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Characterizing release mechanisms of leuprolide acetate-loaded PLGA microspheres for IVIVC development I: *In vitro* evaluation

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ABSTRACT

Release testing of parental controlled release microspheres is an essential step in controlling quality and predicting the duration of efficacy. In the first of a two-part study, we examined the effect of various incubation media on release from leuprolide-loaded PLGA microspheres to understand the influence of external pH, plasticization, and buffer type on mechanism of accelerated release. PLGA 50/50 microspheres encapsulating ~5% w/w leuprolide were prepared by the double emulsion-solvent evaporation method with or without gelatin or by the self-healing encapsulation method. The microspheres were incubated at 37 °C up to 56 days in various media: pH 5.5, 6.5, and 7.4 phosphate buffered-saline (PBS) containing 0.02% Tween 80; pH 7.4 PBS containing 1.0% triethyl citrate (PBStc); and pH 7.4 HEPES buffered-saline containing 0.02% Tween 80 (all media contained 0.02% sodium azide). The recovered release media and microspheres were examined for released drug, polymer molecular weight (Mw), water uptake, mass loss, and BODIPY (green-fluorescent dye) diffusion coefficient in PLGA. After the initial burst release, release of leuprolide from acid-capped PLGA microspheres appeared to be controlled initially by erosion and then by a second mechanism after day 21, which likely consists of a combination of peptide desorption and/or water-mediated breakage of pore connections. PBStc and acidic buffers accelerated degradation of PLGA and pore-network development and increased BODIPY diffusion coefficient, resulting in faster release. Release of leuprolide from the end-capped PLGA showed similar trends as found with acid capped PLGA but with a longer lag time before release. These data provide a baseline mechanistic signature of *in vitro* release of leuprolide for future comparison with corresponding *in vivo* performance, and in turn could lead to future development of rational *in vitro-in vivo* correlations.

1. Introduction

Long-acting release drug products (LARs) are important tools in our arsenal to improve drug therapy when sustained exposure of the drug is desired. Apart from non-biodegradable implants, the most often used LARs are microspheres prepared from biocompatible polymers such as poly(lactic-*co*-glycolic) acid (PLGA), as represented by the Lupron Depot® [1]. Release testing of these controlled release microspheres is essential for predicting the duration of efficacy and monitoring batch-to-batch variation in product quality.

In vitro release of LAR PLGA formulations has been examined in various ways under conditions mimicking the physiological environment [2–7]. However, the *in vivo* environment is complicated due to the presence of the foreign body response, exposure with endogenous components, and poorly defined hydrodynamics [8,9], each of which may contribute to differences in the *in vivo* release kinetics relative to that observed *in vitro* for PLGA microspheres [10]. A majority of reports have shown that degradation kinetics and drug release kinetics of PLGA microspheres tend to occur faster *in vivo* than *in vitro* [11,12]. Therefore, it is important to understand the mechanisms causing faster polymer degradation and drug release *in vivo*, leading to development of rational mechanism-based *in vitro-in vivo* correlations (IVIVCs).

Drug release mechanisms from PLGA microspheres are typically governed by an interplay of water absorption, hydrolysis, erosion, and diffusion through the PLGA polymer matrix [13]. Direct measurement of these mechanistic parameters *in vitro* and *in vivo* could reasonably lead to a mechanism-based IVIVC approach beyond current empirical

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IVIVC practices [12,14,15]. In this study, in order to begin our mechanistic approach, we examined the effect of various incubation media on release kinetics, and the associated mechanistic indicators of release, from 3 formulations of leuprolide-loaded PLGA microspheres *in vitro*. Leuprolide was selected, as it is a suitable model peptide drug, which has been used clinically in numerous PLGA depots for many years. The peptide also represents the luteinizing hormone-releasing hormone analogs, which are small, highly water-soluble and cationic peptides with excellent physical and chemical stability. Leuprolide/PLGA depots are also actively being developed as complex generic drug products. Release conditions were selected based on hypothesized potential mediators of accelerated release *in vivo* relative to *in vitro* of PLGA microspheres, such as plasticization, local acidity, and different buffering species. In future follow-on studies, *in vivo* experiments with the formulations described here will be used to develop mechanism-based IVIVCs.

2. Materials and methods

2.1. Materials

Leuprolide acetate with purity >98% by HPLC analysis was purchased from Shanghai Soho-Yiming Pharmaceuticals Co. Ltd. (Shanghai, China). Free-acid terminated PLGA with a 50/50 ratio of lactic to glycolic acid (L/G ratio) and an inherent viscosity (i.v.) 0.37 dL/g (RESOMER RG503H) and ester end-capped PLGA with an L/G ratio 50/50 and an i.v. 0.60 dL/g were obtained from Evonik Nutrition & Care GmbH (Essen, Germany) and LACTEL Absorbable Polymers (Birmingham, AL, USA), respectively. Gelatin (type A derived from porcine skin with ~300 Bloom number) was purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA). Polyvinyl alcohol (25 kDa, 88% hydrolyzed) was purchased from Polysciences, Inc. (Warrington, PA, USA). BODIPY FL was purchased from Life Technologies Corporation (Grand Island, NY, USA). All other chemicals used were of analytical grade or purer.

2.2. Preparation of PLGA microspheres loaded with leuprolide

Leuprolide PLGA microspheres were prepared by two methods, the double emulsion-solvent evaporation method that is employed to manufacture commercially available Lupron Depot® and the more recently developed novel self-healing microencapsulation method [16]. The former method was used to prepare leuprolide microspheres from free-acid terminated PLGA in this study. Briefly, 400 mg of PLGA dissolved in 1.75 mL of methylene chloride and 170 μL of aqueous solution containing leuprolide at 300 mg/mL with or without 8 mg of gelatin warmed at 60 °C were homogenized at 10,000 rpm for 1 min by using a Tempest IQ^2 homogenizer (The VirTis Co., Gardiner, NY, USA) to form W/O emulsion. Then, this emulsion was cooled to 15 °C and then was vortexed with 4 mL of 2% PVA using a Vortex-Genie 2 (Scientific Industries, Inc., Bohemia, NY, USA) at speed 7 for 20 s to formulate W/O/W emulsion. This emulsion was stirred in 100 mL of 0.5% PVA bath for 3 h to allow methylene chloride to evaporate. Hardened microspheres were harvested and screened to between 63 and 90 μm, washed with ddH_2O and then freeze-dried with approx. 12% (w/w) mannitol as a cryoprotectant.

To prepare microspheres by the self-healing microencapsulation method, blank microspheres were initially prepared from ester end-capped PLGA by using the double emulsion-solvent evaporation method. Four hundred milligrams of ester end-capped PLGA and 12.5 mg of zinc carbonate, as a pore-forming agent, were dissolved/suspended in 1.0 mL methylene chloride and were emulsified with 200 μL of 10 mM phosphate buffered saline (pH 7.4) aqueous internal phase containing 625 mg/mL trehalose using the homogenizer at 10,000 rpm for 1 min. Then, the single emulsion was vortexed with 4 mL of 5% PVA at maximum speed for 15 s to form a double emulsion. After stirring in 0.5% PVA solution for 3 h to harden the microspheres, the microspheres were harvested and screened to between 90 and 125 μm, washed with ddH_2O and then freeze-dried. Loading leuprolide to the preformed blank microspheres was performed by incubating 200 mg of the microspheres in 600 μL of 0.1 M HEPES buffer (pH 7.4) containing 250 mg/mL leuprolide at 4 °C for 42 h. Then, 600 μL of HEPES was added to the suspension and the microspheres were further incubated at 43 °C for 48 h to complete self-healing. The subsequent microspheres were washed with ddH_2O and the 63–125 μm fraction was collected and freeze-dried. For selection of this formulation, it is important to note that $ZnCO_3$, $Mg(OH)_2$, and $MgCO_3$ belong to a special class of excipients for PLGA, which under suitable compositions, have pH-modifying, release-enhancing, and pore-forming functions [17,18]. As recently reviewed [19], during release of drug the co-encapsulated poorly soluble bases react with water-soluble degradation products to form water-soluble osmotic salts (*i.e.*, Mg^{2+} or Zn^{2+} carboxylates), which steadily create much higher water levels and porosity in the polymer to initiate continuous drug release [17]. We found that when incorporating $ZnCO_3$ into self-healing PLGA microspheres encapsulating leuprolide that continuous release *in vitro* release and testosterone suppression *in vivo* was observed for 6–8 weeks, respectively [16].

Formulations prepared by the double emulsion method with or without gelatin or by the self-encapsulation method are abbreviated as GLUP, LUP, and SM, respectively, unless otherwise noted.

