA-COOH. The difference in ionizable moieties compared to leuprolide and octreotide may also affect peptide absorption as vasopressin contains one reactive amine and arginine, while octreotide has two reactive amines, and leuprolide contains arginine and histidine, both of which are non-reactive.

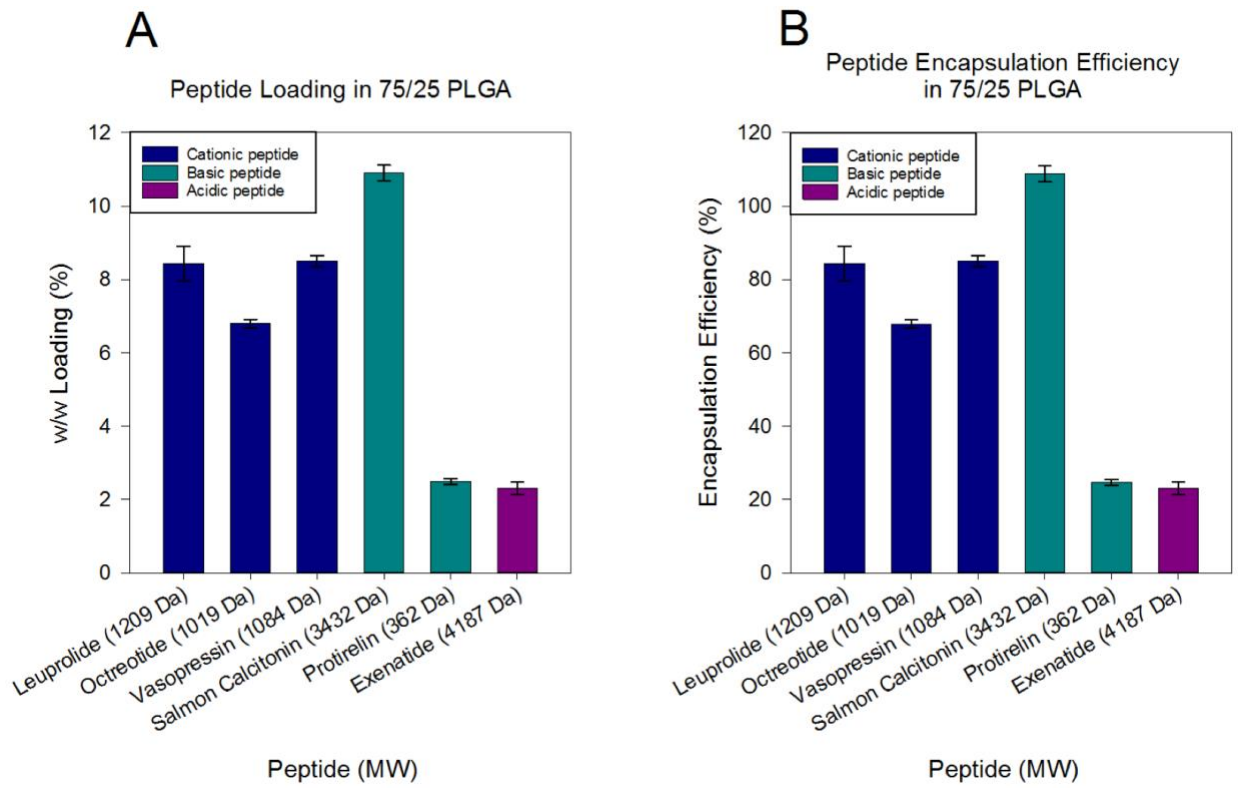

Figure 5.2 Comparison of peptide loading and encapsulation efficiency at 180 mg/mL microsphere concentration to leuprolide

Microspheres incubated with salmon calcitonin loaded 10.9 ± 0.2 % w/w peptide with an encapsulation efficiency of 108.8 ± 2.2 %, as determined by nitrogen analysis (Table 5.2). Salmon calcitonin is a 32-amino acid linear peptide of molecular weight 3431.9 g/mol, with a pI = 9.7. Salmon calcitonin can be classified as a large, basic peptide with both positive (arginine, histidine, lysine, N-terminal hydrogen) and negative (glutamic acid) charged amino acid side chains. As a result of its positive charge salmon calcitonin was able to absorb to the PLGA microspheres with high efficiency. The greater than 100 % encapsulation efficiency as determined by nitrogen analysis may be due to the zwittterionic HEPES from the loading solution which can also bind to PLGA-COOH. HEPES that bound to the microspheres can contribute some nitrogen content during this analysis (data not shown), thus overestimating the nitrogen content. Mass

loss analysis of salmon calcitonin lost from loading solution and washes after loading still shows high encapsulation efficiency (98.8 ± 0.14 %) and loading (9.9 ± 0.01 %) and does not overestimate loading as when analyzed by nitrogen content (Table 5.2). As there are no commercial PLGA products for vasopressin and salmon calcitonin *in vitro* release was not studied for a direct comparison and such studies are a subject of future work.

