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Research Article

Unstirred Water Layer Effects on Biodegradable Microspheres

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This study explores the mechanistic aspects of *in vitro* release from biodegradable microspheres with the objective of understanding the effect of the unstirred water layer on polymer degradation and drug release. *In vitro* drug release experiments on Leuprolide PLGA microspheres were performed under "static" and "continuous" agitation conditions using the "sample and separate" method. At specified time intervals, polymer degradation, mass loss, and drug release were assessed. While molecular weight and molecular number profiles for "static" and "continuous" samples were indistinct, mass loss occurred at a faster rate in "continuous" samples than under "static" conditions. *In vitro* results describe a fourfold difference in drug release rates between the "continuous" and "static" samples, ascribed to the acceleration of various processes governing release, including elimination of the boundary layer. The findings were confirmed by the fourfold increase in drug release rate when "static" samples were subjected to "continuous" agitation after 11 days. A schema was proposed to describe the complex *in vitro* release process from biodegradable polymer-drug dosage forms. These experiments highlight the manner in which the unstirred water layer influences drug release from biodegradable microspheres and stress the importance of selecting appropriate conditions for agitation during an *in vitro* release study.

1. Introduction

Interest in biodegradable polymeric drug delivery systems has grown significantly over the past few decades. Attributes like biocompatibility, biodegradability, and an extensive safety profile make these polymers the preferred choice for the development of sustained release injectable dosage forms that can deliver therapeutic levels of drug for weeks to months [1–4]. Attempts to characterize the various stages of biodegradation, whether *in vitro* or *in vivo*, have greatly contributed to an appreciation of the complex phenomena involved in the drug release process from dosage forms that utilize these polymers. However, there has been limited research investigating the effect of mechanical parameters on an *in vitro* study involving biodegradable polymeric drug delivery systems, and its impact on the overall release profile. On the other hand, such studies are routinely performed with conventional dosage forms like tablets. Indeed, it is well known that agitation, a mechanical parameter, increases release rate from tablets by eliminating boundary layer effects [5]. Consequently, dissolution, a kinetic parameter, is significantly influenced by altering agitation conditions. As such, studies to evaluate the influence of mechanical agitation during dissolution testing of oral dosage forms are relatively straightforward and can be performed using compendial methods and well established study conditions within a short period of time (less than 24 hours).

On the other hand, the drug release process for dosage forms like biodegradable polymeric microspheres is far more complex [6]. For instance, when introduced into a release medium, the presence of water molecules disrupts the hydrogen bonding between polymer chains of the microsphere, leading to a breakdown in bond rotation and polymer chain mobility. Since drug is entrapped inside the polymeric matrix, water incursion will not only facilitate polymer breakdown, but also ensure drug dissolution and open diffusional pathways for drug release through fluid filled pores in the polymer matrix. In this regard, polymer hydration is a key element to understanding the *in vitro* and possibly *in vivo* drug release processes. It follows that any parameter in an

in vitro release study that influences the number of water molecules colliding with the biodegradable polymer will impact polymer hydration, degradation, and, subsequently, drug release.

Unlike tablets and other conventional dosage forms, several methods, compendial and noncompendial, have been utilized to assess *in vitro* release from biodegradable microspheres. This is primarily due to the fact that no standardized method has been approved for such evaluations [7]. Of the various methods utilized to assess *in vitro* release, the "sample and separate" method is the most popular [8]. With the "sample and separate" method, drug loaded microspheres are introduced into a container containing release media at physiological temperature under a specified agitation regimen (i.e., continuous, intermittent, or no mechanical agitation) and drug release is monitored from the slowly degrading polymer. Due to the lack of a standardized method and the fact that drug release occurs over an extended interval, studies to assess the influence of agitation during *in vitro* release of therapeutic agents encapsulated in biodegradable microspheres are difficult to investigate, especially in the early stages of drug development.

In addition to the prolonged study times required, challenges with *in vitro* studies involving any mode of agitation are further compounded by the fact that mechanical agitation approaches are varied and will impact study results. For example, the following choices for continuous agitation of release media contents are cited in the literature: (a) the USP paddle apparatus, (b) magnetic stirrer at a fixed speed, (c) wrist-shaker rotating at 360°, (d) incubator-shaker, (e) shaking water batch, tumbling end-over-end, or (h) high speed stirring/revolution of bottles [8]. Given that the hydrodynamics of each agitated system is different, varying results are to be expected when slight modifications are introduced into an *in vitro* technique employed in a release study. For instance, a comparison of release profiles using different modes of continuous agitation and *in vitro* release methods from Indomethacin polylactide microspheres was assessed by Conti et al. [9]. Drug release from the USP dissolution test apparatus, rotating bottle apparatus, shaker incubator, and a recycling flow through cell was evaluated under continuous agitation. Drug release with the recycling flow through cell was rapid, with similar release profiles noted with the USP dissolution paddle apparatus, shaker incubator, and rotating bottle apparatus. However, the rate or extent of release depended on the mode of agitation employed in the study. Hence, proper selection of the *in vitro* release technique and mode of agitation is critical to an *in vitro* study.

Therefore, the goal of this study was to provide insight into the effects of mechanical agitation on the drug release profiles from a biodegradable polymer with the "sample and separate" set-up. The "sample and separate" method was selected as it is the most widely reported technique in the literature [8]. Microspheres of a model drug, Leuprolide, a water-soluble peptide, were prepared using a poly(lactide-co-glycolide) (PLGA), a biodegradable polymer. Leuprolide, a superagonist of luteinizing hormone releasing hormone (LHRH), was selected as the model peptide due to its solubility in various media and stability at the physiological temperature [10–12]. Experimental tests involving *in vitro* release, polymer degradation, mass loss, and polymer hydration were performed at 37°C at two agitation regimens: (a) static and (b) continuous, and eventually compared with a hybrid agitation scenario (c) "static-continuous." The results and discussions describe: (i) the kinetics involved as well as the effects of mechanical agitation on *in vitro* release profiles and (ii) propose a schema that describes the complex *in vitro* release process from biodegradable polymer-drug dosage forms. It is hoped that findings from this study will provide a guideline to aid in the design of a more robust *in vitro* release test for novel dosage forms like biodegradable microspheres.

2. Proposed Schema for *In Vitro* Release

A scheme outlining the mechanism of drug release from biodegradable microspheres is presented in Figure 1. While it is well known that drug release kinetics from biodegradable microspheres can be tailored by several factors including morphology, polymer molecular weight, drug solubility, copolymer ratio, and polymer hydrophilicity; the mechanism of drug release *in vitro* or *in vivo* is primarily governed by two factors: polymer hydration and subsequently, degradation [13–15].

