Effect of Agitation Regimen on the *In Vitro* Release of Leuprolide from Poly(Lactic-Co-Glycolic) Acid Microparticles

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ABSTRACT: Because of the importance of *in vitro* release tests in establishing batch-to-batch reproducibility and *in vitro–in vivo* correlation, this study investigated the influence of agitation regimen on the *in vitro* release behavior of leuprolide from poly(lactic-co-glycolic) acid microparticles. Leuprolide-loaded microspheres were prepared using Resomer® RG502H and RG503H as polymers. Leuprolide *in vitro* release was performed in phosphate buffer solution under continuous or once-a-week agitation. At predetermined intervals, leuprolide release, polymer mass loss, and degree of hydration were investigated. Leuprolide release and polymer mass loss were higher under continuous agitation with respect to that under intermittent agitation. Using a modified version of Koizumi equation, it was possible to fit leuprolide release profiles. Similarity factor comparison showed a high level of similarity between experimental and modeled data in the case of once-a-week agitation regimen. This work highlights the importance of the *in vitro* release conditions on peptide release behavior from polyester microparticles. © 2011 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 101:1212–1220, 2012
Keywords: leuprolide acetate; microencapsulation; PLGA microparticles; peptide delivery; *in vitro* release; agitation regimen; polymer biodegradation; molecular weight determination; Koizumi equation; controlled release/delivery

INTRODUCTION

In the last three decades, interest for carriers able to control the release of active pharmaceutical ingredients (API) temporally and spatially has grown enormously. For this reason, numerous drug delivery systems such as implants, polymeric microparticles and nanoparticles, liposomes, and solid–lipid nanoparticles have been studied for these applications.[1] Among these, biodegradable microparticles prepared using poly(lactic acid) (PLA) and/or poly(lactic-co-glycolic) acid (PLGA) have been investigated and several formulations have been approved and marketed.[2] Their success can be attributed to the fact that the copolymers of lactic and glycolic acids have been approved by the US Food and Drug Administration for human clinical application due to their excellent biocompatibility, biodegradability, and mechanical strength.[3] Additionally, PLGA microparticles provide ease of administration and the possibility to control drug release kinetics over periods of time ranging from days to months.[4,5] Among marketed formulations, those containing leuprolide were successfully developed for long-term testosterone suppression in the treatment of prostate cancer.[4–6] Leuprolide acetate, a peptide, is a potent agonist of luteinizing hormone-releasing hormone, which inhibits the secretion of pituitary gonadotropin when administered chronically in therapeutic doses.[7,8]

Many studies have been carried out to investigate the influence of the preparation procedure [e.g., stirring, surfactant type and concentration, rate of solvent evaporation, temperature, solvent type and volume, organic–water phase ratio, polymer molecular weight (MW)] on microparticle physicochemical characteristics.[9–14] However, despite the increasing interest addressed to PLA and PLGA microparticles, relatively limited attention has been dedicated to the development of reliable *in vitro* release methodologies for these formulations.[15–17]

Drug release from a bioerodible matrix such as PLGA remains a complex subject in and of itself,

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and the mechanisms governing it are still a matter of investigation. For PLGA microparticles, the mechanisms include API diffusion through the polymer matrix and/or the fluid-filled pores, polymer degradation, and polymer erosion. PLGA degrades through the chemical hydrolysis of the ester bonds generating, first oligomers and later lactic and glycolic acids. When the produced oligomers achieve a certain MW, becoming soluble in the surrounding medium, erosion (with the loss of polymer mass) ensues. It is well known that polyester microparticles start to degrade when entering in contact with water. The rate at which particles absorb water is generally faster[18] than the rate of ester bond hydrolytic cleavage; then, polymer degradation occurs homogeneously within the polymer matrix (bulk erosion).[19,20] However, degradation by-products generate a low-pH microenvironment within the matrix, leading to a phenomenon known as acidic autocatalysis. Autocatalysis may lead to a nonhomogeneous degradation whereby the microparticle core degrades faster than the surface.[21–23] Because drug release is influenced by polymer degradation, autocatalysis may be responsible for accelerated drug release. Nevertheless, the autocatalysis has major impact in devices of larger size than micrometric particles.[19]

Polyester hydrolysis is largely influenced by the composition of the release medium,[24–26] temperature,[27] and the agitation conditions as well.[28] The *in vitro* release profile provides information on the structure of the carrier and on possible interactions between the polymer and the drug. Moreover, *in vitro* release studies are generally performed to obtain reliable information, or at least the best approximation, of the *in vivo* release and batch-to-batch reproducibility.[17] The establishment of optimal *in vitro* release test conditions will provide assurance of good *in vitro–in vivo* correlations (IVIVC).[29] In fact, although for the oral forms, the *in vitro* release tests are generally well established, the same cannot be guaranteed for subcutaneous and intramuscular parenteral formulations. Actually, in the case of these products, it is much more complicated to mimic the *in vivo* conditions and, subsequently, IVIVC is difficult to achieve. In light of these considerations, it appears essential to identify reliable guidelines for more suitable working conditions, for example, apparatus, release media, agitation, temperature, sampling methods, test intervals, microparticle mass–bulk fluid volume ratio, and sink conditions.[16,30–34]

In this study, the effect of the agitation regimen on leuprolide *in vitro* release from PLGA microparticles was studied and a mechanistic explanation was attempted.