2.3. Size analysis

Microspheres were suspended in ddH_2O and imaged under a microscopy. Size of microspheres in the images was quantified using ImageJ software to measure the diameter of a minimum of 300 microspheres for each formulation. Another size analysis using a dynamic light scattering (DLS) (Mastersizer 2000, Malvern Instruments Ltd, Worcestershire, UK) was also performed. The microspheres were dispersed in ddH_2O and measurements were taken 5 times for each sample.

2.4. Determination of loaded leuprolide in microsphere

Loading of leuprolide was determined by two-phase extraction followed by UPLC analysis. Briefly, 5 mg of leuprolide-loaded PLGA microspheres were dissolved with 750 μL of methylene chloride and then 750 μL of 50 mM pH 4.0 sodium acetate buffer was added, and then vortexed for 1 min. After centrifugation at 3400 ×*g* for 4 min, 500 μL of the aqueous phase was collected and replaced with the same volume of fresh buffer solution. This extraction was repeated with the same sodium acetate buffer 5 times and then with 50 mM sodium acetate buffer pH 4.0 containing 1 M sodium chloride for additional 6 times (11 total extractions). The amount of leuprolide in the combined aqueous phase was determined using ultra performance liquid chromatography (UPLC) (Acquity, Waters, Milford, MA, USA) composed of a BEH C18 column (Waters, Milford, MA, USA) and a UV detector (UV wavelength = 280 nm). A gradient elution using acetonitrile with 0.1% (v/v) trifluoroacetic acid (TFA) and ddH_2O with 0.1% TFA at a flow rate of 0.5 mL/min was performed as follows: 0 min (25:75), 2 min (35:65) and 2.5 min (25:75), and a 1 min recovery.

2.5. Release kinetics of leuprolide from PLGA microspheres

Ten milligrams of leuprolide-loaded PLGA microspheres were suspended in 1 mL of the following releasing media; 10 mM phosphate-buffered saline (PBS) with 0.02% Tween 80 (PBST) at pH 7.4 (PBST7.4), 10 mM PBST at pH 6.5 (PBST6.5), 10 mM PBST at pH 5.5 (PBST5.5), 10 mM PBS with 1.0% (w/w) triethyl citrate (TC) (PBStc) and 10 mM HEPES-buffered saline (HBS) with 0.02% Tween 80 (HBST) at pH 7.4 (all contained 0.02% sodium azide). Then, the microspheres were incubated at 37 °C with agitation at 240 rpm (KS 130 basic, IKA Works Inc., Wilmington, NC, USA) and after centrifugation at 6000 ×*g* for 5 min, the entire media were collected and replaced

with 1 mL of the same buffer at days 1, 3, 7 and every 7 days up to day 56. To avoid particle loss during sampling, the supernatant was carefully removed using a fine tip pipette and if any visible microspheres were taken up, the supernatant was placed back in the tubes and the centrifugation/sampling was repeated. In some cases where some floating microspheres were visible, the tubes were tilted to one side to move the particles away from the sampling tip. The amount of leuprolide released to the release media was determined by UPLC. After the entire incubation, the remaining leuprolide was extracted by the method mentioned in the Section 2.4 and was determined by UPLC. A leuprolide solution at 50 μg/mL dissolved in each releasing medium was incubated at 37 °C in parallel with the release experiment without media replacement, and aliquots were subjected to UPLC analysis to examine the peptide stability during the release study.

2.6. Determination of Mw decline

Each formulation was incubated in the same manner including replacement of media as performed in the release study. After collected from the release media, microspheres were washed with ddH_2O and dried at room temperature under reduced pressure. Tetrahydrofuran (THF) was added to dissolve the microspheres to obtain approx. 2 mg/mL polymer concentration and then supernatants were subjected to gel permeation chromatography using two styragel columns (HR 1 and HR 5E columns, Waters, Milford, MA, USA) with Waters 1525 HPLC system. Samples were eluted with THF at 0.5 mL/min and monitored by refractive index detection. Breeze software was used to obtain weight-averaged molecular weight (Mw). The Mw was calculated using monodisperse polystyrene standards.

2.7. Quantification of microsphere water uptake and mass loss

Microspheres were incubated as in the release kinetics study and collected on pre-weighed nylon membrane filters under vacuum and washed off with ddH_2O. Then, the surface water was removed by further filtration under vacuum for 5 s and the wet weight of the microspheres was immediately measured. The samples were dried at room temperature under reduced pressure to a constant weight and then the dry weight was recorded. To correct for the interparticle water, dry microspheres were suspended in each buffer solution at 4 °C and the wet and dry weights were measured after filtering and drying, respectively, as described above. The weight difference between wet and dry particles is used to estimate the fraction of interparticle water (W_i), as defined by

$$W_i \equiv \frac{W_1' - W_2'}{W_2'},$$

where W_1' and W_2' are the weights of wet microspheres and dry microspheres, respectively, after immediate collection at $t = 0$ [20]. The water uptake of microspheres at time t ($W_P(t)$) was thus estimated by

$$W_p(t) = \frac{W_1 - W_2 - W_2 \times W_i}{W_2},$$

where W_1 and W_2 are the wet and dry microsphere weights after incubation at 37 °C in the various buffers at time t. The percent mass loss was calculated according to the following equation:

$$\%\text{mass loss} = \frac{W_0 - W_2}{W_0} \times 100,$$

where W_0 is the initial weight of dry microspheres excluding the cryoprotectant.

2.8. Imaging of BODIPY uptake and analysis of effective BODIPY diffusion coefficients in PLGA microspheres

PLGA microspheres were incubated under different release media for various times as described in the release study and then transferred into 1 mL of the same media but containing BODIPY FL at 5 μg/mL, which was pre-warmed at 37 °C, followed by incubation at 37 °C for 3 h under the same agitating condition. Probe distribution in PLGA microspheres was imaged by confocal laser scanning microscopy (Nikon A1, Tokyo, Japan). Pixel intensities of BODIPY (I) at position (r) between the edge of the microspheres to the center of the microspheres (radius, a) were extracted using ImageJ software and were fit by the following equation of Fick's second law of diffusion:

$$\frac{C}{C_0} = \frac{1}{r/a}\sum_{n=0}^{\infty}\left(erfc\frac{(2n+1)-r/a}{2\sqrt{Dt/a^2}} - erfc\frac{(2n+1)+r/a}{2\sqrt{Dt/a^2}}\right),$$

where t is time exposed with dye, D is the effective diffusion coefficient in the microspheres, and C and C_0 are the dye concentration in the microspheres and at the surface, respectively (C/C_0 is equivalent to I/I_0). The fitting was carried out using DataFit software (Version 9) to obtain the value of D ($R^2 > 0.8$) as reported previously [21].

2.9. Quantification of water-soluble acid released from PLGA microspheres

At predetermined time points prior to active release of leuprolide, such as days 1, 3, 7, and 14 for PBStc and the same schedule with additional day 21 for PBST7.4, PBST5.5, and HBST, 800 μL of supernatant of various incubation buffers were collected into 2-mL glass vials and then freeze-dried, followed by further drying under reduced pressure at room temperature for a day. The dried water-soluble acid samples were reconstituted with 800 μL of dehydrated acetonitrile and 500 μL of the solution was subject to derivatization with *p*-bromophenacyl bromide (*p*BPB) [22]. Before the derivatization, water-soluble acid was titrated with methanolic potassium hydroxide using a color indicator methanolic phenolphthalein. After drying the solution with nitrogen gas blow at 40 °C, 500 μL of 10 mM *p*BPB dissolved in dehydrated acetonitrile was added and immediately vortexed. Derivatization was performed at 50 °C for 3 h in a dark place. Derivatized water-soluble acid samples were subjected to UPLC analysis with a BEH C18 column and following conditions: elution, gradient with acetonitrile and water at a ratio of 40:60 to 0.8 min, 50:50 to 2.5 min, 60:40 to 3.5 min, and 70:30 to 4.5 min, followed by 100% acetonitrile wash for 3 min and equilibration at the initial solvent ratio for 3.5 min; flow rate, 0.3 mL/min; detection wavelength, 254 nm. For the preparation of monomers or dimer standards, L-lactic acid, glycolic acid, and their mixture were heated at 175 °C for 3 h under reduced pressure. Monomers and dimers were derivatized with *p*BPB in the same manner for peak identification. Water-soluble acids were quantified using a standard curve of L-lactic acid with 98% purity.

2.10. Determination of hydrated glass transition temperature (Tg)

LUP microspheres (10 mg) were incubated in 1 mL of PBST and PBStc at 37 °C for one day. The samples were centrifuged to remove supernatant and washed by water 3 times. After the final washing step, a small volume (~100 μL) of water was left to suspend the microspheres and 50 μL of the suspended microspheres were transferred to DSC aluminum pans followed by sealing with hermetic lids. The hydrated Tg was determined with a modulated differential scanning calorimeter (MDSC) (Discovery, TA Instruments, New Castle, DE) as previously reported [23]. Briefly, temperatures were ramped between 5 °C and 75 °C at 3 °C/min, with modulation amplitude of ±1 °C/min and a period of 60 s. All samples were subjected to a heat/cool/heat cycle.