Water plays a key role in the hydration and eventual degradation of the PLGA polymer. Indeed, water is a well known plasticizing agent and its role has been investigated and cited in several publications that discuss PLGA degradation [16–19]. As an example, at *in vitro* or *in vivo* conditions, water incursion is partly responsible for initial drug release (burst release) due to solubilization of easily accessible drug from either the porous network in the microsphere or its surface. After initial drug release, water penetrates the polymer matrix and causes polymer relaxation and swelling [20]. Subsequently, the water-swollen relaxed PLGA polymer begins to degrade via ester hydrolysis. Thus, constant diffusion of water into the interior of the microsphere leads to drug solubilization and its transport through the water-swollen polymeric matrix. The hydrolytic reaction leads to cleavage of the polymer backbone causing a decrease in molecular weight (bulk hydrolysis). This phenomenon increases the number of carboxylic end groups in PLGA, causing further acidification of the internal microenvironment. Further, the continual drop in molecular weight causes a loss in the mechanical strength of the polymer, but not its integrity. As autocatalytic degradation ensues, erosion of polymer integrity leads to mass loss and the generation of water-soluble fragments [21]. These collective mechanisms play a critical role in the drug release process from biodegradable microspheres.

Thus, the mechanism of polymer hydration and degradation leading to drug release can be categorized as a summation of the following steps.

Step 0: initial hydration of the biodegradable microsphere allowing dissolution of easily accessible drug (peptide), that is, drug associated with the surface of the microsphere and/or easily accessible pores, leading to "burst release" of drug. Since burst release is relatively instantaneous, the time

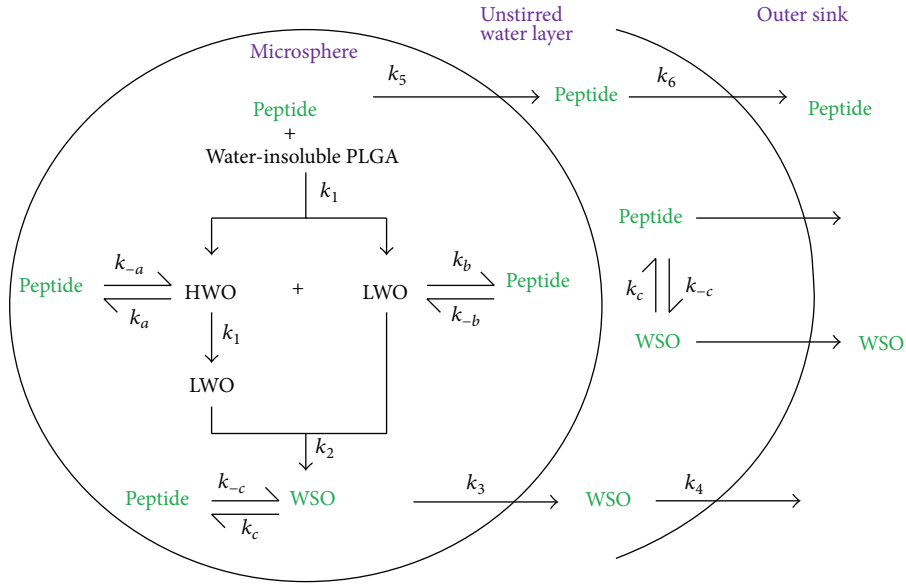


Figure 1: Degradation scheme for peptide-PLGA microspheres. Note: HWO = high molecular weight oligomers, LWO = low molecular weight oligomers are water-insoluble (black) and WSO = water soluble oligomers, peptide are water soluble (green).

required for the initial hydration phase is very short, that is, minutes to few hours. Significant polymer degradation is not expected to occur during this phase.

Step 1: hydration of the water-insoluble polymer leading to hydrolysis, that is, rate of formation of variable molecular weight water-insoluble oligomers (rate = k_1).

Step 2: breakdown of the high molecular weight water-insoluble oligomers (HWO) into low molecular weight water-insoluble oligomers (LWO) (rate = k_1).

Step 3: possible adsorption/desorption of the variable molecular weight oligomers with drug molecule (rates = k_a, k_{-a}, k_b, and k_{-b}).

Step 4: ester hydrolysis of the water-insoluble oligomers (HWO and LWO) to water-soluble oligomers (WSO) (rate = k_2).

Step 5: diffusion of water-soluble oligomers (WSO) to the surface (rate = k_3).

Step 6: movement of drug molecule through the polymer matrix to the surface (rate = k_4).

Step 7: possible adsorption/desorption of the WSO with drug molecule inside the polymer matrix or the unstirred water layer (rates = k_c, k_{-c}).

Step 8: passage of water-soluble oligomers (WSO) through the unstirred water layer (UWL) into the outer sink (rate = k_5).

Step 9: passage of water-soluble peptide through the unstirred water layer (UWL) into the outer sink (rate = k_6).

Steps 1–9 enumerate the various barriers that a drug encapsulated inside a biodegradable polymeric carrier needs to surmount for *in vitro* drug release to occur. From literature, despite several discussions on *in vitro* release from biodegradable microspheres, there has been no attempt to systematically categorize the various processes and steps involved, thereby aiding in a more fundamental understanding of a complex phenomenon.

Further, this schema (Figure 1) not only illustrates the diverse yet simultaneously occurring processes that occur due to the incursion of water inside a single peptide-loaded polymeric microsphere, but also details the various moieties that are directly or indirectly responsible for drug release from the said microsphere. Additionally, the schema provides a guideline for correlating the impact of minor changes to an *in vitro* technique and with drug release behavior. Any parameter that increases or hinders the rate of a single step (e.g., k_1, k_2, k_a, k_{-a}, etc.) will alter drug release kinetics. Proper identification of the parameter involved, its implications, and a mitigation strategy will enable the development of a robust *in vitro* release technique for these complex dosage forms. Additionally, it will provide more insight into the understanding of polymer degradation kinetics and mechanisms, both *in vitro* and *in vivo*. Further, it may also shed light on possible interactions that lead to incomplete drug release from these dosage forms.

3. Materials and Methods

3.1. Materials. Leuprolide Acetate was purchased from Bachem Inc., Torrance, CA. PLGA polymer (RG503H, Mw 30 kDa) was obtained from Boehringer Ingelheim Inc., Germany. All other chemicals used were of analytical reagent grade.

3.2. Preparation of Microspheres. PLGA microspheres were prepared by a dispersion method followed by solvent extraction/evaporation [22, 23]. Briefly, a solution of Leuprolide in methanol was added to a 22% (w/w) solution of polymer in methylene chloride to form a homogeneous dispersed phase. The dispersed phase was added to an aqueous solution containing 0.35% polyvinyl alcohol (continuous phase) under stirring with a Silverson L4R mixer (Silverson machines, MA, USA) at a predetermined speed. The organic solvents were removed by stirring for 2 hours. The resulting microspheres were recovered by filtration, washed to remove traces of polyvinyl alcohol and residual solvent, dried under vacuum at room temperature for 3 days to ensure low moisture and then stored in a dessicator.