MATERIALS AND METHODS

Materials

Leuprolide acetate was purchased from Bachem (Torrance, CA). Polyvinyl alcohol (PVA, MW ~70–100 kDa) was obtained from Sigma–Aldrich (St Louis, Missouri). PLGA Resomer® RG502H (MW ~10,000 Da) and Resomer® RG503H (MW ~27,000 Da) were supplied by Boehringer Ingelheim (Ingelheim, Germany). All other chemicals and reagents were of the highest purity grade commercially available.

Leuprolide Acetate-Loaded PLGA Microparticle Preparation

Leuprolide-loaded PLGA microparticles were prepared by solvent diffusion/evaporation method with a 15% theoretical loading. In brief, a solution of leuprolide in methanol was added to a 31% (w/w) solution of polymer in methylene chloride to form a clear solution. The resulting solution was then slowly added into 0.35% (w/v) aqueous PVA solution while mixing with a Silverson L4R mixer (Silverson Machines, East Longmeadow, Massachusetts) at 7000 rpm. After mixing, the resulting suspension was transferred to a larger volume of 0.35% (w/v) aqueous PVA solution under stirring at 900 rpm. The solvents were removed by increasing the temperature of the preparation up to 40°C and stirring at this temperature for 1 h. The suspension was then cooled to 25°C and the solidified microparticles were recovered by filtration using a 5-μm Millipore SMWP filter (Millipore, Billerica, Massachusetts). The microparticle suspension was lyophilized overnight and dried particles were stored in a desiccator at room temperature.

Particle Size Determination

Particles were sized by laser diffractometry using a Malvern 2600 laser sizer (Malvern 2600c Particle Sizer; Malvern Instruments Ltd., Malvern, Worcestershire, UK). The average particle size was expressed as the mean volume diameter.

Morphological Analysis and Surface Area Determination

Microparticle morphology was evaluated using scanning electron microscopy (Hitachi model S800; Hitachi, Chiyoda, Tokyo, Japan). Samples were prepared by placing microparticle powder onto an aluminium specimen stub and were sputter coated with palladium/gold prior to imaging. The Brunauer–Emmett–Teller surface areas of the samples were determined by N_2 adsorption at–196°C on Micromeritics

ASAP 2010 equipment (Micromeritics Instrument Corporation, Norcross, Georgia). All samples were degassed at 70°C for 24 h prior to adsorption.

Leuprolide Acetate Quantitative Determination

Leuprolide acetate was analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC). RP-HPLC analysis was carried out by means of a Bondclone 10 C18 column ($150 \times 3.9\,mm^2$; Phenomenex, Torrance, California). Elution was performed in a gradient manner (flow rate 1.5 mL/min) with mobile phase A, water with 0.1% trifluoroacetic acid (TFA) as an ion-pairing agent; and mobile phase B, water–acetonitrile (50:50, v/v) with 0.1% TFA, at a gradient of 70% A and 30% B to 23% A and 77% B over 15 min. The injection volume was 30 μL and the ultraviolet (UV) detector was set at 220 nm. Column, mobile phase, and samples were equilibrated at room temperature. A calibration curve for leuprolide RP-HPLC analysis was constructed with six solutions in the concentration range of 15–300 μg/mL.

Encapsulation Efficiency Determination

In order to determine the amount of leuprolide per unit weight of microparticles, 10 mg of microparticles were dissolved in 1 mL of dimethyl sulfoxide. Once a clear solution was obtained, 2 mL of 0.1 M acetate buffer (pH 4) were added to the solution and mixed thoroughly for 5 min.[35] The cloudy solution was then centrifuged in a glass tube to get a clear supernatant, which was assayed for leuprolide concentration by RP-HPLC.

In Vitro Drug Release Studies

In vitro leuprolide release was performed in 10 mL of 0.1 M phosphate buffer solution (pH 7.4) containing 0.02% sodium azide at 37°C under continuous or once-a-week magnetic agitation (1-min duration). Individual samples of 10 mg were transferred in flat-bottom vials for each assay point. At predetermined intervals, 1 mL supernatant was withdrawn and analyzed by RP-HPLC. In order to determine the mass balance relationship, at each time point, microparticles were filtered, dried under vacuum, and leuprolide was extracted from the microparticles and assayed for leuprolide as previously described.