The results were analyzed by using TA TRIOS software, and all measurements were performed in duplicate for each sample.

2.11. Data analysis

The nonlinear regression function of SigmaPlot (Version 13) was used to perform curve fitting to obtain times for 50% release ($t_{50,release}$) and 50% mass loss ($t_{50,erosion}$). The $t_{50,release}$ and $t_{50,erosion}$ were determined by 4 parameters sigmoidal curve fitting and 3 parameters sigmoidal curve fitting, respectively, and the $t_{50,erosion}$ of SM was determined by cubic curve fitting.

Statistical analysis for diffusion coefficient of BODIPY was performed using Prism (Version 6). Data were analyzed with the nonparametric one-way analysis of variance (ANOVA) and *post hoc* Dunnett's multiple comparison tests compared with a standard PBST7.4. A probability level of 5% ($p < 0.05$) was considered to be statistically significant.

3. Results and discussion

3.1. In vitro release

PLGA microspheres commonly degrade and release encapsulated contents faster *in vivo* than *in vitro* and this accelerating reaction is speculated to be due to the inflammatory response against the microspheres and local environmental changes by accumulated endogenous components, such as enzymes and acidic matter [8,9]. We prepared GLUP, LUP, and SM having 77.2 ± 0.6 μm, 77.3 ± 0.5 μm, and 84.2 ± 0.6 μm (mean ± S.E.M.), respectively, determined by microscopic observation, and 81.1 ± 0.1 μm, 79.9 ± 0.1 μm, and 85.4 ± 0.1 μm (volume median diameter ± S.E.M.), respectively, by DLS analysis. Loading of leuprolide (w/w %) in GLUP, LUP, and SM were determined as 5.4 ± 0.1%, 6.3 ± 0.1%, and 4.8 ± 0.1% (mean ± S.E.M., n = 3), respectively. The loading values translated to encapsulation efficiencies of 63.1 ± 1.0%, 62.0 ± 1.6% and <5%, for each of the size-controlled formulations, respectively. Note that the low efficiency of the passive self-healing encapsulation is expected for this formulation [16].

Several studies use a dialysis bag for measuring continuously release of encapsulated drug from PLGA microspheres [14,24]. However, the dialysis membrane typically delays diffusion of the drug to the bulk release buffer and may result in a longer induction phase (between initial burst and active releasing phase) than the method of suspending microspheres in tubes as we employed in this study [24]. In addition, *in vivo* release of leuprolide-loaded PLGA microspheres lacks an induction phase and proceeds in roughly a zero order manner [25]. Since the purpose of this study is to move toward rational IVIVCs, we adopted the simpler method suspending in tubes which also provided a continuous release profile. In order to find potential release conditions for acceleration of *in vitro* release and in the future to compare with *in vivo* release, PLGA microspheres were incubated in various conditions where the pH of PBST was set to 5.5, 6.5, and 7.4 and a different salt buffer solution HBST was used. Additionally, the triethyl citrate plasticizer was added to the standard PBS buffer (PBStc) to increase polymer chain mobility and accelerate degradation of PLGA.

As shown in Fig. 1, every formulation exhibited a low initial burst occurring within the first day of incubation. A higher initial burst was observed for GLUP microspheres independent of media type, and this effect was most likely caused by the presence of gelatin inducing water uptake into the micropores of the microspheres [2,26]. Slow and continuous release was observed thereafter for the acid-capped 503H preparations and a lag phase was observed for the SM formulation, with the latter more pronounced than previously reported, likely due to the larger microsphere diameter used here relative to that used in our previous paper (where little lag time was observed owing to the $ZnCO_3$ pore-forming agent) [16]. Both the presence of plasticizer and reduced pH accelerated the release during the active release period for all three formulations in the general order of PBStc > PBST5.5 > PBST6.5 > HBST7.4 > PBST7.4. It is interesting to note that PBStc accelerated release by shifting the initiation point of the active releasing phase (*i.e.*, where very rapid drug release is occurring) approximately 14 days earlier than a standard condition buffer PBST7.4 for all formulations. Swapping HEPES, a zwitterion buffer salt, for phosphate in the media also showed a marginal increase in release in the latter part of the release phase for each formulation. As HEPES (pKa = 7.55) has weaker buffer capacity than phosphate (pKa = 6.82) in mildly acidic region, it is assumed that acid generated by degradation of PLGA probably

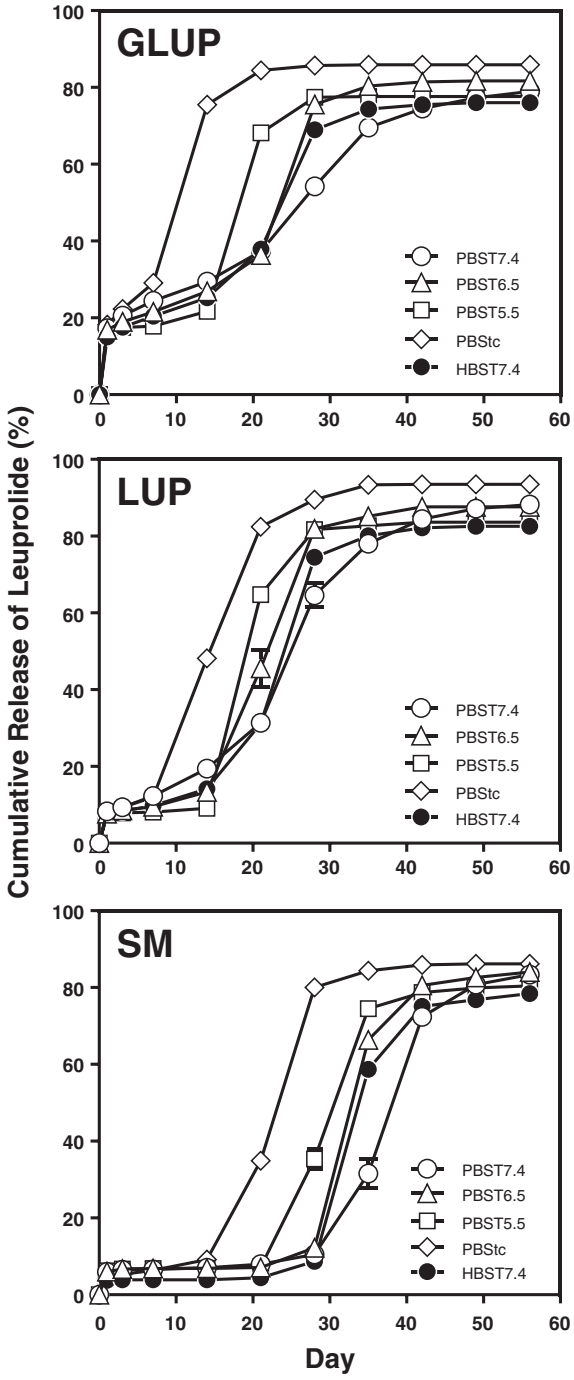


Fig. 1. Cumulative amount of leuprolide released from PLGA microspheres. GLUP, LUP, and SM were incubated in PBST7.4 (○), PBST6.5 (△), PBST5.5 (□), PBStc (◇), and HBST7.4 (●) at 37 °C with mild agitation up to 56 days. At pre-determined time points, the supernatant was collected and subjected to UPLC analysis. Quantified leuprolide amount is expressed as cumulative release. Symbols represent mean ± S.E.M. (n = 3).

resulted in a higher decrease in pH in the HEPES than phosphate media, which could have caused faster release in HBST pH 7.4 than in PBST pH 7.4.

It is important to note that from the early Takeda publications it is known that free leuprolide was not completely stable in PBS, leading the Lupron Depot® innovators to examine release based on the amount remaining in the microsphere [27]. Therefore, we monitored this deterioration in all of the biorelevant media [Fig. 1S in Supplementary data] and confirmed that >95% of free peptide was detected at day 7 and >80% at the last time point of incubation. During release evaluation, the media was replaced every 7 days, and therefore, significant deterioration of released leuprolide in the media during the incubation could be averted, as also evidenced by the near complete release relative to the loaded peptide, indicating a good mass balance (Fig. 1).

The onset of active erosion phase for SM formulation composed of ester end-capped PLGA was delayed compared to that for the formulation composed of acid-end group PLGA. Release from PLGA microspheres is governed by degradation of PLGA polymer and its degradation is associated with hydrolysis until low molecular weight monomers and oligomers can dissolve and release from the polymer [28]. Acid-end group PLGA has a high auto-catalytic reaction to enhance the hydrolysis as compared to ester end-capped PLGA as well as a lower molecular weight providing a constant source of water-soluble oligomer [29]. Even though the SM preparation contained a pore-forming agent, $ZnCO_3$, it appeared that a higher level of $ZnCO_3$ would have been needed to create a more continuous release [16].