3.3. Characterization

3.3.1. Particle Size. Particle size was measured using a laser diffractometer (Malvern 2600c Particle Sizer, Malvern, UK). The average particle size was expressed as the volume mean diameter "V_{md}" in microns.

3.3.2. Particle Morphology. Surface morphology was measured by scanning electron microscopy (SEM) using a Hitachi Model S800 (Japan) after Palladium/Gold coating of the microsphere sample on an aluminum stub.

3.3.3. Peptide Content. Approximately 10 mg of the peptide-loaded microspheres were dissolved in 2 mL dimethyl sulfoxide. The peptide was subsequently extracted from the organic solvent by adding 4 mL of 0.1 M Acetate buffer, pH 4.0, followed by agitation for 1 hour, and assayed using a gradient HPLC method and a C-18 column (3.9 × 300 mm, μBondapak, Waters, USA) at a flow rate of 1 mL/min. The mobile phase contained 30% v/v acetonitrile and 0.1% trifluoroacetic acid in water.

3.3.4. In Vitro Release. *In vitro* release was performed using the "sample and separate" method. About 50 mg of peptide-loaded microspheres were transferred to a 50 mL stoppered glass cylinder and suspended in release media (0.1 M phosphate buffered saline or PBS, pH 7.4) at 37°C (n = 6). Peptide was freely soluble in this media. The peptide-loaded microspheres were subject to three agitation conditions (a) static, (b) continuous, and (c) static-continuous. Under "static" agitation conditions, the microspheres were not agitated throughout the study, while at "continuous" agitation conditions, microspheres were subjected to moderate agitation using a magnetic stir bar to ensure good suspendability of the microspheres in the release media and minimal sedimentation in the release vessel. In the case of "static-continuous" agitation, the microspheres were not agitated (static) for 11 days, after which "continuous" agitation was employed.

Sampling was performed at predetermined intervals by withdrawing 1 mL of release media using a syringe attached to a single use 0.22 μm filter. Any microspheres that adhered to the filter were reintroduced into the release media without any loss during fresh media replacement. Analysis of peptide in the supernatant was assessed by an isocratic reverse phase HPLC method using a C-18 column (3.9 × 300 mm, μBondapak, Waters, USA) using a acetonitrile-water mixture containing 0.1% trifluoroacetic acid at a flow rate of 1.1 mL.

3.3.5. Mass Loss. Mass loss was assessed by gravimetry. Briefly, a weighed amount of peptide-loaded microspheres were transferred to 50 mL stoppered glass cylinder and suspended in release media (0.1 M PBS, pH 7.4) at 37°C in a manner similar to that described in the *in vitro* release study (refer Section 3.3.4). At predetermined intervals, the contents of the release media of the "static" and "continuous" samples were filtered using 0.65 μm PVDF filters (Millipore, USA). The microspheres were collected, dried under vacuum at room temperature for 3 days to ensure complete evaporation of water, and weighed. Mass loss was calculated using

$$\%\ \text{Mass loss} = \frac{(M_0 - M_t)}{M_0} \times 100, \tag{1}$$

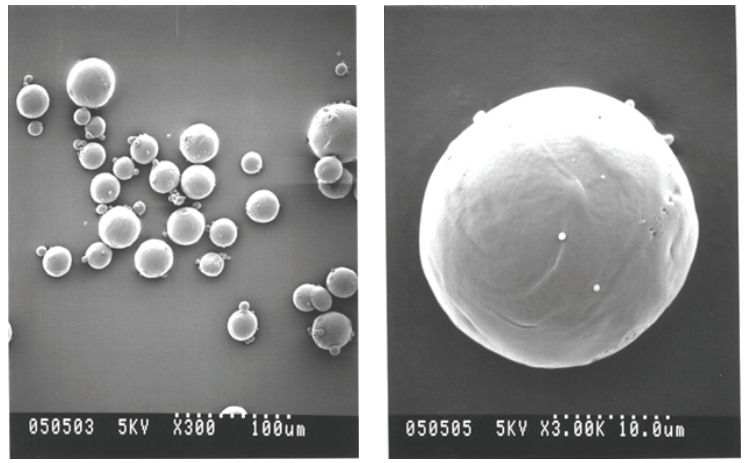
where the variable M_0 describes the initial mass of the microspheres and the variable M_t describes the mass of the microspheres at time "t," respectively.

3.3.6. Polymer Degradation. After measuring mass loss (refer Section 3.3.5), samples from the "static" and "continuous" conditions were analyzed by gel permeation chromatography (GPC) to evaluate polymer degradation. The GPC system consisted of two ultrastyragel columns connected in series (7.8 × 300 mm each, one with 10^4 Å pores and one with 10^3 Å pores), a delivery device (Shimadzu LC-6A, Japan) and software to compute molecular weight distribution (Waters, Maxima 820, Milford, USA). Sample solutions in tetrahydrofuran (THF) at a concentration of 5 mg/mL were filtered through a 0.45 μm filter (Millipore, USA) before injection into the GPC system and were eluted with THF at 0.4 mL/min. The weight-average molecular weight (Mw) of each sample was calculated using monodisperse polystyrene standards, Mw 1,000–50,000 Da.

4. Results and Discussion

4.1. Particle Size and Morphology. The particle size of the peptide-loaded microspheres, as measured by laser light diffraction, had a mean diameter of approximately 25 μm. The scanning electron micrograph images for the peptide-loaded microspheres are depicted in Figure 2. At the lower magnification, it is evident that the peptide-loaded microspheres were spherical, with a relatively nonporous smooth surface containing a few small pores of varying size. A closer examination of the microsphere surface (higher magnification) revealed a nonuniform pore distribution. Additionally, the microspheres could not be fractured, implying that the interior was not hollow.

Given the small particle size of these microspheres, a high surface to volume ratio was to be expected. A consequence of high surface area in microspheres is the phenomenon known



(a) (b)

Figure 2: Scanning electron micrograph of Leuprolide PLGA microspheres.

as initial burst [11]. This instantaneous release or burst of drug from the dosage form *in vivo* or *in vitro* occurs due to release of peptide that is lightly bound to the surface of the microsphere. The initial burst phenomenon has been observed and reported by other researchers that investigated *in vitro* release behavior from microspheres of different sizes [11]. Thus, modulating particle size is an effective approach to ensure initial burst. Burst release may also be used to provide a loading or bolus dose prior to the slow sustained release of drug from the inner matrix of the microspheres, especially with certain classes of therapeutic agents like LHRH superagonists [23]. Hence, in this study, for a water-soluble peptide like Leuprolide in small sized microspheres, a high surface to volume ratio suggested a strong possibility of initial burst release at the early time point(s).