Mass Loss and Hydration Determination

At each time point of the *in vitro* release studies, remaining microparticles were recovered by filtration using a 0.8-μm Millipore AAWP filter (Millipore) and weighed accurately (wet weight, W_w). The microparticles were dried overnight under vacuum at room temperature and weighed again (dry weight, W_d). Mass loss (ML) and degree of hydration (DH) were calculated as follows:

$$ML = \frac{(W_o - W_d)}{W_o} \times 100 \quad (1)$$

where W_o is the initial weight of the microparticle.

$$DH = \frac{(W_w - W_d)}{W_d} \times 100 \quad (2)$$

Polymer MW Determination

To determine polymer MWs, microparticles were dissolved in tetrahydrofuran (THF) at a concentration of 5 mg/mL. After filtration through a 0.45-μm filter (Millipore), MW was determined by gel-permeation chromatography (GPC). The GPC system consisted of two Ultrastyragel columns (Waters, Milford, Massachusetts) connected in series ($7.8 \times 300\,mm^2$ each, one with 10^4 Å pores and one with 10^3 Å pores), a delivery device (Shimadzu LC-6A; Shimadzu, Nakagyoku, Kyoto, Japan), a UV detector set at 210 nm (Shimadzu), and a software to compute MW distribution (Maxima 820; Waters). Samples were eluted with THF at 0.4 mL/min. The weight-averaged MW of each sample was calculated using monodisperse polystyrene standards (MW 1000–50,000 Da).

In Vitro Release Modeling

To model leuprolide *in vitro* release profiles from microparticles under different agitation regimen, Koizumi's model (Eq. 3) was used as a starting point and was subsequently improved[36]:

$$\frac{M_t}{M_\infty} = 4 \times \pi \times a^2 \times \left\{ \sqrt{2 \times (C_0 - C_s) \times C_s \times D \times t} + \frac{4C_s}{9a}\left[\frac{C_S}{(2C_0 - C_s)} - 3\right] \times D \times t \right\} \quad (3)$$

where M_t is the mass of leuprolide released at time t, M_∞ is the mass of leuprolide released as time approaches infinity, a is the radius of the microparticle, C_0 is the initial concentration of the drug in the polymer, C_s is the drug solubility in the polymer, and D is the diffusion coefficient.

This model allows a better agreement than Crank's model, which is suitable to describe the burst phase.[37] Because microparticle ML and polymer MW reduction were followed, the influence of both parameters on the diffusion coefficient was evaluated in Eq. 3, as previously carried out by Faisant and coworkers[38,39] with