3.2. Mw decline of PLGA microspheres

As PLGA erosion is strongly influenced by polymer hydrolysis, which contributes to the release of leuprolide [17,18], we also monitored the kinetics of Mw decline. PLGA microspheres were incubated under the same conditions applied to the study of release kinetics and erosion, and at various times intervals the formulations were subjected to Mw analyses. The initial weight averaged Mw of PLGA with acid- and ester-end caps were 37 kDa and 50 kDa, respectively, and these values were roughly maintained even after encapsulation by double emulsion and self-healing loading procedures, respectively. In Fig. 2, the rate of hydrolysis of PLGA from the acid-end group formulations showed a similar trend in the release and erosion behavior, *i.e.*, PBStc > PBST5.5 > PBST6.5 > HBST7.4 > PBST7.4.

For the ester-end capped preparation there was much less difference among the various media with a slight acceleration in the plasticizing media and a very marginal increase in all the other media by the end of the release period. The presence of TC accelerated degradation of PLGA regardless of terminal properties of PLGA as compared to standard condition PBST7.4. GLUP and LUP, which were composed of acid end-group PLGA, were more susceptible to the effect of external acidity in the buffer than the ester-end capped SM, likely due to differences in permeability to acid and the auto-catalytic reaction by carboxyl ends [29,30]. The Mw decline of GLUP and LUP in PBST5.5 were comparable with that in PBStc. It is important to note that during the rapid release phases (7–14 days for PBStc and 14–21 days for PBST5.5) the polymer MW was approaching a very low oligomeric level (≪5000 by 14 and 21 days, respectively), whereas during this period the microspheres in the other buffers still had appreciable molecular weights. In contrast, in the absence of plasticizer, the SM formulation showed no obvious accelerated Mw decline among different pHs until day 21 when the active release phase started. As degradation-derived acids increased hydration of PLGA, the SM formulation also became susceptible to increased degradation in the presence of acidic media, as observed for GLUP and LUP formulations after the active erosion started.

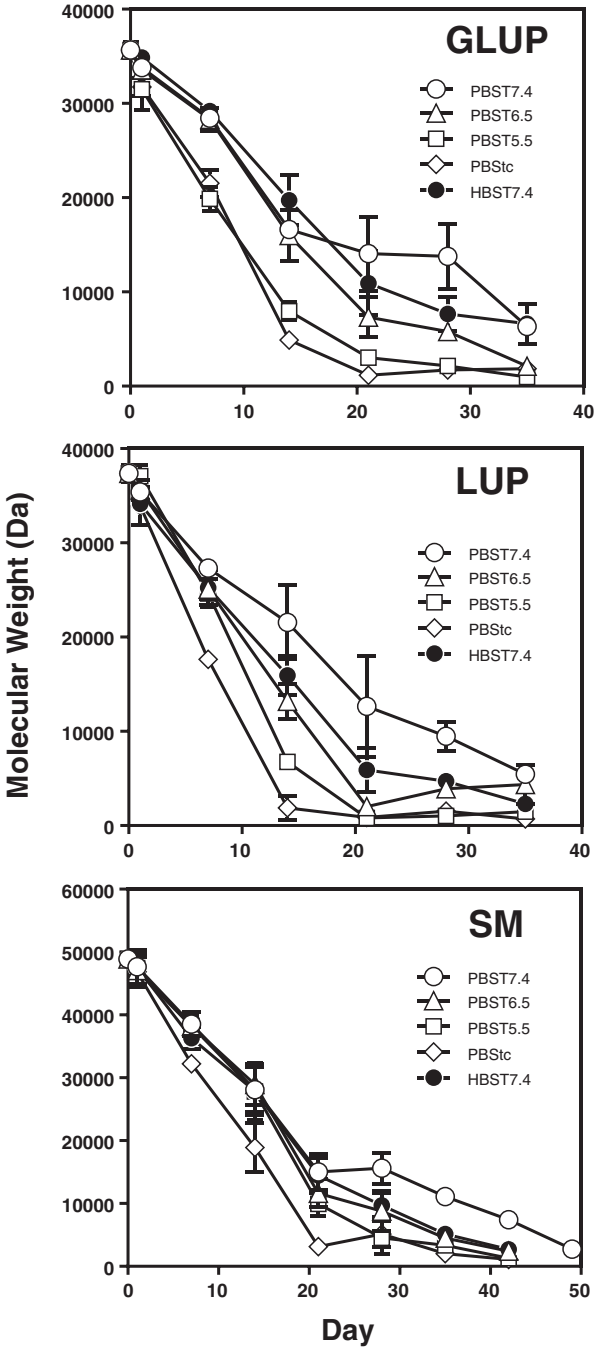


Fig. 2. Kinetics of Mw decline of GLUP, LUP, and SM incubated in PBST7.4 (○), PBST6.5 (△), PBST5.5 (□), PBStc (◇), and HBST7.4 (●). The microspheres were collected and washed with ddH_2O, followed by drying at room temperature under reduced pressure. THF was added to dissolve the microspheres and the supernatant was applied to GPC analysis. Symbols represent mean ± S.E.M (n = 3).

3.3. Water uptake

Water uptake in the microspheres is required to initiate hydrolytic degradation [31]. Acceleration of release in the active release phase under certain conditions has been suggested to be associated with water influx into the water-pore networks of the microspheres [32,33].

It has also been reported that a plasticizer facilitated water invasion into a PLGA film at 1.5 times the amount of a plasticizer-free condition [34]. However, we found that TC, which triggered obvious acceleration

in release from the microspheres and Mw decline, had little impact on the estimated water uptake, as shown in Fig. 3.

Similarly, the acidic conditions minimized any increases in water uptake into the microspheres, owing to higher protonation of the carboxylic acid end groups, making the polymer more hydrophobic. TC and the acidic buffer induced larger degradation, which triggered downstream erosion, diffusion and resulted in subsequent pore-network development by later release times (see particle morphology in Figs. 6 and S2 and later sections).

It is important to note that the water uptake estimated in this section involves both the large amount of water inside the pores within the microspheres and the water in the polymer phase; the former is important for osmotically-induced release and the latter is important for hydrolysis of the polymer. It is also important to note that accurate determination of the water uptake in microspheres requires a correction for interparticle water, which is determined under cold conditions to minimize water uptake by the polymer. This correction means that the actual values in the figure are expected to be slightly different (*e.g.*, slightly

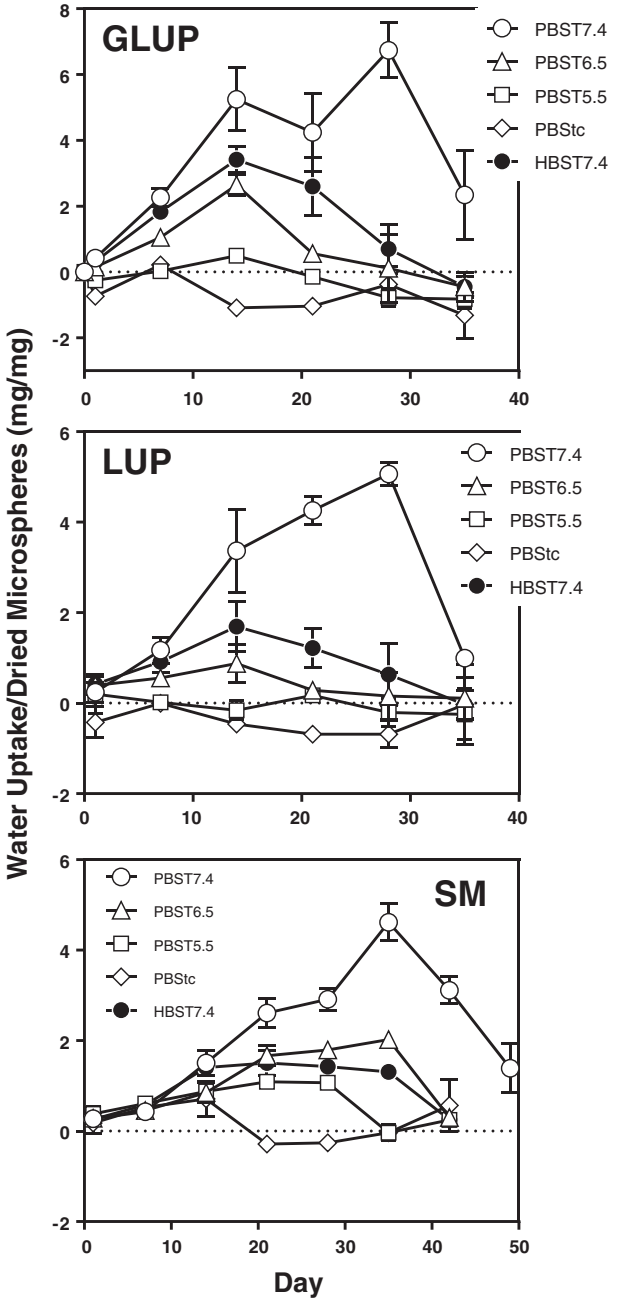


Fig. 3. Kinetics of water uptake into GLUP, LUP, and SM incubated in PBST7.4 (○), PBST6.5 (△), PBST5.5 (□), PBStc (◇), and HBST7.4 (●). The microspheres were collected onto preweighed membrane filters under vacuum. Then, the microspheres underwent washing with ddH_2O and vacuum exposure for 5 s to remove salt and the surface residual water, respectively. The wet weight of the microspheres was immediately measured and then the dried weight was recorded after drying at room temperature under vacuum to a constant weight. Water uptake was corrected with interparticle water and calculated as mentioned in the Section 2.7. Symbols represent mean ± S.E.M (n = 3).

