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Research paper

Mechanistic analysis of triamcinolone acetonide release from PLGA microspheres as a function of varying *in vitro* release conditions

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ABSTRACT

In vitro tests for controlled release PLGA microspheres in their current state often do not accurately predict *in vivo* performance of these products during formulation development. Here, we introduce a new mechanistic and multi-phase approach to more clearly understand *in vitro-in vivo* relationships, and describe the first "*in vitro* phase" with the model drug, triamcinolone acetonide (Tr-A). Two microsphere formulations encapsulating Tr-A were prepared from PLGAs of different molecular weights and end-capping (18 kDa acid-capped and 54 kDa ester-capped). *In vitro* release kinetics and the evidence for controlling mechanisms (*i.e.*, erosion, diffusion, and water-mediated processes) were studied in four release media: PBST pH 7.4 (standard condition), PBST pH 6.5, PBS + 1.0% triethyl citrate (TC), and HBST pH 7.4. The release mechanism in PBST was primarily polymer erosion-controlled as indicated by the similarity of release and mass loss kinetics. Release from the low MW PLGA was accelerated at low pH due to increased rate of hydrolysis and in the presence of the plasticizer TC due to slightly increased hydrolysis and much higher diffusion in the polymer matrix. TC also increased release from the high MW PLGA due to increased hydrolysis, erosion, and diffusion. This work demonstrates how *in vitro* conditions can be manipulated to change not only rates of drug release from PLGA microspheres but also the mechanism (s) by which release occurs. Follow-on studies in the next phases of this approach will utilize these results to compare the mechanistic data of the Tr-A/PLGA microsphere formulations developed here after recovery of microspheres *in vivo*. This new approach based on measuring mechanistic indicators of release *in vitro* and *in vivo* has the potential to design better, more predictive *in vitro* release tests for these formulations and potentially lead to mechanism-based *in vitro-in vivo* correlations.

1. Introduction

Poly(lactic-*co*-glycolic) acid (PLGA) is the most commonly used biodegradable polymer used to achieve long-term controlled drug release over weeks to months [1]. A wide range of therapeutic molecules, such as peptides, proteins, and poorly soluble small molecules, have been encapsulated in PLGA formulations [2,3]. During the development of these formulations, *in vitro* experiments are performed to measure drug release kinetics, which are used to predict *in vivo* release and pharmacokinetics of the encapsulated drug. Although these tests are routine, no formal FDA guidelines currently exist for assessing *in vitro* release from injectable controlled release (CR) formulations to establish *in vitro-in vivo* correlations (IVIVCs) for accurately predicting *in vivo* performance. This has resulted in inconsistencies in the methods used for *in vitro* release tests reported in the literature and in many cases, poor prediction of *in vivo* release [4,5].

Drug release rates measured during *in vitro* tests can change depending on the experimental setup and/or the media used [6–11]. Ideally *in vitro* release tests can be designed to accurately

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predict *in vivo* release by selecting both the proper setup and media, ultimately resulting in IVIVCs. This need has been thoroughly discussed in recent years in the literature and during regulatory-scientific meetings [4,12–19]. Discussion of IVIVC development for CR products has focused on the need to design *in vitro* tests to mimic not only the rate of drug release *in vivo*, but the underlying mechanistic factors responsible for drug release *in vivo*.

To develop our mechanistic analysis, we selected the poorly soluble corticosteroid triamcinolone acetonide (Tr-A) as a model drug. Tr-A was chosen for this mechanistic study for several reasons, including (1) its low susceptibility to polymorphism, (2) good stability, and (3) ease of quantitation using standard analytical techniques. It also represents poorly soluble small molecules in BCS classes II and IV for which traditional formulations (*e.g.* oral tablets or capsules) may be difficult to develop but represent a growing number of new chemical entities. Various drugs in this class have been developed in commercial PLGA controlled release products. These drugs also often (1) have a wide therapeutic index, (2) require a low daily dose, and (3) are for long-term treatment of chronic disease, all conditions favorable for a PLGA controlled release strategy.