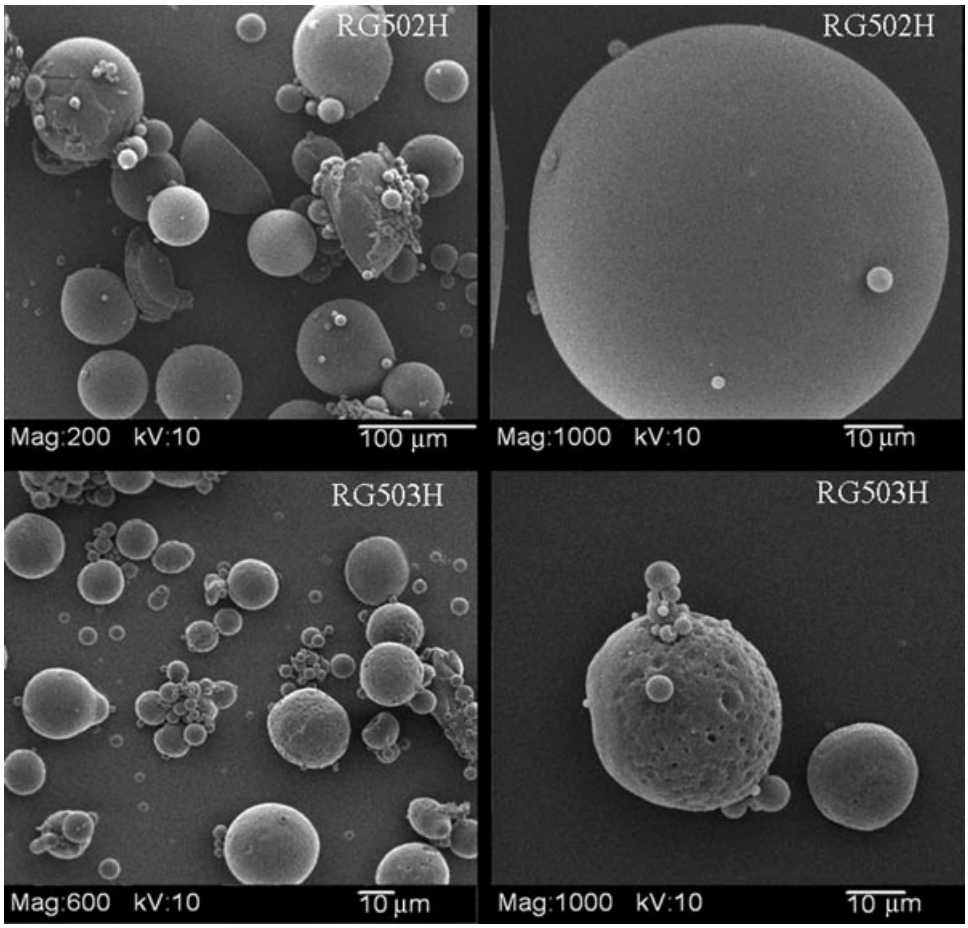

Figure 1. Leuprolide acetate-loaded microparticles prepared with polymers RG502H and RG503H observed by scanning electron microscopy.

polymer MW. Therefore, D_t was determined using the following equations:

$$D_t = D_0 + \frac{k}{\mathrm{MW}} \tag{4}$$

$$D_t = D_0 \; + \; \frac{k}{1 \; - \; \mathrm{ML}} \tag{5}$$

where D_t is the diffusion coefficient at time t, D_0 is the initial diffusion coefficient in PLGA, k is the degradation rate constant of the polymer, expressed as MW reduction or microparticle ML as shown in Eq. 6:

$$\mathrm{MW}_t = \mathrm{MW}_0 \; \times \; \exp\left(-k_{\mathrm{degr}} \; \times \; t\right) \tag{6}$$

Equation 6 describes PLGA degradation kinetics following contact with the release medium where MW_t is the molecular weight at time t, MW_0 is the initial molecular weight, and k_{degr} is the degradation rate constant. The degradation constant has been alternatively obtained from the microparticle ML profiles.

A model independent mathematical approach[40–43] was used to fit the experimental *in vitro* release profiles of leuprolide-loaded microparticles both under continuous and once-a-week agitation regimen. For comparing the profiles, similarity factor f_2 was computed using the following equation: fontsize79

$$f_2 = \; 50 \times \; \log\left\{\left[1\left(\frac{1}{n}\right)\sum_{t=1}^{n}(R_t \; - \; T_t)^2\right]^{-0.5} \; \times \; 100\right\} \tag{7}$$

where f_2 is the similarity factor and R_t and T_t are the cumulative percentages of the fitted and experimental drug released at each of the selected n time points, respectively. f_2 has been determined for different agitation regimen and two polymers.

RESULTS AND DISCUSSION

Microparticle Characterization

Leuprolide-loaded microparticles prepared using single emulsion solvent diffusion/evaporation technique were spherical, but presented a relatively nonporous surface in the case of RG502H and a slightly porous

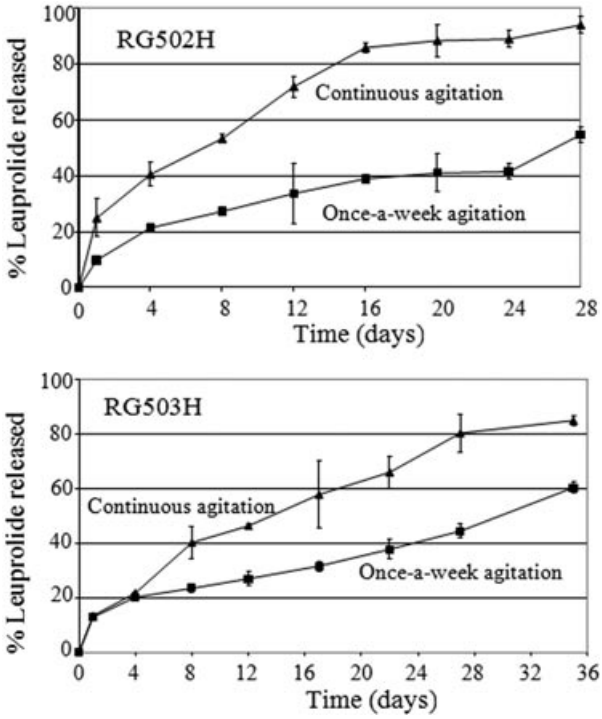

Figure 2. Leuprolide *in vitro* release profiles under continuous or once-a-week agitation at 37°C in 0.1 M phosphate buffer solution (pH 7.4) from microparticles prepared with polymers RG502H and RG503H.