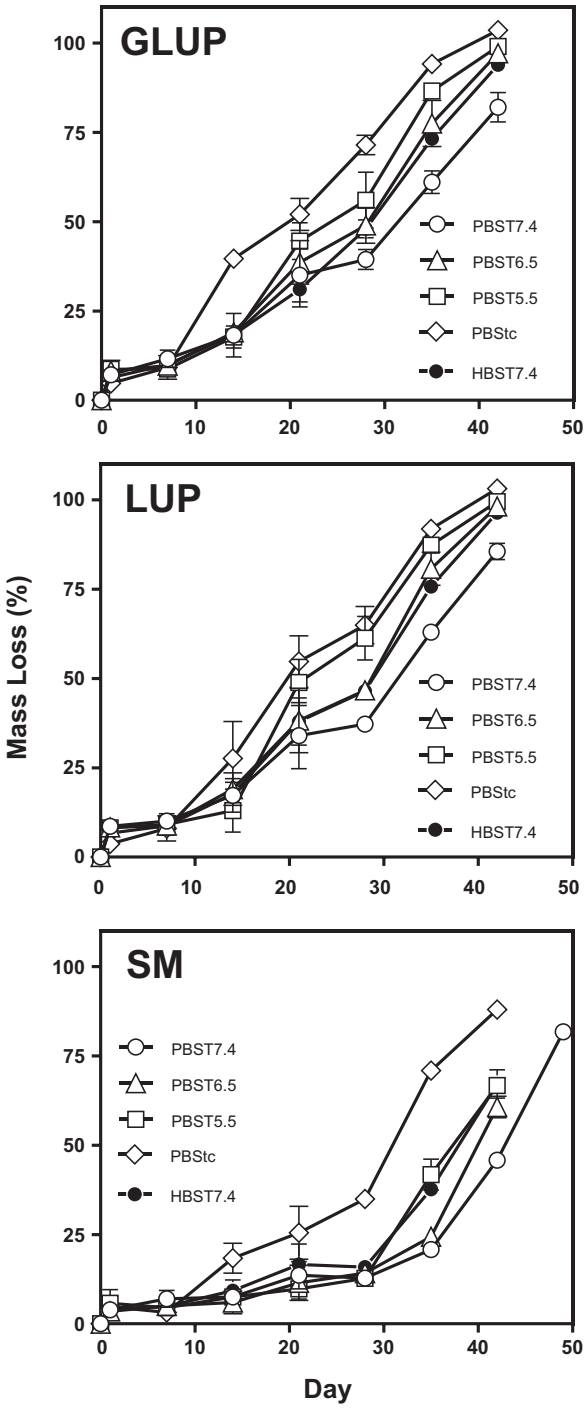


Fig. 4. Kinetics of mass loss of GLUP, LUP, and SM incubated in PBST7.4 (○), PBST6.5 (△), PBST5.5 (□), PBStc (◇), and HBST7.4 (●). The dried weight of the microspheres obtained in the water uptake study was used for calculation of percentage of mass loss as mentioned in the Section 2.7. Symbols represent mean ± S.E.M (n = 3).

different interfacial tension and water viscosity at the higher temperature and during erosion), although any error associated with this correction should be similarly reflected for the water uptake values in each media used.

PBST7.4 induced the largest water uptake in the PLGA microspheres among various incubation media in every formulation and it peaked at day 28 for GLUP and LUP and at day 35 for SM. Those periods were in the middle of the active release phase, as shown in Fig. 1. In addition, the onset of active releasing phase in PBST7.4 corresponded to the moment when water uptake was higher than that in other media conditions, at day 14 for GLUP and LUP and at day 21 for SM.

3.4. Mass loss

If erosion were the only mechanism governing release and peptide is uniformly distributed inside the polymer matrix, the peptide molecules would be expected to be liberated at the similar rate as those polymer degradants. Therefore, release kinetics is commonly correlated to erosion kinetics as an indicator of the degree to which the polymer erosion is controlling the release from the polymer. As shown in Fig. 4, no obvious differences among various incubation media on mass loss were observed during the first week of incubation. Then, from the onset of the active release phase, mass loss proceeded continuously until the end of the incubation.

The presence of TC and external acidity in the buffer accelerated mass loss in the similar trend as the two conditions enhanced the release and Mw decline. In addition, like the trends with degradation kinetics, the erosion kinetics in the acid-capped PLGA microspheres (GLUP and LUP) showed more pronounced effects from these media than observed for the ester-capped formulation (SM).

Fig. 5 shows a relationship between release and mass loss in order to visualize mechanistic progress in comparison to an ideal erosion-mediated release, which is presented as a dotted line. Until the onset of the active release phase where approximately 30% or 10% of leuprolide was released from GLUP and LUP or SM, respectively, in all media except for PBStc, the release proceeded in an erosion-dependent manner. Then, the release was accelerated as compared to the progress of mass loss. By contrast, TC penetrated into the microspheres and caused relaxation of polymer chains, resulting in faster release than mass loss in comparison to other media. TC is well known to plasticize PLGA, as it rapidly reduces Tg of the polymer [34] and enhances healing of the polymer when incorporated [16]. To confirm that the plasticizer rapidly enters the polymer, LUP microspheres were incubated in PBST and PBStc at 37 °C for one day, and then the hydrated Tg was determined to be 30.2 ± 0.1 and 25.0 ± 0.2 (n = 3, mean ± SEM), respectively. The rapid plasticization beyond what occurred in common PBST release media would not be possible without rapid penetration of TC in the polymer from the external solution [34,35].

Table 1 summarizes times for 50% release ($t_{50,release}$) and 50% mass loss ($t_{50,erosion}$) calculated by performing curve fitting as stated in the Section 2.9 and shows $t_{50,release}/t_{50,erosion}$ to visualize the dependency of release on mass loss. As mentioned above, the presence of TC brought a unique acceleration of release and exhibited a lower ratio of $t_{50,release}/t_{50,erosion}$ than any other media. Differences in pH and buffer composition had little impact on the $t_{50,release}/t_{50,erosion}$ ratio, which were all <1, which strongly suggests an additional release mechanism besides erosion contributed to the release of peptide.

Determination of $t_{50,release}$ and $t_{50,erosion}$ of GLUP, LUP, and SM incubated in PBST7.4, PBST6.5, PBST5.5, PBStc, and HBST7.4 was performed by nonlinear regression curve fitting, as mentioned in the Section 2.9, for the results obtained in Figs. 1 and 4, respectively. Both $t_{50,release}$