Table 5.2 Comparison of salmon calcitonin loading and encapsulation efficiency by total nitrogen analysis and mass loss.

Analysis Method	w/w Loading (%)	Encapsulation Efficiency (%)
Total Nitrogen Analysis	10.9 ± 0.22	108 ± 2.2
Mass Loss	9.9 ± 0.01	98.8 ± 0.14

5.4.4 Remote Loading of Exenatide

The large acidic peptide, exenatide demonstrated low absorption to PLGA microspheres as only 2.3 ± 0.2 % loaded with 23.1 ± 1.7 % encapsulation efficiency in 180 mg/mL of 75/25 PLGA microspheres. Compared to both leuprolide and octreotide exenatide is ~4 times as large (MW= 4186.6 g/mol) which may have an effect on the potential for peptide absorption, or how quickly the peptide absorbs to the microspheres. Exenatide also has a low isoelectric point of pI = 4.9 and negative side chains as it has both negative (aspartic acid and glutamic acid) and positive (arginine, histidine, lysine, N-terminal hydrogen) charges. At pH 7.4 the peptide has a net negative charge (pH > pI) which may result in repulsive forces between the peptide and polymer decreasing peptide absorption.

To test if exenatide had not fully absorbed to PLGA microspheres in the 24 h incubation, microspheres were incubated with exenatide for 48h hours. However, the

longer incubation time did not improve loading or encapsulation efficiency as slight decreases in these dependent parameters were observed (Table 5.3).

Table 5.3 Exenatide loading and encapsulation in 75/25 PLGA microspheres incubated for 24 h and 48 h.

Loading condition	w/w Loading (%)	Encapsulation Efficiency (%)
24 h, pH 7.4	2.3 ± 0.2	23.1 ± 1.7
48 h, pH 7.4	1.56 ± 0.13	15.6 ± 1.3
24 h, pH 6	0.29 ± 0.04	2.87 ± 0.42
24 h, pH 6.7	0.21 ± 0.02	2.13 ± 0.19

This trend was observed previously for 50/50 PLGA microspheres loaded with leuprolide for 48h compared to 24h incubation, and similar mechanisms may be at play here. In attempts to improve loading and encapsulation, the pH of the loading solution was altered in order to decrease the repulsive negative charge on the peptide. However, decreasing the pH to 6 and 6.7 did not appear to have a positive effect on peptide interaction with the PLGA (Table 5.3) as loading decreased instead of increased.

Despite the low loading and encapsulation efficiency of exenatide, continuous release was seen *in vitro* (Figure 5.3), for microspheres loaded for 24 h, with a low initial burst release of <10 % in 1 day. Microspheres also appear to still be releasing although release was only monitored for 28 days.