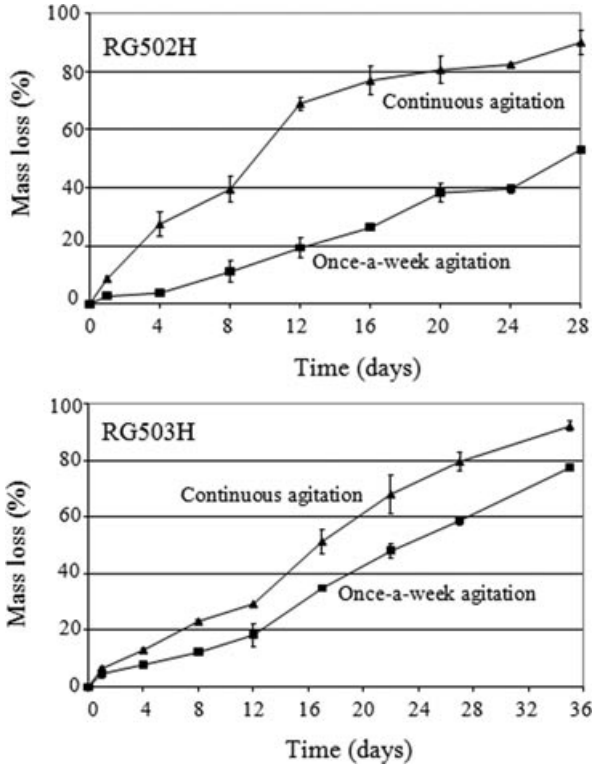

Figure 3. Mass loss profiles of leuprolide acetate-loaded microparticles prepared with polymers RG502H and RG503H, incubated under continuous or once-a-week agitation at 37°C in 0.1 M phosphate buffer solution (pH 7.4).

surface for RG503H (Fig. 1). This has been sometimes ascribed to the presence of peptide.[11] Practical content was 10.5% and 11.5% for RG502H and RG503H microparticles, respectively. The preparation method yielded a high encapsulation efficiency (70%–77%) as well as a good recovery because about 70% of the solid material was recovered as microparticles. RG502H particles have a mean size of 49.8 μm [d(v,0.5), 32.9; d(v,0.9), 78.0; d(v,0.1), 9.7], whereas RG503H particles measure 13.8 μm [d(v,0.5), 13.6; d(v,0.9), 36.7; d(v,0.1), 5.0], almost four times smaller than the former ones. This difference in particle size, together with the higher surface porosity observed for RG503H (Fig. 1), justified the larger surface area (2.9 vs. $<0.5\ m^2/g$) determined for the smaller particles.

In Vitro Release Studies

The *in vitro* release profiles of leuprolide acetate-loaded microparticles under continuous or once-a-week agitation are shown in Figure 2. In the case of microparticles prepared with RG502H, only about 10% of leuprolide was released under once-a-week agitation, whereas 25% of leuprolide was released under continuous agitation during the first 24 h. In the case of continuous agitation, the release was complete after 28 days, whereas only approximately 60% of leuprolide was released under once-a-week agitation (Fig. 2). For microparticles prepared with RG503H, the *in vitro* release profiles were similar during the first 4 days, whereas after the day 4, leuprolide release under continuous agitation was higher than the release under once-a-week agitation. This behavior can be ascribed to the smaller particle size, narrower distribution width, and larger surface area of RG503H microparticles with respect to RG502H. In fact, the large surface area may allow an easy leuprolide diffusion at early stages, impairing the release acceleration effect observed in the case of RG502H with continuous agitation.[44] The release under continuous agitation was practically complete after 35 days, whereas it reached only 60% under intermittent stirring.

Leuprolide mass balance was considered satisfactory for both microparticle formulations even if a slight deviation from 100% mass balance was observed (data not shown). This behavior was assumed to be the result of technical difficulties in recovering all the material from the vial and the filter.

Microparticle ML and Hydration

The ML profiles of leuprolide-loaded microparticles are shown in Figure 3. Within 4 days, RG502H microparticles were characterized by MLs of 3.7%