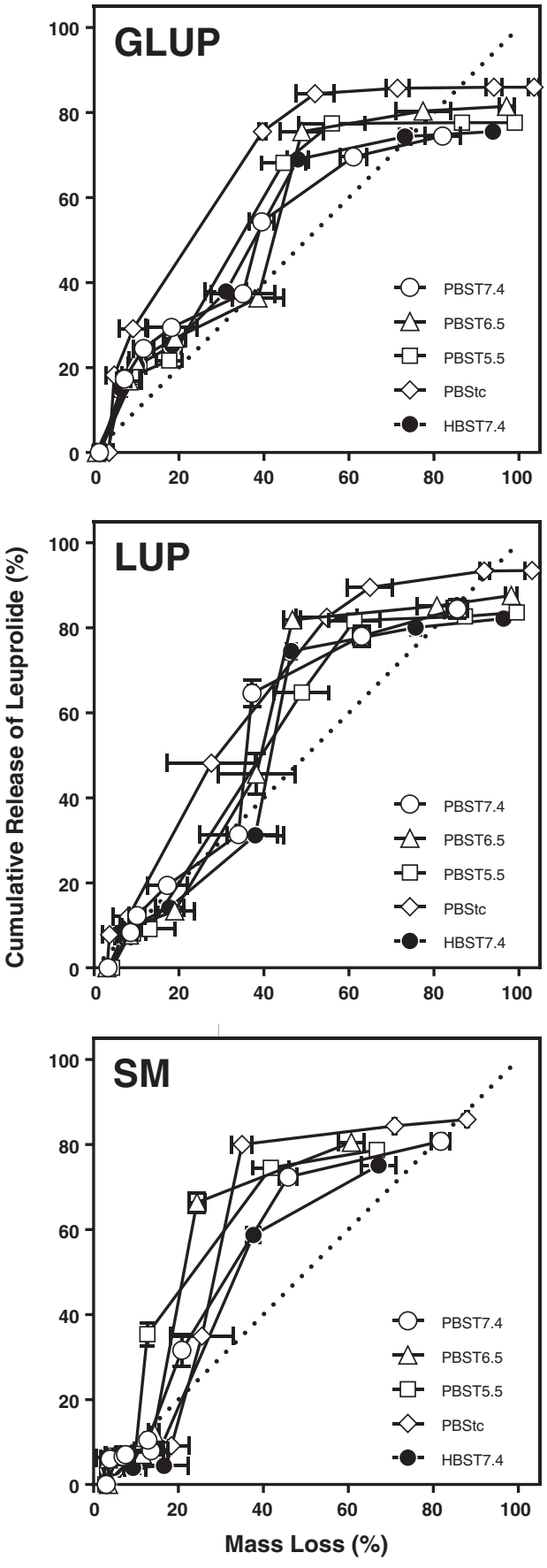


Fig. 5. Relationship between cumulative release of leuprolide and mass loss of GLUP, LUP, and SM incubated in PBST7.4 (○), PBST6.5 (△), PBST5.5 (□), PBStc (◇), and HBST7.4 (●). Dotted lines indicate an ideal erosion-mediated release, y = x, as a reference. Symbols represent mean ± S.E.M (n = 3).

Table 1
Characteristic times of release and erosion, $t_{50,release}$ and $t_{50,erosion}$ (in days).

Formulation	Item	PBST7.4	PBS6.5	PBS5.5	PBStc	HBST7.4
GLUP	$t_{50,release}$	25.5 ± 0.5	23.3 ± 0.3	18.2 ± 0.1	10.5 ± 0.1	22.7 ± 0.3
	$t_{50,erosion}$	37.7 ± 9.5	32.2 ± 4.1	26.9 ± 2.1	21.6 ± 1.3	33.2 ± 2.6
	$t_{50,release}/t_{50,erosion}$	0.68	0.72	0.68	0.49	0.68
LUP	$t_{50,release}$	24.5 ± 0.4	21.3 ± 0.2	19.4 ± 0.2	14.3 ± 0.1	23.0 ± 0.2
	$t_{50,erosion}$	42.9 ± 12.3	32.5 ± 4.3	24.7 ± 1.7	22.9 ± 1.9	33.0 ± 3.6
	$t_{50,release}/t_{50,erosion}$	0.57	0.66	0.79	0.62	0.70
SM	$t_{50,release}$	37.0 ± 0.2	32.6 ± 0.2	29 ± 0.1	22.3 ± 0.1	33.0 ± 0.1
	$t_{50,erosion}$	43.2 ± 0.6	40.3 ± 0.9	38.4 ± 0.9	30.7 ± 2.0	38.5 ± 1.0
	$t_{50,release}/t_{50,erosion}$	0.86	0.81	0.76	0.73	0.86

and $t_{50,erosion}$ are reported as mean ± S.D. with exception for $t_{50,erosion}$ of SM, which is represented as mean ± 95% confidence interval. The ratio $t_{50,release}/t_{50,erosion}$ was calculated based on mean values.

3.5. BODIPY uptake

In order to analyze the status of the polymer for molecular diffusion as a function of release time, PLGA microspheres were incubated briefly with BODIPY dye, which is known to diffuse through solid PLGA over reasonable time scales easily, and monitored by laser fluorescence confocal microscopy [21]. In addition, as a water-soluble compound, the dye can also diffuse into aqueous pores inside microspheres, which provides an image of both polymer microstructure and pore-interconnections inside microspheres (in the cases when dye uptake was carried out long enough) [36]. Fig. 6 shows the representative morphology and dye distribution of the microspheres 7 days after incubation.

In regions where the dye has penetrated the polymer matrix during the brief incubation, the dark regions are associated with aqueous pores since the dye has a very favorable partition for the polymer phase, and the bright regions denote the polymer phase [21]. The presence of PBStc and PBST5.5 caused numerous pores at the vicinity of the surface. The BODIPY uptake micrographs for each of the buffers and formulations are shown as a function of time up to day 28 in Fig. S2. Evidence for more rapid degradation in the presence of PBStc is clearly seen for each of the formulations, with the dye entering more extensively with time, and by day 28 for GLUP and LUP (Fig. S2A and B) the microspheres appeared to be porous (*i.e.*, little or no dark regions) with fully equilibrated dye penetration (see also very low MW of the polymer at this time point in Fig. 2). We speculate that in these formulations when the dye reached deep into the polymer, dye diffusion appeared to be limited *via* polymer-phase diffusion as no discontinuities of dye were observed (which would have suggested pore-diffusion). This is in contrast to those confocal micrographs for the SM microspheres at days 7 and 14 in PBStc, suggesting some degree of a percolating pore network with a noncontinuous dye penetration (Fig. S2C). Similar observations for GLUP and LUP were observed in the PBST5.5 buffers but slightly delayed. These observations suggest that the presence of TC and acidic buffer made the polymer mobile and caused significant changes to the polymer microstructure. By contrast, at times when the BODIPY dye reached deep into the polymer matrix for the higher pH buffers without plasticizer an interconnected pore network seemed more likely (*e.g.*, LUP in PBST and HBST in Fig. S2B).

Based on these images, the effective diffusion coefficient of BODIPY dye was calculated according to Fick's second law of diffusion, whenever diffusion was Fickian and easily fit to the diffusion law in the porous polymer matrix [21], and these values are shown in Fig. 7.

The presence of TC or the acidic pH buffer significantly increased diffusion coefficient as compared to a standard PBST7.4 for all formulations. Particularly for GLUP and LUP formulations, TC in the media resulted in a sustained high diffusion coefficient level, while the acidic pH buffer caused a lowering in diffusion coefficient as incubation proceeded. The SM formulation was composed of a medium Mw PLGA end-capped with ester and the effective diffusion coefficient of BODIPY in this polymer was lower than the other formulations, resulting in no significant accelerating impact on release until day 14.

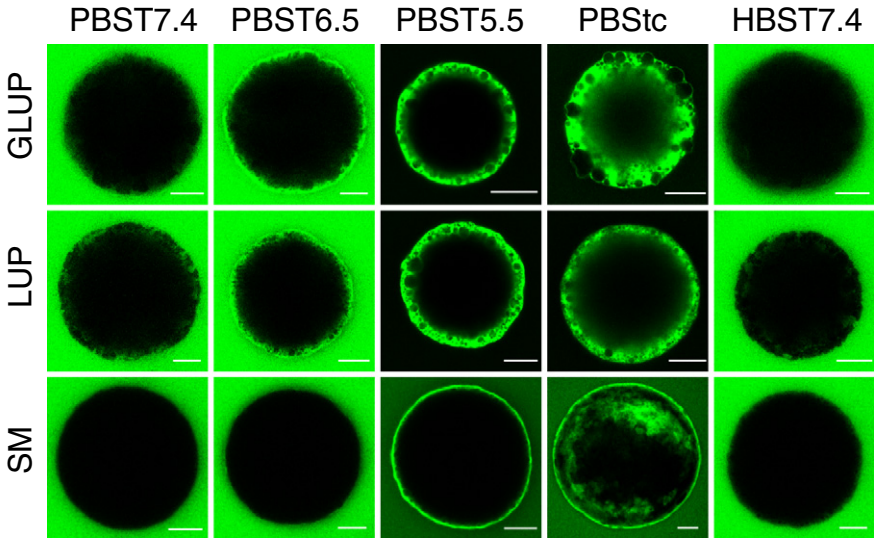


Fig. 6. Representative confocal microscopic images of GLUP, LUP, and SM incubated in PBST7.4, PBST6.5, PBST5.5, PBStc, and HBST7.4 for 7 days at 37 °C and for an additional 3 h with 5 μg/mL BODIPY dye under the same saline solutions. The white bar in bottom right corner of images corresponds to 20 μm.