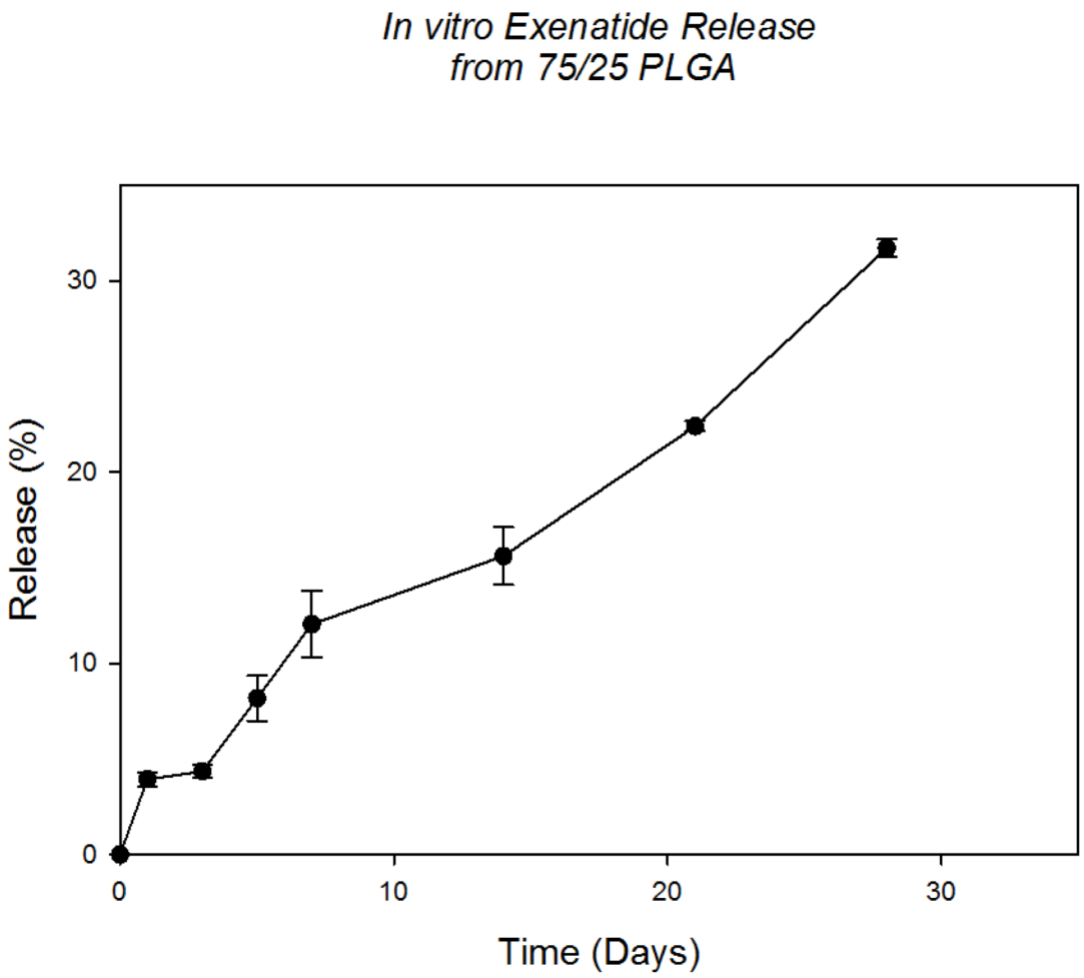

Figure 5.3 In vitro release of exenatide from 75/25 PLGA microspheres

5.4.5 Remote Loading of Protirelin

Low molecular weight PLGA microspheres incubated for 24 h with protirelin (thyrotropin-releasing hormone) at a peptide concentration of 20 mg/mL showed low encapsulation efficiency (24.7 ± 0.8 %) and loading (2.47 ± 0.1 %). Protirelin is a small, 3 amino acid, basic peptide of molecular weight 362.4 g/mol with an isoelectric point of 10.5. Due to is low molecular weight compared to both leuprolide and exenatide it would be expected that protirelin would more easily partition into the microsphere. Protirelin contains the amino acid histidine which has a side chain pKa of ~6 and is positively

charged at low pH. Since the loading solution pH (7.4) is close to the pKa of the histidine side chain not all of the histidine will be positively charged. As a result, this may result in less peptide absorption to PLGA-COOH. Loading from a lower pH (6.7 and 6), however, like with exenatide did not improve peptide absorption (Table 5.4) for unknown reasons. Loading at a pH lower than the pI could improve peptide encapsulation however, at more acidic pH the PLGA microsphere microclimate becomes more acidic which can result in faster PLGA degradation and thus faster drug release [19]. As seen with exenatide, incubation for 48 h did not improve encapsulation efficiency or loading of protirelin, indicating that incubation time over 24 h does not affect peptide absorption for protirelin.

Table 5.4 Loading and encapsulation efficiency of protirelin in 75/25 PLGA microspheres at pH 7.4 (24 h and 48h incubation), 6.7, 6 and 8.

Loading condition	w/w Loading (%)	Encapsulation Efficiency (%)
24 h, pH 7.4	2.47 ± 0.08	24.7 ± 0.8
48 h, pH 7.4	2.25 ± 0.07	22.5 ± 0.68
24 h, pH 6.7	0.32 ± 0.13	3.2 ± 1.3
24 h, pH 6.0	0.16 ± 0.01	1.6 ± 0.1

5.5 Conclusion

This work attempts to apply the aqueous remote loading technique to 5 different peptides of various molecular weights, size, and isoelectric points using parameters optimized in previous chapters with leuprolide. Peptides were incubated with low molecular weight 75/25 PLGA microspheres at 180 mg/mL. Cationic peptides, octreotide and vasopressin, and the large basic peptide salmon calcitonin were able to absorb to PLGA microspheres with over 60 % efficiency and over 6 % w/w loading. Peptides containing arginine (vasopressin and salmon calcitonin) were able to load with

high encapsulation efficiency most comparable to leuprolide. However, octreotide which only contains 2 reactive amino groups from the N-terminal amine and the lysine side chain did not show as high an encapsulation efficiency. The lower peptide sorption seen with octreotide compared to leuprolide is, however, consistent with previous studies using 50/50 PLGA [1].