Drug release from PLGA microspheres can be controlled principally by at least three rate-limiting release mechanisms or combinations thereof; (1) diffusion through the polymer matrix, *i.e.,* drug release by diffusion either through pores of the matrix or the polymer phase itself; (2) water-mediated transport processes, *i.e.*, release triggered directly by the influx of water, which can cause swelling and new pore formation, osmotic pumping and convective mass transfer, and (3) bulk erosion as is well established for PLGA, *i.e.,* polymer mass loss causing new pores of the polymer to form allowing the drug to rapidly diffuse out of the polymer matrix. It is important to note that drug release is often the result of more than one of these mechanisms and that these mechanisms are not independent of each other. For example, water uptake in the polymer matrix will cause hydrolysis of the PLGA chains and is involved in the erosion of the polymer.

To begin developing IVIVCs for PLGA microspheres, we have devised a multi-phased mechanistic approach to the understanding of drug release *in vitro* and *in vivo.* The approach has 3 phases: Phase 1 (*i.e.,* this paper) – mechanistic evaluation of *in vitro* release of a model drug, which involves the simultaneous monitoring of physical chemical processes (or mechanistic indicators of release), namely, degradation, erosion, water uptake, and polymer matrix diffusion, which are collectively known to be coupled and responsible to drug release from PLGA; Phase 2 – development and validation of an implantable system to allow recovery and mechanistic evaluation of microspheres during *in vivo* release, which involves pharmacokinetic and histological analysis of the implant relative to standard subcutaneous injection; and Phase 3 – measuring the drug release and mechanistic indicators of drug release *in vivo* and comparing those to that recorded *in vitro*. Phase 4 and beyond will focus on elucidating the underlying causes for *in vitro* release and *in vivo* differences, and devising release media and conditions that minimize mechanistic and rate differences. The purpose of this work was to develop the Phase 1 for Tr-A by studying the mechanisms of *in vitro* drug release from two PLGA microsphere formulations in various bio-relevant media. This analysis is critical to develop our multiphase mechanistic approach.

2. Materials

Triamcinolone acetonide (Tr-A) and PLGA RESOMER® 502H (i.v. = 0.19 dL/g, free acid terminated) were purchased from Sigma-Aldrich. Poly vinyl alcohol (PVA, 88% hydrolyzed, MW ~ 25,000) was purchased from Polysciences, Inc. (Warrington, PA). PLGA (i.v. = 0.61 dL/g, ester terminated) was purchased from Lactel (Birmingham, AL). BODIPY® FL (4,4-Difluoro-5,7-Dimethyl-4-Bora-3a,4a-Diaza-s-Indacene-3-Propionic Acid) was purchased from Life Technologies. All solvents used were HPLC grade and were purchased from Fisher Scientific and unless otherwise noted, all other chemicals were purchased from Sigma-Aldrich.

3. Methods

3.1. Microsphere preparation

Microspheres were prepared by using solid-in-oil-in-water (s/o/w) double-emulsion solvent evaporation methods. Two polymers were used to encapsulate Tr-A to result in two formulations. The first (Tr-A_1) used a low molecular weight, free acid terminated PLGA 502H (18 kDa); Tr-A_2 used a moderate molecular weight, ester terminated (e.t.) PLGA (54 kDa). Control studies from a wide number of formulations were used to select these two formulations for further evaluations (see Supplementary Information). Tr-A was first micronized in a cryo-mill (Retsch®, PA, USA) prior to formulation to obtain drug particle sizes <10 μm. Micronized or unmicronized drug (5% w/w theoretical Tr-A loading) was mixed with PLGA dissolved in methylene chloride (Tr-A_1: 1000 mg/mL; Tr-A_2: 400 mg/mL) and homogenized at 10,000 rpm for 1 min to form a s/o suspension. Next, 4 mL of a 5% PVA solution was added and vortexed at high speed for 1 min. This s/o/w emulsion was rapidly transferred to 100 mL 0.5% PVA stirring bath. The methylene chloride was allowed to evaporate for 3 h and the hardened microspheres were collected by sieve (63–90 μm) and washed thoroughly with ddH_2O. Microspheres were then lyophilized and stored at −20 °C before use.

3.2. Scanning electron microscopy

Prior to imaging, lyophilized microspheres were mounted by using double sided carbon tape and coated with a thin layer of gold under vacuum. Scanning electron microcopy (SEM) was performed on a Hitachi S3200N scanning electron microscope (Hitachi, Japan). Images were captured by EDAX® software.

3.3. Determination of Tr-A loading and encapsulation efficiency

Prepared microspheres (~5 mg) were dissolved in 20 mL acetonitrile. The resulting solution was filtered and analyzed for Tr-A content by ultra-performance liquid chromatography (UPLC), as described below. Drug loading was calculated from the ratio of the mass of drug in the microspheres to the mass of the microspheres. Encapsulation efficiency was calculated by the measured drug loading divided by the theoretical loading.