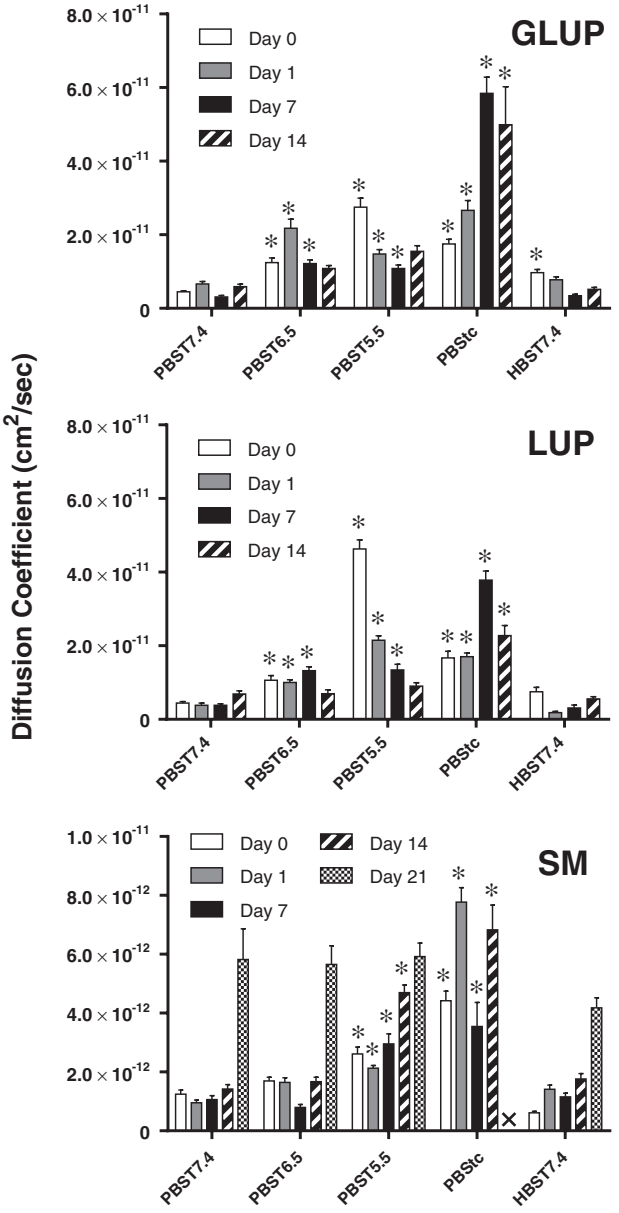


Fig. 7. Diffusion coefficient of BODIPY dye in GLUP, LUP, and SM incubated in PBST7.4, PBST6.5, PBST5.5, PBStc, and HBST7.4 for 0 day, 1 day, 7 days, 14 days and 21 days. Consecutive pixel intensities of BODIPY from the edge of the microspheres to the center of the microspheres were fit by the integrated solution of Fick's second law of diffusion. Each bar represents mean ± S.E.M. (n = 18, except for GLUP and LUP in PBStc on day 14 where n = 12, and for SM in PBStc on days 7 and 21 where n = 12, and no result indicated by cross mark, respectively, due to poor fit by the solution to Fick's second law of diffusion). Asterisks (*) indicates statistically significant difference from PBST7.4 at $p < 0.05$ as determined by the nonparametric ANOVA and following the Dunnett's test.

At day 21 where active release began, diffusion coefficient sharply increased in PBST7.4, PBST6.5, and HBST. Salt difference had little effect on diffusion coefficient for all formulations.

All formulations showed high diffusion coefficients for the BODIPY dye with dye saturation at day 21. However, TC caused BODIPY dye diffusing thoroughly in microspheres regardless type of PLGA and resulted in no loss of Fickian diffusion. In addition, at later phases of erosion diffusion was also not well behaved with highly non spherical shaped microspheres making calculation of effective BODIPY diffusion coefficient of the polymer matrix in all incubating media difficult.

3.6. Correlating leuprolide release with water-soluble acid release

Leuprolide is a positively charged peptide at neutral pH or lower, potentially interacting with negatively charged carboxylic acids in PLGA, in which the microclimate pH is predicted often to become acidic [37]. Moreover, we have shown that leuprolide can rapidly penetrate low MW acid-capped PLGAs by absorption, which depends on the ionic interaction between the PLGA carboxylate end groups and the arginine and/or histidine side chains on the peptide [38]. This interaction appears to be quite strong as indicated by the rapid absorption and slow desorption under neutral pH conditions at 37 °C [38]. Since peptide is absorbed into the polymer phase under neutral pH conditions at 37 °C, therefore a potential trigger for peptide desorption may be related to either a) the cleavage of the polymer chain ionically bound to leuprolide to make a water-soluble acid or b) the availability of water-soluble acids that can bind to the peptide in place of the polymer end-group. Also relevant would clearly be the viscosity of the polymer phase to resist solid-state diffusion.

To test this hypothesis, release of water-soluble acids from PLGA microspheres was monitored in the release media of microspheres by pre-derivatization HPLC (*i.e.*, after derivatization with pBPB) [22]. Glycolic acid, lactic acid, dimers of glycolic acid and lactic acid, and a dimer of lactic acid were detected and reported as total water soluble acid levels. The dimer of glycolic acid is not measurable and likely unstable in water [22]. As shown in Fig. 8, there was an apparent relationship between released leuprolide and water-soluble acid even at day 14 or 21 when the release became faster than mass loss. Consistent with our hypothesis of peptide desorption, leuprolide release in GLUP and LUP formulations proceeded proportionally along with generation of water-soluble acid with reasonably high R^2 values. Buffer types had no significant impact on the slope of leuprolide and water-soluble acid correlations.

3.7. Physical-chemical analysis of the factors governing release

When acid-end group PLGA microspheres are prepared with solvent evaporation process, two key questions need to be answered: whether the interaction between the polymer carrying acid-end group and the peptide carrying cationic amine groups is formed during manufacturing process and whether the presence of gelatin affects above interaction, if any. Whether extensive salt formation happens or not will affect whether the peptide remains largely in the pores or has migrated into the polymer phase [38]. As the polymer microspheres are hydrated and the polymer pores are occupied by water rapidly, hydrophilic peptide interacts with the polymer carboxylic acid groups, which results in a decrease in peptide mobility. During incubation, local peptide partition equilibrium between pores and polymer phase is presumed to exist in the polymer matrix. In the presence of plasticizer in the release media, the polymer chains should become much more mobile because of lower Tg as the plasticizer rapidly partitions into the polymer phase. Increased polymer-chain mobility is also expected to increase polymer hydrolysis. There was an apparent paradox that, as water uptake rate increased in the more neutral pH buffers, the polymer hydrolysis rate actually tended to decrease (Figs. 2 and 3). This contradiction may be explained if the vast majority of water uptake remains inside pores instead of polymer phase, the latter fraction of which is typically rather low [37,39], and additional protons entering the polymer would also be expected to increase acid-catalyzed ester hydrolysis. The very low water content of GLUP and LUP in the neutral pH buffer containing the plasticizer may have been caused by a) the increased osmotic pressure of the PBStc solution and b) the possibility that the additional mobility of polymer chains increased ion-pairing with the peptide neutralizing the water-sorbing peptide cation (*i.e.*, if the peptide is bound in the polymer phase, osmotic pressure in the pore would be expected to be reduced).