4.2. Peptide Content and In Vitro Release. For the Leuprolide microspheres investigated, peptide content was determined to be 14%. *In vitro* peptide release studies were performed at 37°C in 0.1 M PBS, pH 7.4, using the "sample and separate" method, and are shown in Figure 3 (previously reported in reference [22]).

As illustrated in Figure 3, the *in vitro* release profiles for the "static" and "continuous" agitation conditions were drastically different. However, as predicted in Section 4.1, the small sized Leuprolide PLGA microspheres exhibited an initial burst release of peptide. The value of this instantaneous burst release was comparable for the "continuous" and "static" systems at 37°C and ranged between 6.5 and 8% of the total release. Since initial burst occurs due to easily accessible drug (e.g., drug molecules associated with the microsphere surface), it is a stand-alone phenomenon. In other words, the extent of burst release should be unaffected by the type of agitation or lack thereof in an *in vitro* experiment. The nondependence of burst release on agitation from the current study is in excellent agreement with a previously published report where initial burst in continuously agitated systems was found to be independent of temperature [24].

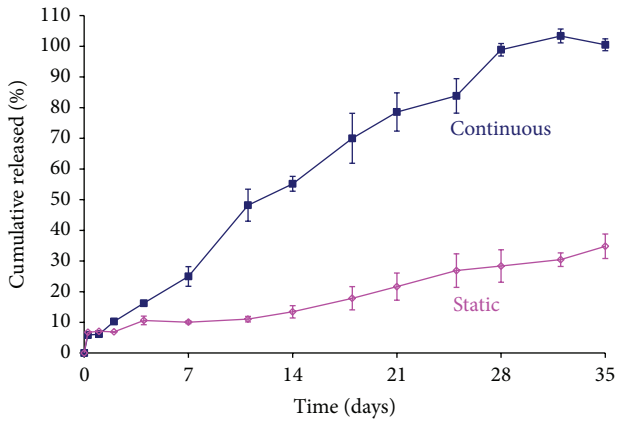


Figure 3: *In vitro* release of Leuprolide from PLGA microspheres at 37°C in 0.1 M PBS, pH 7.4 (reproduced from [22] with kind permission of Springer Science + Business Media).

After the initial burst and a brief polymer hydration period of 1-2 days where drug release was essentially unchanged, peptide release was linear throughout the course of the *in vitro* study. For the samples subjected to "static" conditions, 35% peptide release was observed in 35 days suggesting an estimated net release rate of approximately 1% per day. A good mass balance, noted as approximately 60% of the unreleased peptide was found remaining inside the "static" microspheres after termination of the *in vitro* study (day 35, data not shown). In comparison, complete release was achieved in approximately 30 days for the "continuous" samples implying that the overall release rate could be estimated around 3.33% per day.

Since peptide release was linear after initial burst, a zero-order release was modeled for the "continuous" and "static" conditions. From the slope of the best fit line, peptide release rate was determined to be 3.3936%/day ($R^2 = 0.9847$) for the "continuous" samples and 0.8175%/day ($R^2 = 0.9429$) for the "static" samples, with a strong statistical correlation obtained

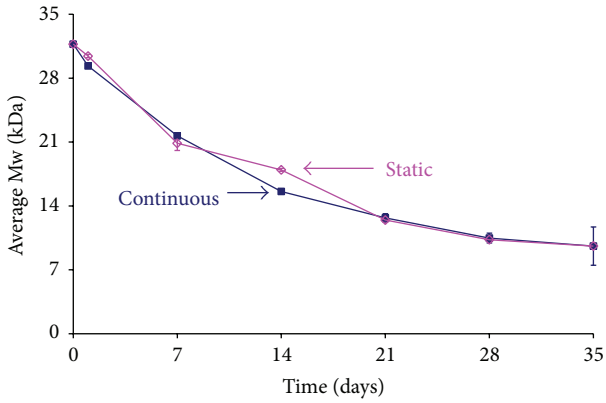


Figure 4: Average Mw of Leuprolide PLGA microspheres.

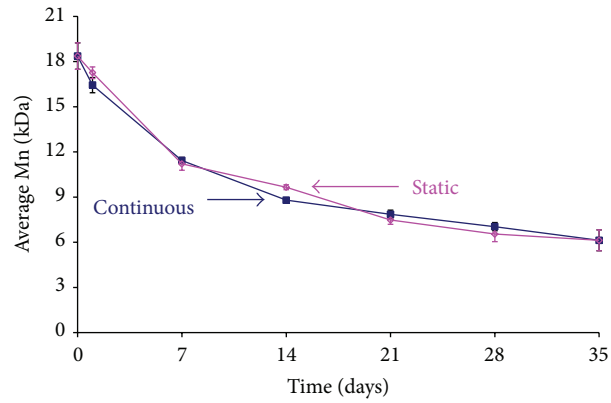


Figure 5: Average Mn of Leuprolide PLGA microspheres.

for the data analysis. The actual release rates obtained from the zero-order fit are in excellent agreement with the estimated release rates. Further, a comparison of the zero-order peptide release rates indicates a greater than fourfold difference between the "continuous" and "static" conditions, suggesting that the kinetics of drug release is much faster when "continuous" agitation is employed. Since the peptide was freely soluble in the media, and hydration of the polymer is not a limiting factor due to an abundance of the *in vitro* release buffer, the slower rate of release in the "static" samples could be ascribed to: (a) peptide adsorption to the water-insoluble and soluble oligomers (described as rates = k_a, k_b, and k_c in Figure 1) and/or (b) unstirred water layer effects. The effect of peptide adsorption and/or unstirred water layer on *in vitro* release is discussed in Section 4.5.

4.3. Polymer Degradation. Degradation of the PLGA polymer under "static" and "continuous" conditions, as measured by GPC, is detailed in Figure 4. From the plot, it is evident that the initial degradation rate of the polymer was low, that is, the drop in molecular weight from day 0 to day 1 was between 4 and 7%. Between day 1 and day 7, the degradation rate surged greatly, ranging between 26–31%. After this, polymer degradation continued in a sustained fashion through the course of the experiment reaching a molecular weight that had dropped 67% from the initial. From literature, it is well known that biodegradation of water-insoluble PLGA follows pseudo first order kinetics [25]. Plots of the natural logarithm of the remaining weight-average molecular weight (Mw) versus time were linear throughout the 35-day period (plot not shown). Using the slope of the straight line, the degradation rate constant (k_1) as calculated for the "continuous" and "static" systems was found to be similar (0.082/day and 0.081/day for "continuous" and "static" conditions, resp.). A good correlation (R^2 of 0.96 for "continuous" and 0.97 for "static") was obtained for the linear regression analyses, confirming that findings from the current study corroborated previously published reports documenting pseudo first order degradation kinetics with the PLGA polymer [25].