(once-a-week agitation) and 27.4% (continuous agitation). ML was complete after 28 days with continuous stirring, whereas it reached approximately 60% under intermittent agitation (Fig. 3). The ML rate constants were 0.080 and 0.025 day^{-1} under continuous and once-a-week agitation, respectively. From the low initial ML observed, it can be speculated that the initial leuprolide release was caused mainly by diffusion of the peptide located on the particle surface and in particle pores accessible by water and not by polymer degradation and/or erosion (ML is an indicator of polymer erosion once a critical MW is achieved).[44] RG503H microparticle ML profiles were similar during the first 4 days under both release conditions, whereas later, the ML was greater under continuous stirring than under a once-a-week agitation. ML was complete after 35 days under continuous agitation, whereas it reached almost 80% under once-a-week agitation (Fig. 3). For RG503H, ML rate constants were 0.068 and 0.039 day^{-1} under continuous and intermittent agitation, respectively. RG502H and RG503H ML profiles were similar to the *in vitro* release pattern.[45] As observed in Figure 3, the differences in ML for the two agitation regimens were much more evident in the case of RG502H than in RG503H. This behavior can be ascribed to the different microparticle characteristics influencing the diffusion of soluble oligomers and monomers in the bulk medium and, therefore, the ML. In particular, RG502H microparticles were characterized by a mean diameter larger than that of RG503H microparticles and, consequently, by a smaller surface area. Both features generate unfavorable conditions for fast by-product diffusion from the polymer matrix in the release medium. In fact, RG502H microparticle size confers longer diffusion pathway and a lower surface area in contact with the buffer solution. Then, as experimentally observed (Fig. 3), a different stirring regimen may generate important differences in ML. On the contrary, the agitation conditions did not affect RG503H ML profiles so deeply because by-product diffusion is facilitated even without stirring by the smaller size and larger exposed surface area.

Additionally, the possible contribution of the acidic microenvironment cannot be excluded *a priori* and should be discussed. In fact, a pH drop has been observed within RG503H microparticles incubated in phosphate-buffered saline at 37°C under continuous shaking. In this specific case, the acidic microenvironment is dependent on the particle sizes; large (38 μm), medium (24 μm), and small (14 μm) particles showed an average pH of 5.4, 6.2, and 7.2, respectively.[22,46] In large and medium particles, the central part reached pH values as low as 1.5.[22] Because RG503H microparticles are characterized by small dimensions, the internal microenvironment should not be very acidic. On the basis of these estimations,[22] RG502H microparticles, should develop an acidic microenvironment, but because the dimensions are smaller than 200–300 μm,[47] the autocatalysis phenomenon should be too mild to lead to heterogeneous degradation.[19] Nevertheless, a complete understanding of this process is still missing and contrasting results have been reported.[19,48]

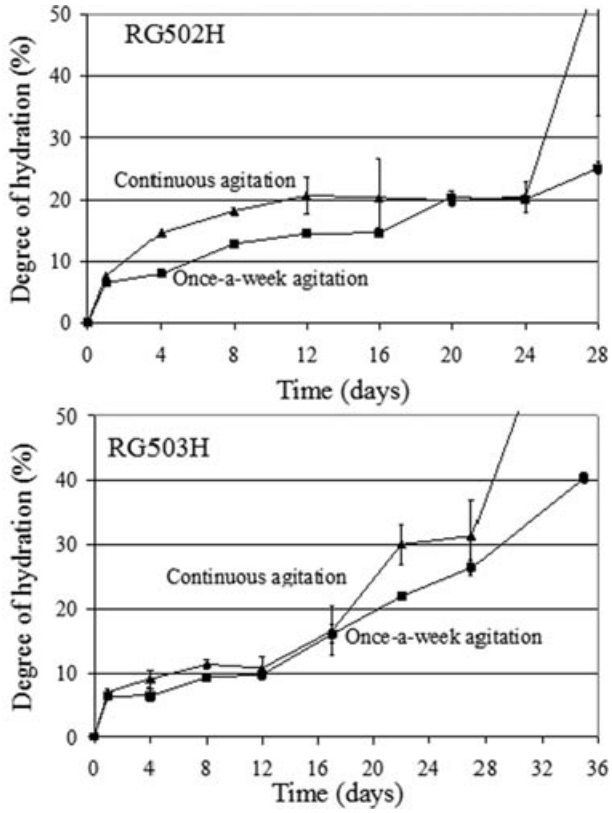

Figure 4. Hydration profiles of leuprolide acetate-loaded microparticles prepared with polymers RG502H and RG503H, incubated under continuous or once-a-week agitation at 37°C in 0.1 M phosphate buffer solution (pH 7.4).