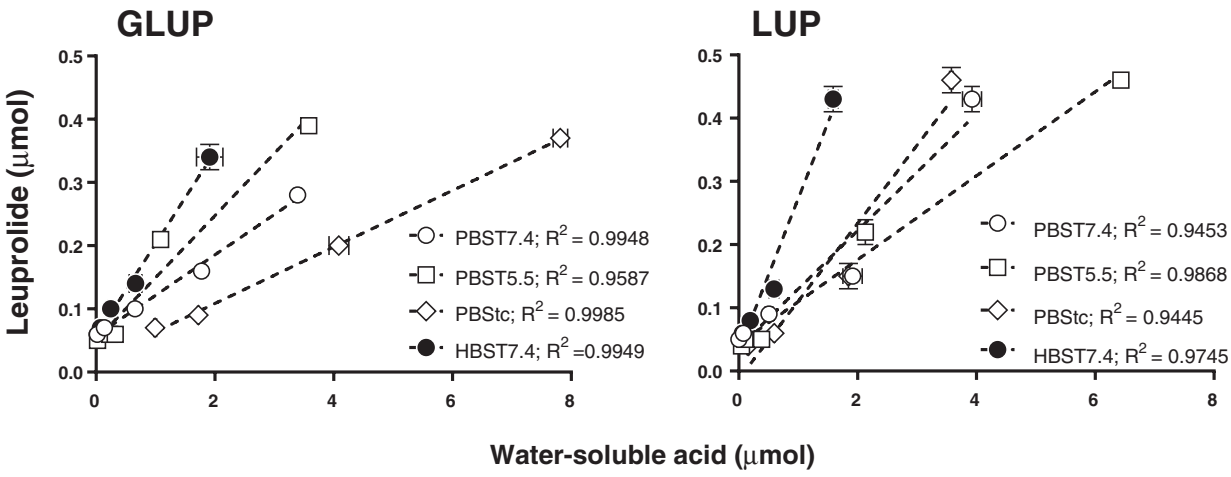
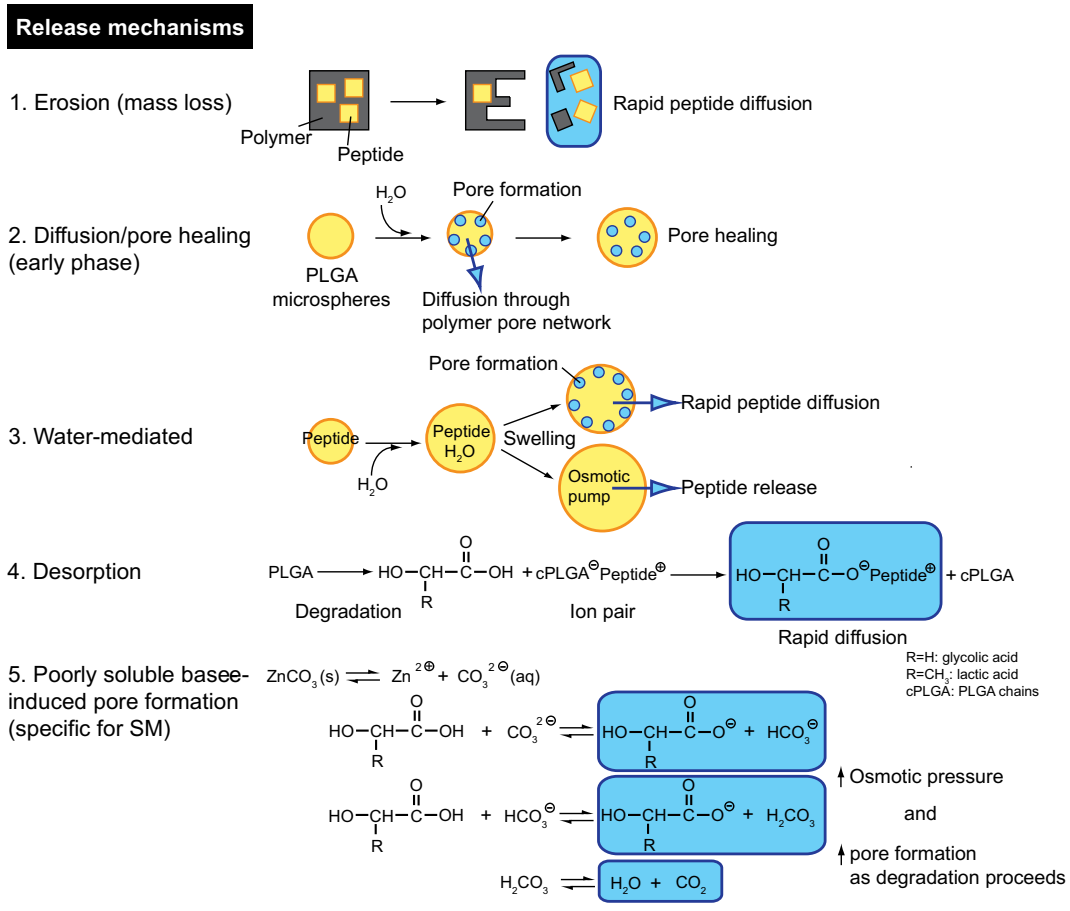


Fig. 8. Correlation of leuprolide release with water soluble acid levels in incubation media, PBST7.4 (○), PBST5.5 (□), PBStc (◇), and HBST7.4 (●) at days 1, 3, 7, 14, and 21 except for PBStc which was examined until day 14. Curve fitting results for each sample are indicated by dotted lines with R^2 values shown in legend notes. Each plot represents mean ± S.E.M (n = 3).

In order to identify the mechanism governing the release in various buffer solutions, it is important to summarize the data we have generated. The accelerated release of leuprolide in other media in comparison to PBST7.4 standard media was found to mirror the accelerated degradation of the polymer (Figs. 1 and 2). Second, the degree to which that release mirrored mass loss was limited to ≤40% of release (Fig. 5).



Scheme 1. Description of hypothesized release mechanisms of leuprolide-loaded PLGA microspheres, which are largely interrelated. Principal rate limits are believed to occur owing to, for example: 1) erosion – the polymer barrier to encapsulated peptide is physically removed as the polymer loses mass, which allows rapid peptide diffusion out of the polymer pore network; 2) diffusion through the polymer matrix and pore healing – (mostly during the initial burst phase) the peptide diffuses through new (see #3 below) and existing pores before the polymer heals, sealing off the pores for peptide exit [5]; 3) water-mediated solute transport – water penetrates the polymer causing swelling of the polymer, which can result in new pore formation and rapid peptide diffusion out of the polymer and/or osmotic pumping pore liquid out of the polymer and convective peptide transport [40]; 4) desorption of peptide from polymer phase – the peptide bound to the polymer phase by salt formation [38] desorbs by ion-exchange with degraded water-soluble monomer to allow rapid diffusion of the soluble peptide-ion pair; 5) Poorly soluble base-induced pore formation (relevant to SM microspheres containing $ZnCO_3$) – the poorly soluble base, $ZnCO_3$, dissolves in response to the presence of water soluble acids and forms water-soluble salts and CO_2 which cause osmotic pressure and cause pore-formation as degradation proceeds to facilitate continuous release of peptide [18].

Therefore, we must consider alternatives to simple mass loss that accelerated release. As mentioned above, this peptide is known to be capable of binding the free-acid terminated polymer [38], suggesting the potential for desorption/diffusion of the peptide. The linear relationship between the rate of peptide release and the rate of acid release supports this hypothesis. Similarly, the upswing in release rate relative to mass loss just after 40% release/mass loss (Fig. 5A and B) appears to precede by ~1 week a strong increase in microsphere uptake of BODIPY throughout the polymer (see for example in Fig. S2B: 14 days in PBStc; 21 days in PBST5.5; 28 days in PBST6.5; 28 days in PBST7.4; 28 days in HBST). In this case, the increased permeation appeared to be either a strong increase in pore diffusion (*e.g.*, Fig. S2B: 21 days in PBStc) or diffusion through the highly degraded polymer phase (*e.g.*, Fig. S2B: 28 days in HBST7.4), the former may in fact be caused by break down of the inter-pore connections caused by osmotic pressure. Described in Scheme 1 are the above hypothesized three operative release mechanisms (mass loss, water-mediated, and desorption) occurring after the initial burst phase, as well as diffusion/pore healing, which are expected to be operative during release in the first 1–3 days after incubation. For the SM microspheres the release observed was likely a result of water-mediated breakdown of the pore-network when the polymer molecular became low, although only in the case of PBStc could the BODIPY dye be seen as early as 7 days penetrating the pore polymer pores (*e.g.*, Fig. S2C 7–28 days), as the active release phase in most of the other media proceeded after the observation period for the BODIPY dye. Note also, the presence of $ZnCO_3$ in the SM formulation is expected to cause pore formation and more continuous release by protecting auto-catalytic hydrolysis, than if left out of the formulation (Scheme 1).

4. Conclusions

Analysis of acid end-group PLGA microspheres formulated here strongly suggest that after the initial burst the *in vitro* release of encapsulated leuprolide occurs by erosion-control over the first part of release. Thereafter, a second mechanism becomes operative causing release kinetics to be faster than erosion kinetics. The second mechanism is expected to involve either desorption of leuprolide from the polymer and/or water-mediated pore-opening, followed by rapid diffusion out of the matrix depending on which release media was used. Ion-pairing with water-soluble acids as degradation proceeds may facilitate desorption of the peptide, as suggested by similar release rates of peptide and water-soluble acids. The presence of TC significantly increases diffusion coefficient of BODIPY, polymer chain mobility and polymer hydrolysis rate, and strongly reduces water uptake. Reducing external pH causes similar but more modest effects as that caused by TC with the exception of the effects on BODIPY diffusion. Changing phosphate buffer to HEPES resulted in only a very marginal increase in release and associated erosion parameters. Influence of the buffer on the release of leuprolide from ester end-capped self-healing PLGA microspheres containing pore-forming agent $ZnCO_3$ followed similar trends, although in this case the acidic buffers accelerated release relative to erosion more than did TC plasticizer. The approach described here provides a detailed mechanistic signature as a function of various media with the ultimate goal of matching both release kinetics and the mechanism of that observed *in vivo*, which will be reported with the acid-end group formulations for leuprolide in follow-on studies. Hence, this research is promising to understand the underlying molecular basis for both *in vitro* and *in vivo* release kinetics, and to establish mechanism-based IVIVCs.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2016.08.023.

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