Figure 5 depicts a plot of the number-average molecular weight (Mn) as a function of time for the "continuous" and "static" samples. As reported by previous authors, degradation process of a PLGA polymer involves a decrease in weight-average molecular weight (Mw), which leads to the formation of smaller polymer chains [21]. In a manner similar to Figure 4 where the change in weight-average molecular weight (Mw) is described, polymer Mn values dropped over the course of the study and profiles were essentially indistinct for the "continuous" and "static" samples. As with the weight-average molecular weight (Mw), the initial degradation rate for Mn was low (6–10%), but higher than what was observed with Mw at the corresponding time period (Figure 5). Between day 1 and day 7, the degradation rate shot up between 30 and 35% and continued through day 35, thus ensuring the formation of smaller polymer chain lengths to finally reach a value that had dropped 67% from the initial Mn. Semilog plots of the remaining number-average molecular weight (Mn) versus time were linear throughout the 35-day period (not shown), confirming that decrease in polymer Mn followed pseudo first order kinetics. Using the slope of the straight line, the degradation rate constant (k_1) as calculated for the "continuous" and "static" systems was found to be similar (0.069/day and 0.073/day for "continuous" and "static" conditions, resp.), slightly lower than that observed with Mw. A good correlation (R^2 of 0.92 for "continuous" and 0.93 for "static") was obtained for the linear regression analyses.

From an initial value of 1.73, polydispersity (Figure 6) showed a marginal increase to 1.76–1.78 by day 1. By day 7, the value rose to nearly 1.9 for the "continuous" samples after which it dropped steadily to reach 1.5 by day 28. At the 35 day time point, polydispersity for the "continuous" sample rose once again to reach 1.55. In contrast, polydispersity values for the "static" samples were nearly constant from day 7 to day 14 after which a sharp drop was observed (1.58) followed by a subsequent increase to 1.67 with a final value 1.55 at the 35-day time point. Polydispersity is the ratio of Mw to Mn and describes the broadness of molecular weight distribution within a polymer. An increase in polydispersity is indicative of a greater drop in Mn relative to Mw. For example, the

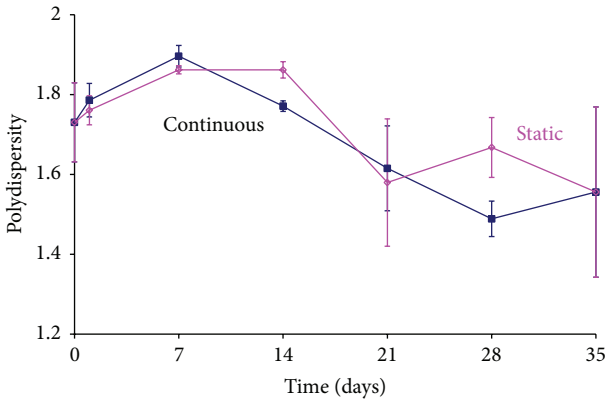


Figure 6: Polydispersity of Leuprolide PLGA microspheres.

initial increase in polydispersity (day 0 to day 1) suggests that a smaller weight fraction (Mn) of the polymer was formed instantaneously under conditions of the *in vitro* experiment. Conversely, a narrowing of the polydispersity is attributed to the simultaneous disappearance of high molecular weight (Mw) polymer chains coupled with the diffusion of low molecular weight (Mw) fragments out of the matrix into the bulk media and has been observed by other researchers [26]. A similar polydispersity profile, that is, an initial rise followed by a drop with a subsequent increase in polydispersity, was observed by Chlopek et al. and ascribed to the fact that Mn depends more on the fraction of low molecular weight than on the fraction of high molecular weight [27].

4.4. Mass Loss. Mass loss from biodegradable microspheres, routinely evaluated by a gravimetric measurement of the remaining drug-polymer mass at predetermined time points during the biodegradation process, is illustrated in Figure 8. During the initial hydration period (day 0 to day 1), there was no mass loss in the "static" and "continuous" samples. At day 7, the "static" and "continuous" microspheres lost about 6–8% of mass due to hydrolysis. From day 7 to 14, mass loss values with the "continuous" samples demonstrated a fourfold increase, illustrating the highest drop over the duration of the study. After day 14, mass loss with the "continuous" samples was steady culminating in a total value of 67% over the 35-day period, suggesting an estimated net loss of mass of approximately 2% per day. In comparison, mass loss between day 7 and day 14 with the "static" samples was significantly slower, with only a twofold increase over the previous time point. After 14 days, mass loss for the "static" samples, continued to increase gradually and reached a total value of 48% within 35 days, suggesting an estimated loss of 1.37% per day. Overall, the amount of mass lost through 35 days for the "continuous" samples was greater than that for the "static" samples.

Since mass loss from the "static" samples followed relatively linear behavior, it was described by the following equation: % Mass loss = $1.45t - 2.84$, where "t" = time, with a strong correlation ($R^2 = 0.98$) for the analyses. The calculated rate (1.45%) for the "static" samples is in good agreement with the estimated value (1.37%). In comparison, mass loss for the "continuous" samples was nonlinear and followed a biphasic profile, slow through day 7, after which mass loss values increased to reach nearly 67% in 35 days.

Studies involving mass loss are an indicator of the extent of polymer erosion, during which the polymer undergoes autocatalytic degradation leading to a loss in molecular weight, mechanical strength, and integrity. During this phase, the water-insoluble polymer and oligomers degrade to form water-soluble oligomers. From the drug release scheme outlined in Figure 1, it is clear that hydration is critical to the degradation process. In the polymer hydration phase, the first step entails a breakdown of the water-insoluble PLGA to high molecular weight water-insoluble oligomers (HWO) and low molecular weight water-insoluble oligomers (LWO), rate = k_1. The HWO may be considered as intermediates of the ester hydrolysis reaction of the PLGA polymer after which they are converted to low molecular weight water-insoluble (LWO) and water-soluble oligomers (WSO) and, finally, to lactic and glycolic acids. Of particular note is that during the formation of HWO, a drop in molecular weight causes a loss in the mechanical strength of the polymer, but not its integrity [25, 28]. Once degradation continues, continual cleavage of the polymer chain leads to a rapid decrease in polymer molecular weight, with the consequent loss of its integrity, a phenomenon termed as mass loss.