The hydration degree of leuprolide-loaded microparticles under both agitation regimens increased gradually over time, as shown in Figure 4. However, there was no significant difference in the hydration profiles under different stirring conditions. In fact, profiles were almost overlapping until day 24 for RG502H (20.48%, continuous; 20.02%, once-a-week) and 27 for RG503H (31.34%, continuous; 26.41%, once-a-week). A difference was observed on day 28 (RG502H) and 35 (RG503H), even though the large standard deviations observed limit the significance of this result. It can be stated that DH did not play a so significant role as did agitation and particle surface area on peptide release. In fact, PLGA, due to its hydrophobicity, absorbs only small amounts of water within the matrix (e.g., RG503H ~3%), which is responsible for glass–rubber transition temperature depression and polymer hydrolysis.[49,50] Because

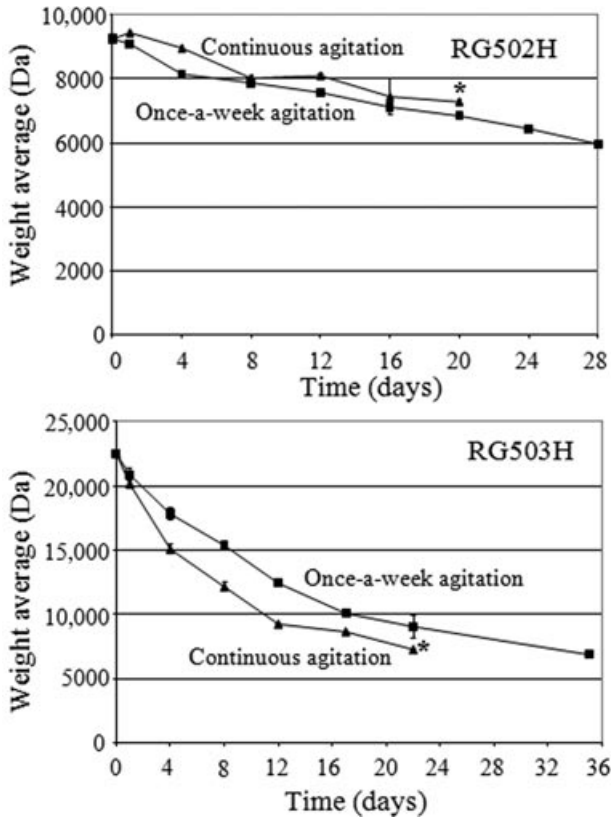

Figure 5. Polymer mean molecular weight of microparticles prepared with polymers RG502H and RG503H and incubated under continuous or once-a-week agitation at 37°C in 0.1 M phosphate buffer solution (pH 7.4). *, insufficient material to perform the analysis.

the DH, extrapolated with the present experimental setup, takes into account the total water wetting the system (i.e., within the matrix, filling the pores, and on the surface), one could expect, contrarily to the obtained results, particles bearing a larger surface taking up more water.

Polymer Degradation

Polymer mean MW during *in vitro* incubation is shown in Figure 5. There were no considerable differences in polymer MW reduction rates when microparticles were subjected to continuous (RG502H, 0.035 day^{-1}; RG503H, 0.050 day^{-1}) or once-a-week (RG502H, 0.030 day^{-1}; RG503H, 0.038 day^{-1}) agitation. Obviously, RG502H is expected to degrade faster and to reach critical MW (soluble oligomers) sooner than RG503H, but because of smaller size and larger surface area (approximately six times higher), the rate of MW decrease is similar under once-a-week agitation for the two polymers (RG502H, 0.030 day^{-1}; RG503H, 0.038 day^{-1}) and even higher for RG503H under continuous stirring (k, 0.050 day^{-1}). Theoretically, different stirring conditions could provoke a change in the rate of by-product diffusion out of the microparticles. As previously described, degradation by-product accumulation produces an acidic microenvironment, which may lead to heterogeneous degradation. This event should not occur due to the small diffusion path length.[19] In addition, in a previous work it was shown that PLGA MW reduction rate was equal when particles (30–70 μm) were incubated in bulk water or in 90% relative humidity environment.[49] This means that even in the absence of bulk water surrounding the microparticles, the acidic by-product accumulation within the particle matrix is not appreciably affecting the rate of the degradation process. However, pockets of larger MW changes due to microenvironment cannot be excluded. Polymer degradation profiles (MW reduction) did not follow similar trends of microparticle ML or leuprolide *in vitro* release profiles (Fig. 3). In fact, stirring regimen has a small effect on degradation rate, whereas the diffusion of the soluble oligomers and then the release are strongly affected. The loss of polymer mass creates porosity in the microparticle matrix, enhancing the peptide diffusion coefficient.[51] Peptides are (generally) high-MW hydrophilic compounds characterized by a very low diffusion coefficient through the hydrophobic polymer matrix.[51] Then, as previously mentioned, only a fraction of the API is released by diffusion, whereas the majority of release is due to matrix erosion consequent to polymer degradation. Subsequently, it is speculated that pore formation (necessary for protein and peptide release) is largely influenced by the agitation regimen.