In the case of the large acid peptide exenatide and small basic peptide protirelin we see much lower encapsulation efficiency and loading despite loading with parameters that were previously optimized. This lower potential for absorption may be due to the net negative charge of the peptide or not all of the histidine being positively charged at the loading pH, in the case of protirelin, and the negative charge of the acid terminated PLGA, preventing peptide absorption by ionic interaction. Protirelin also has a lower molecular mass resulting in lower loading on an equivalent mole absorption basis compared with leuprolide. It might be possible to improve encapsulation efficiency for these two peptides by loading from a lower pH and including a base in the microspheres to help mitigate acidic microclimate pH and PLGA degradation rate. From these initial studies of remote loading with exenatide and protirelin we see that there much more work to be done to achieve high loading and encapsulation consistent with cationic peptides and the large basic peptide and indicates some of the challenges in the development of PLGA products. We have shown that in initial studies, aqueous remote loading can be applied to octreotide, vasopressin and salmon calcitonin to achieve encapsulation efficiencies and loading in the same batch of low molecular weight PLGA-COOH microspheres. Indicating that there exists the potential to develop

multiple products from the same input of microspheres, which could help reduce manufacturing costs for such peptide controlled release products.

5.6 References

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Chapter 6: Conclusions, Significance, and Future Directions

The work presented in this thesis aims to further the understanding and applicability of the aqueous remote loading technique for cationic peptide encapsulation in PLGA microspheres. Previous work has shown that cationic peptide can interact with ground acid terminated PLGA (PLGA-COOH) via absorption, but these results suffered from low encapsulation efficiencies and high initial burst release *in vitro*. In order to validate the full potential and application of remote loading, these shortcomings were addressed by: 1) probing the mechanistic interaction of PLGA-COOH and cationic peptides by calorimetry, 2) theoretically predicting encapsulation and loading, 3) experimentally applying theoretical analysis to *in vitro* and *in vivo* studies compared to commercial formulations, and 4) applying optimized parameters to other cationic peptides.

From the derived model to theoretically predict encapsulation efficiency and loading, we see that encapsulation efficiency is dependent on the initial polymer concentration ($C_{p,i}$), the polymer water uptake (φ), the binding capacity (κ), and a binding constant (α). We also see that loading is dependent on the encapsulation efficiency and the ratio of initial peptide mass/polymer mass. Using this theory, we were able to optimize leuprolide absorption to PLGA microspheres in Chapter 3. However, there are a few limitations to this prediction as experimentally determined values for

binding strength and capacity were used in the model and the water uptake value was determined for 50/50 PLGA microspheres and was not determined for 75/25 PLGA prior to encapsulating leuprolide. Despite these limitations, when applying the theory to 75/25 PLGA microspheres, in Chapter 4, we were able to improve EE to > 80 %, compared to initial formulations, with over 1 month controlled release *in vitro*. *In vivo* experiments with leuprolide loaded 75/25 PLGA microspheres at 180 mg/mL, 240 mg/mL, and irradiated 240 mg/mL microsphere concentration showed comparable efficacy to 1 month Lupron Depot®. Rats administered 100 μg/kg/day of leuprolide achieved and maintained castration levels with monthly dosing. Thus, demonstrating that microspheres can be exposed to radiation prior to incubation with aqueous leuprolide to achieve loading and *in vivo* release comparable to the commercial product.

In initial studies to determine the potential for a broad application of aqueous remote loading, 5 peptides of differing charges were tested. We see that for cationic peptides (octreotide, salmon calcitonin, vasopressin) we are able to load low molecular weight 75/25 PLGA microspheres with high encapsulation efficiency and loading comparable to initial studies with leuprolide. However, for negatively charged peptides we see less than 30 % encapsulation efficiency.

Future studies will determine the corresponding leuprolide concentrations from *in vivo* samples (Chapter 4). It may also be of interest to test leuprolide loaded 75/25 microspheres *in vivo* in a prostate cancer rat model. The achievement and maintenance of castration can then be monitored as well as tumor size regression compared to Lupron Depot®. Additional studies will be done using the 5 peptides discussed in Chapter 5. For the cationic peptides (octreotide, salmon calcitonin, vasopressin) studies

to determine optimal *in vitro* release conditions will be determined and release monitored to determine if 1 month controlled release can be achieved. For exenatide and protirelin, studies will be done to improve loading and encapsulation by remote loading. This could be achieved by encapsulating in a lower pH loading solution, which may require a base to be added to the microsphere formulation in order to mitigate acidic microclimate pH of the microsphere, or by testing remote loading in ester terminated PLGA. Once optimized for loading and release *in vitro, in vivo* tests for release may be considered.

The potential for aqueous remote loading of peptides is significant in that it allows for encapsulation of peptides without exposure to potential degradants during microsphere development and allows for encapsulation on a small scale prior to scale-up. This is significant in that it can reduce large batch failure and loss of expensive peptide as microspheres can be tested for optimal specifications prior to the development of large batches and the addition of peptide. Lastly, remote loading can reduce the cost of manufacturing as microspheres do not have to be manufactured aseptically and can simply be incubated with sterile peptide solution after development. While this work has shown the potential of remote loading with leuprolide, if applicable to peptides of any size or charge, this encapsulation technique could revolutionize the development of new and generic PLGA LAR products.