4.5. Correlating Mass Loss with Polymer Degradation and In Vitro Release. Figures 4, 5, and 7 describe the behavior of the degrading polymer and its corresponding loss of mass. Even though decrease in polymer molecular weight (Mw) and molecular number (Mn) were essentially identical for the "continuous" and "static" conditions, mass loss profiles were vastly different. Further, in comparison to polymer degradation, there was a significant lag in the onset of mass loss. Indeed, in the initial stages of incubation, the polymer experienced its highest drop in molecular weight, much more rapid than mass loss. For example, polymer molecular weight dropped from an initial value of 32 kDa to 21 kDa within 7 days (~34%) whereas mass loss was a mere 6–8%. However, at later time points, the drop in polymer molecular weight was more gradual.

At closer glance, when compared to molecular weight, mass loss profiles (Figure 7) were a better indicator for *in vitro* release behavior (Figure 3). Despite the fact that the rate of mass loss lagged behind *in vitro* release, the profiles followed a similar trend, that is, "continuous" samples exhibited faster mass loss and drug release whereas the converse was observed with the "static" samples. Thus, based on the data, the relationship between mass loss and *in vitro* release was clearly evident and can be explained by understanding the changes in the polymer matrix during the hydrolytic degradation process. Prior to hydrolysis, the polymer matrix contains unhydrolyzed PLGA and the water-soluble peptide (Figure 1). During the initial stage of hydration, degradation of the polymer primarily involves formation of high and low molecular weight water-insoluble oligomers (HWO and LWO). Thus, the polymer matrix composition in the early

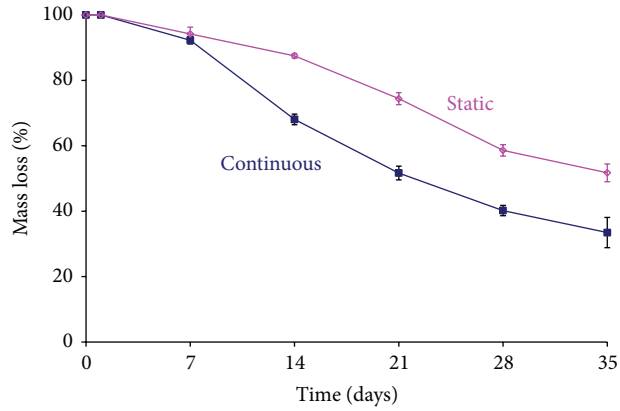


Figure 7: Mass loss of Leuprolide PLGA microspheres.

stages of degradation is largely HWO, LWO, unhydrolyzed PLGA polymer, and the encapsulated water-soluble peptide. These HWO and LWO are large chains with shorter lengths than the original PLGA polymer, are hydrophobic in nature, contain carboxylic end groups, and are surrounded by water molecules as well as the water-soluble peptide. The presence of these oligomers with acidic functionalities causes a lowering of pH inside the polymer matrix. Thus, the continued attack by water molecules and increased acidity inside the polymer matrix accelerates the hydrolytic breakdown of HWO to LWO and LWO to WSO [29–31]. These hydrolysis reactions have been reported to play an important role in the complex bulk degradation mechanism of PLGA polymers [32–34]. Eventually, ester bond cleavage of the water-insoluble oligomers (HWO and LWO) leads to formation of WSO, that is, water-soluble oligomers. Finally, WSO and peptide diffuse through the matrix and escape from the surface of the rapidly degrading microsphere or from regions of the matrix that are easily accessible to the *in vitro* release medium.

Using the data from Figure 7 (% mass remaining) and Figure 3 (% peptide released), the composition of the Leuprolide PLGA microspheres over time was determined and plotted (Figures 8 and 9). From these plots and the scheme proposed in Figure 1, the relationship between polymer degradation, mass loss, and *in vitro* release can be explained as follows.

4.5.1. Fate of HWO. In the current study, formation and breakdown of HWO in the polymeric matrix were determined by GPC analysis and are shown in Figures 4 and 5 (rate = k_1 in Figure 1). Assessment of the low molecular weight water-insoluble PLGA oligomers (LWO) was difficult as they are known to be partially soluble in THF, the solvent used for analysis [35]. Due to their molecular weight and insolubility in water, the unhydrolyzed PLGA polymer, HWO, and LWO retain a presence and their integrity in the polymeric matrix. This fact is clearly evident by the lack of any mass loss through day 1 at the "static" and "continuous" conditions (Figure 7), despite a drop in molecular weight during that time period (Figures 4 and 5). Further, GPC analysis attests that the average molecular weight of HWO obtained from "continuous" and "static" samples are indistinct throughout the study duration, suggesting that these insoluble oligomers retained in the matrix do not impact mass loss (Figures 8 and 9). Another interesting aspect that should be considered is that hydrolytic degradation of PLGA to HWO and its breakdown to LWO (rate = k_1) occurred independently of the amount of HWO, LWO, WSO, and peptide in the polymer matrix. This negates any influence of peptide adsorption or desorption (rate = k_a and k_{-a}) on polymer degradation. Indeed, the formation of HWO for "continuous" and "static" samples was similar through the entire duration of the study (Figures 4 and 5), despite their mass loss profiles being different.

4.5.2. Fate of LWO. Once formed, the low molecular weight water-insoluble oligomers (LWO) undergo ester hydrolysis to form water-soluble oligomers (WSO) (rate = k_2). Unlike k_1, the breakdown of the LWO to form WSO (k_2) when agitated under "static" or "continuous" conditions could be slightly different and three possible scenarios can be envisaged.

(i) Rate of breakdown of LWO in "static" samples is greater than "continuous" samples.

(ii) Rate of breakdown of LWO in "static" samples is equal to "continuous" samples.

(iii) Rate of breakdown of LWO in "static" samples is lesser than "continuous" samples.

Each of scenarios (i)–(iii) will be explored in Section 4.5.3 below.

4.5.3. Fate of Water-Soluble Moieties (WSO and Peptide). Compared to their more hydrophobic counterparts, the WSO and peptide are water-soluble hydrophilic entities. Of the two, the WSO contains carboxylic end groups, similar to that of the HWO and LWO. In the early stages of hydration, results from Figures 4 and 5 indicate that the amount of WSO generated (rate = k_2) is negligible.