Mathematical Modeling

The mathematical model used is applicable to spherical PLGA particles where drug loading is greatly higher than the drug solubility in the polymer (dispersed drug system). In fact, leuprolide acetate can be considered practically insoluble in PLGA, even considering the small amount of water absorbed in the microparticles upon incubation. In fact, this water fraction has been seen to correspond to unfreezable water[49] strongly bound to polymer hydrophilic portions.[52] Anyway, leuprolide acetate solubility should be lower in RG503H than in RG502H. Moreover, leuprolide diffusion coefficient increased with time as a result of MW decrease and polymer ML. Polymer degradation constants of both batches according to MW reduction or ML were determined. The best fitting between calculated and experimental data was obtained combining Eqs. 5 and 3. This suggests that the diffusion coefficient increase was better described by ML rather than by polymer MW decrease. In fact, when Eq. 4 was combined with Eq. 3, f_2 values were 46.61 (RG502H) and 15.07 (RG503H) under continuous agitation and 31.30 (RG502H) and

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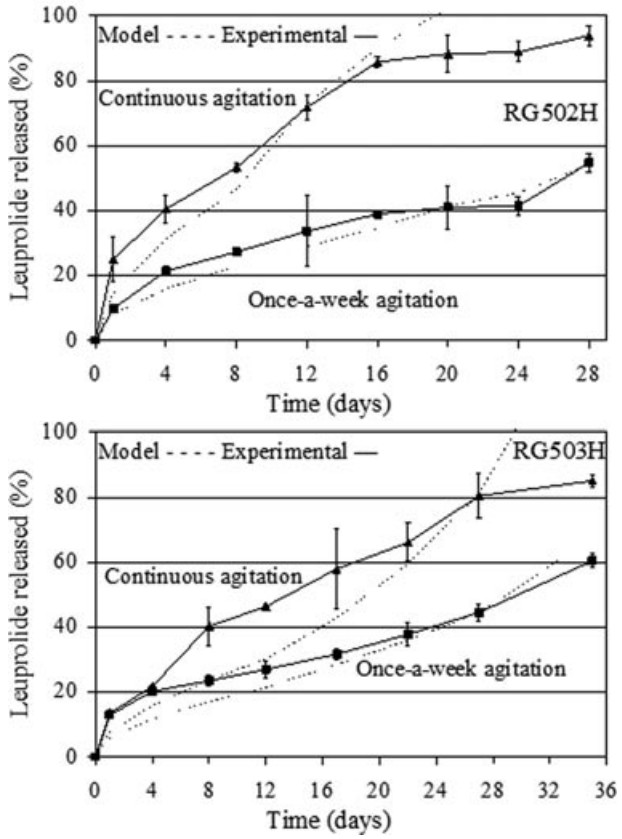

Figure 6. Modeled and experimental leuprolide *in vitro* release profiles under continuous or once-a-week agitation at 37°C in 0.1 M phosphate buffer solution (pH 7.4) from microparticles prepared with polymers RG502H and RG503H.

26.16 (RG503H) under once-a-week agitation. An f_2 value smaller than 50 means that curves are not equivalent. In Figure 6, the fitting of the model integrated with Eq. 6 to the experimental data is reported and it shows a better agreement with RG502H than with RG503H. However, f_2 values were greater than 50 under once-a-week agitation for both polymers (72.14 and 63.23 for RG502H and RG503H, respectively), whereas they were below 50 under continuous agitation regimen (42.72 and 33.55 for RG502H and RG503H, respectively).[39] An f_2 value equal to or greater than 50 ensures profile similarity and the sameness or equivalence of the two curves. The data provide reasonable support, albeit not convincing, that *in vitro* release profiles correlate well with the model under once-a-week agitation, whereas the same similarity cannot be established under continuous agitation. Previous *in vivo* studies[53] with leuprolide-loaded microparticles prepared using the same polymers have shown testosterone suppression duration compatible with the *in vitro* release profiles obtained under once-a-week agitation regimen presented in this study. This finding can be explained if it is considered that PLGA microparticles, once injected subcutaneously or intramuscularly, will not experience the same motion and turbulence occurring in a continuously stirred vial.

CONCLUSIONS

This work highlights the importance of *in vitro* release conditions on peptide release behavior. In particular, the agitation regimen used in the release studies appeared to influence, to a large extent, the polymer ML, which, in this specific case, showed a trend similar to that of the drug release profiles. This is consistent with the fact that perfect sink conditions should be achieved only in the continuously stirred vial. It can be speculated that a liquid boundary layer surrounding the microparticles stirred weekly, slowing down both peptide and oligomer/monomer diffusion, is relevant in polymer erosion and drug release. These findings are of noteworthy importance because *in vitro* release studies are commonly used to assess batch-to-batch reproducibility and to predict the *in vivo* formulation behavior.[54]

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