Due to their size and water solubility, the WSO and peptide can readily diffuse out of the polymeric matrix into the sink (rates = k_3, k_4, k_5, and k_6). Hence, it can be hypothesized that disappearance of WSO and peptide from the polymeric matrix be termed as mass loss. Indeed, findings from Figures 8 and 9 confirm this hypothesis. In the current study, data from Figures 8 and 9 illustrate that loss of mass from the polymer matrix in the early stages of hydration is negligible. This is an expected outcome due to the fact that WSO formation and diffusion is practically nonexistent in the "static" and "continuous" samples, and peptide release is minimal. In the later stages of polymer degradation, the total polymer mass as well as the water-soluble peptide content remaining in the "continuous" samples decreases rapidly (Figure 8) but exhibits a slower decline in the "static" samples (Figure 9). Thus, the mass loss with both samples is due to the diffusion of WSO and peptide from the microsphere into the release media. From the data, it could be inferred that greater mass loss with "continuous" samples was probably due

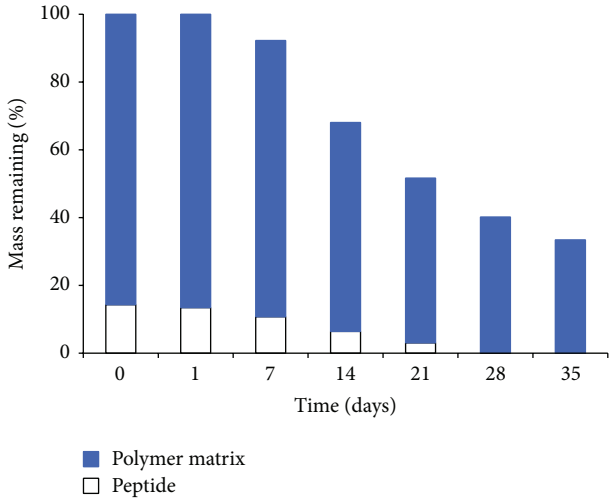


Figure 8: Composition of Leuprolide PLGA microspheres (% mass remaining) under continuous agitation.

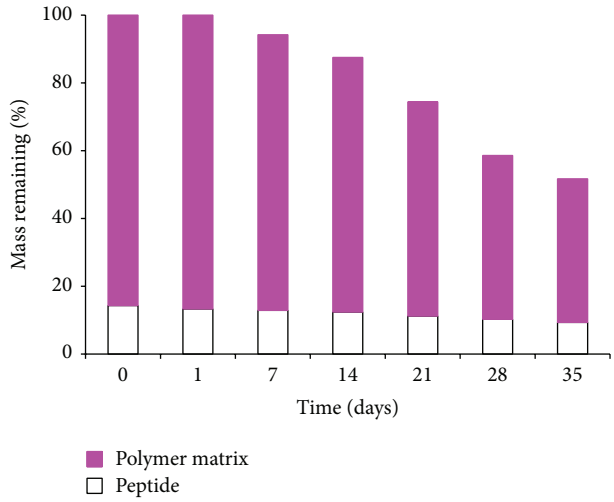


Figure 9: Composition of Leuprolide PLGA microspheres (% mass remaining) under static agitation.

to higher rate of diffusion of WSO (rate = k_3 and k_4) and peptide (rate = k_5 and k_6) through the polymer matrix and UWL. Since diffusivity depends on path length, the distance that WSO or peptide molecules would need to traverse to reach the microsphere's external surface in the early or middle stages of hydration is the same, irrespective of whether the sample is "static" or "continuously" agitated. Low values of k_3, k_4, k_5, and k_6 would imply that the WSO or peptide moieties do not rapidly escape out of the microspheres, and/or through the UWL. From first principles, it is clear that rates k_4 and k_6 are much higher for "continuous" samples, due to elimination of UWL (boundary layer) effects. Further, mass balance results for unreleased peptide in the "static" samples suggest a low value for rate k_5. Since the WSO is a hydrophilic entity, similar to the peptide, a low rate of diffusion (k_3) can be inferred for this species in the "static" samples. This would not be wholly unexpected, given the minimal diffusion of the other hydrophilic species inside the microspheres, that is, water-soluble peptide (Figure 9).

Since k_1 is the same for "static" and "continuous" samples, but k_3, k_4, k_5, and k_6 are lower with the former, three possible scenarios can be envisioned for k_2.

(i) Rate of breakdown of LWO (formation of WSO) in "static" samples is greater than "continuous" samples. If this were true, then the number of acid end groups in the microsphere would increase. Given that k_3 (diffusion of WSO) for "static" samples is less than k_3 for "continuous," the increased number acidic moieties trapped inside the microsphere would be high, resulting in autocatalysis of the polymer and any other oligomers (HWO, LWO) under "static" conditions. Since k_1 is the same in "static" and "continuous" samples (Figure 4), it follows that k_2 for "static" is not greater than that for "continuous" samples.

(ii) Rate of breakdown of LWO in "static" samples is equal to "continuous" samples. Once again, if the number of acidic functionalities generated in the "static" and "continuous" samples is the same, autocatalytic effects in the "static" samples are to be expected. From the data in Figure 8, it is evident that large amounts of WSO are generated/escape from the "continuous" samples. However, if this situation was to occur with the "static" samples, the acidic WSO would be entrapped in the polymer matrix and k_1 values for the "static" samples would be larger than that for the "continuous" samples, which is not the case (Figures 4 and 5). Hence, the rate of formation of WSO (k_2) in "static" samples cannot be considered as equal to that in the "continuous" samples.

(iii) Rate of breakdown of LWO in "static" samples is lesser than "continuous" samples. In this instance, less number of acid end groups are generated in the "static" samples when compared to the "continuous" samples. Indeed, autocatalytic effects in the "static" samples would not be as predominant due to slower breakdown of the LWO to form WSO, and k_1 would be unaffected for "static" and "continuous" samples. With the "continuous" samples, higher rate of formation of WSO is expected due to the increased number of collisions with water molecules and also faster depletion of the soluble acidic functionalities. This scenario seems most likely and affirmed by the data on the water-soluble peptide in Figure 9.

Therefore, "continuous" agitation accelerates the formation of WSO (k_2) and diffusion of WSO (k_3, k_5) and peptide (k_4, k_6) by increasing collisions between water molecules which catalyze ester hydrolysis causing mass loss and consequently, drug release.

4.5.4. Adsorption Effects. As shown in Figure 1, there is a possibility that slower rate of release in the "static" samples could potentially be due to peptide adsorption or desorption to the water-insoluble (HWO, LWO) and soluble oligomers

(WSO), described as rates = k_a, k_{-a}, k_b, k_{-b}, k_c, and k_{-c}. From the discussion in Section 4.5.2 (fate of HWO), it is clear that peptide adsorption or desorption (rate = k_a and k_{-a}) has no influence on degradation of unhydrolyzed PLGA or the HWO. In fact, studies have shown that Leuprolide is rapidly desorbed from PLGA [36]. Hence, adsorption/desorption of peptide was not expected to play a role in polymer degradation.

Overall, the results from Figures 8 and 9 also explain the slow release of drug during the hydration stage, that is, drug release after the "initial burst." This phase, often termed as "diffusional," occurs during a period of low mass loss. In the current study, diffusional release occurred within 1-2 days of the *in vitro* experiment, during which time the polymer experienced a drop in molecular weight due to hydrolytic attack by water molecules leading to the formation of predominantly water-insoluble moieties (Figures 4 and 5). Results from the present study also confirm findings from a previous report where low release accompanied by a drop in molecular weight was observed prior to the onset of mass loss [21].

Concurrent with the generation of WSO and its diffusion to the release media, the drug entrapped inside the degrading polymeric matrix also undergoes dissolution and transport. From first principles, this dissolved drug will move from a region of higher concentration to a lower concentration, that is, *in vitro* release medium. Thus, the mass of the polymer decreases significantly because degraded polymer chains (and peptide) can leach out of the polymer microspheres. This phase, often termed as "erosional" component of drug release has been previously reported to occur during a period of high mass loss. For instance, Vey et al. demonstrated that significant molecular weight reduction with little mass loss was observed during the initial phase (~9 days) of a degradation study. However, once mass loss commenced after day 9, molecular weight reduction was slower [25]. In the current study, erosional release occurred nearly 7 days after the initiation of the *in vitro* experiment, during which time the polymer experienced a moderate drop in molecular weight (Figure 4) with significant mass loss due to predominantly water-soluble moieties and peptide escaping the polymer matrix (Figure 7). Based on these results, it seems highly plausible that diffusion of the WSO (and peptide) is responsible for mass loss from the polymer matrix and, therefore, the erosional phase of drug release.

4.6. UWL Effects. Given that peptide release from PLGA microspheres is regulated by the polymer, factors that influence polymer hydrolysis and degradation will impact formation and diffusion of WSO as well as release kinetics of the water-soluble peptide. Since PLGA polymer degradation occurs in two phases (refer Section 4.5), namely, (a) initial hydration resulting in polymer molecular weight reduction and (b) continued hydration resulting in polymer mass loss, it follows that altering the rate of either of these processes will alter kinetics of LWO degradation (formation of WSO) and drug release from the microspheres (Figures 8 and 9). To confirm the hypothesis that agitation minimized UWL

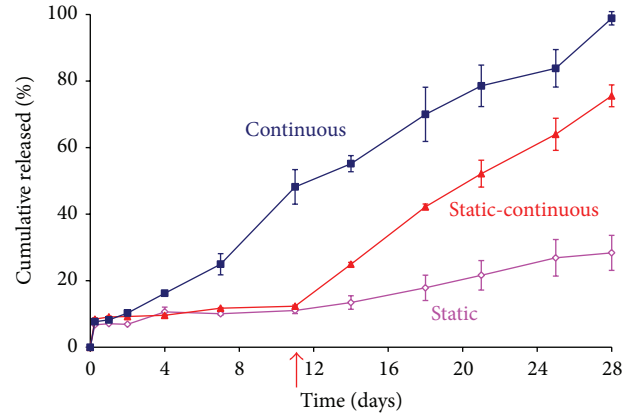


FIGURE 10: Effect of agitation on *in vitro* release of "static-continuous" samples of Leuprolide PLGA microspheres.

effects, *in vitro* release on Leuprolide PLGA microspheres was initially performed under "static" conditions, after which, the samples were "continuously" agitated ("static-continuous" samples). From the plot in Figure 3, a period of 11 days was deemed sufficient to demonstrate a significant difference in the *in vitro* release in the "static" and "continuous" samples.

In a manner comparable to what was observed previously with the continuously "static" and "continuous" samples (Figure 3), results from the "static-continuous" samples (Figure 10) once again reaffirmed that agitation or lack thereof does not impact initial burst release from microsphere dosage forms. After an initial burst, release data from the "static-continuous" samples were essentially identical to the "static" samples through 11 days (approximately 12% peptide release), also confirming the reproducibility of the "sample and separate" *in vitro* technique employed in this study. At the day 11 time point, the samples were subjected to "continuous" agitation. By the next sampling interval (day 14), the release rate shot up approximately 100% to reach 24%. In general, the *in vitro* release profile for the "static-continuous" samples from day 11 to 14 mimicked that of the "continuous" samples, albeit with a lower extent of peptide release. This acceleration in water-soluble peptide release with the "static-continuous" samples continued through the end of the study (day 28). Thus, once agitation commenced and UWL effects were minimized, peptide release rate for the "static-continuous" samples mimicked those of the "continuous" samples. This observation confirms that agitation accelerates rates k_4 and k_6 (peptide diffusion), and, similarly, rates k_2, k_3, and k_5 (formation and diffusion of WSO).

As described in the introduction section, there are varied choices regarding the mode of agitation to minimize UWL. In this study, a magnetic stirrer based continuous agitation mechanism was utilized. Advantages of this mode of agitation over others include ease of set-up, and simplicity of sample withdrawal and buffer replacement. While it is speculated that continuous agitation using different apparatus should provide similar release profiles, it is not always the case. For example, Bain et al. compared the *in vitro* release of spray-dried Rifampicin microspheres formulated using a

blend of PLA polymers using two modes of agitation at the same ratio of microspheres to dissolution media: (i) bottles shaken horizontally in a water bath and (ii) USP XII dissolution apparatus [37]. In both modes of continuous agitation, the microspheres remained as individual particles and were continually suspended in the media. However, faster release was observed with the USP paddle apparatus and attributed to a greater degree of agitation with the paddle which prevented the microspheres from forming aggregates at the base of the vessel [37]. Similar to a previous study with Indomethacin microspheres, the rate and extent of drug release depended on the mode of agitation employed with Rifampicin microspheres [9].

In addition to preventing microsphere aggregation, results from the study confirm that "continuous" agitation minimizes unstirred water layer (UWL) effects. Further, data from the "static-continuous" study confirm that agitation plays a key role in influencing the rate of various mechanistic processes that impact the degradation and mass loss in biodegradable polymers like PLGA and PLA (polylactide). Thus, a variation in the agitation regimen at any point of time in an *in vitro* study will provide skewed results. Given that *in vitro* experiments with biodegradable microspheres extend for the duration of action of the dosage form, that is, weeks to months, any modifications to the agitation regimen will alter the data generated. Apart from "static" and "continuous" agitation regimens, "intermittent" agitation has been used by other researchers [38]. This type of agitation regimen could be beneficial in periodically minimizing UWL effects; however, the impact of the type and duration of agitation should also be investigated.

5. Conclusions

Polymer degradation, mass loss, and drug release rates from PLGA based dosage forms are influenced by the presence or absence of an unstirred water layer. The use of agitation, a mechanical parameter, employed during *in vitro* release testing of sustained release injectable dosage forms, minimizes unstirred water layer effects. Given the complex nature of drug release from PLGA based dosage forms, studies to understand the effect of agitation will enable development of a robust *in vitro* method that can accurately identify the causes of complete or incomplete drug release. Special attention should also be paid to the type and nature of agitation used and understanding its implications on the data generated.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

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