3.4. Tr-A Quantification by UPLC

Tr-A content in loading solutions and release media was determined using UPLC (Acquity UPLC, Waters, USA). The mobile phase was composed of either 40: 60 v/v (acetonitrile: ddH2O) or 70: 30 v/v (methanol: water) and the flow rate was set to 0.5 mL/minute. Samples and standards prepared in either acetonitrile or PBST were injected onto a C18 (Aqcuity BEH C18, 1.7 μm, 2.1 × 100 mm) column maintained at 30 °C. Tr-A was detected at 254 nm.

3.5. Assessment of drug release in vitro

Drug release and mechanistic analyses of microspheres was carried out using a sample-and-separate method in four types of release media: PBS (137 mM NaCl, 3 mM KCl, 7.74 mM Na_2HPO_4,

2.26 mM NaH_2PO_4) at pH 7.4 and 6.5; 10 mM HBS (HEPES-buffered saline) at pH 7.4; and PBS with 1.0% triethyl citrate (TC) at pH 7.4. PBS pH 7.4, PBS pH 6.5, and HBS all contained 0.02% Tween 80 and all media contained 0.05% NaN_3. These media were chosen to determine the effects of various pH, buffering species, and presence of plasticizer. Microspheres (3–5 mg) were placed in 30 ml of media to maintain sink conditions and shaken mildly at 37 °C for the duration of the experiment. At each time point (1, 3, 7 days and weekly thereafter), the microspheres were separated from media by centrifugation at 4000 rpm (3200*g*) for 5 min (Eppendorf 5810 R; Eppendorf, Hamburg, Germany) and the media was completely removed and replaced. Drug content was measured by UPLC, as previously described.

3.6. Mass loss and water uptake of microspheres

During *in vitro* release, mass loss and water content of the PLGA microspheres was determined weekly. Microspheres were separated from release media by filtration and washed with ddH_2O to remove salts. The wet weight of microspheres was recorded and then the microspheres were dried under vacuum to constant weight. Mass loss and water uptake were estimated as previously described [20].

3.7. Molecular weight of PLGA

During *in vitro* release, weight-averaged molecular weight (Mw) of degrading PLGA was measured by gel permeation chromatography (GPC) weekly. The Waters 1525 GPC system (Waters, USA) consisted of two styragel columns (HR 1 and HR 5-E columns), a binary HPLC pump, waters 717 plus autosampler, waters 2414 refractive index detector and Breeze software to obtain the molecular weight. Samples were dissolved in tetrahydrofuran (THF) at a concentration of ~1 mg/ml, filtered through a 0.2 μm hydrophobic filter and eluted with THF at 0.35 ml/min. The molecular weight of each sample was calculated using monodisperse polystyrene standards, Mw 820–450,000 Da.

3.8. BODIPY uptake and Laser Scanning Confocal Microscopy (LSCM)

At certain time points during *in vitro* release, a small aliquot of microspheres was removed from release media and incubated in a solution of the diffusion probe, BODIPY FL (5 μg/mL), in the same media at 37 °C for 10 min or 3 h under mild agitation. This dye was used due to its preferential partitioning into the polymer phase, making it a suitable marker for solid-state diffusion, as previously described by our lab [20,21]. BODIPY distribution in degrading microspheres and subsequent image analysis was determined as previously described [21]. Briefly, microspheres were imaged using a Nikon A1 spectral confocal microscope (Nikon, Tokyo, Japan) to observe dye distribution and microsphere morphology. The images were then analyzed using ImageJ software (National Institutes of Health, USA). Normalized dye intensity (I/I_0) – position (r/a) pairs were then fit to the solution of Fick's second law of diffusion using DataFit software (Oakdale Engineering, USA) to determine the effective solid-state diffusion coefficient of BODIPY (D_{bodipy}), as described previously [21].

3.9. Estimation of Tr-A diffusion in blank PLGA microspheres

Blank_1 (free acid terminated PLGA, 18 kDa) and Blank_2 (ester terminated PLGA, 54 kDa) microspheres were prepared using the same methods as described above, omitting the addition of the Tr-A during formation of the first emulsion. These microspheres were suspended in PBST pH 7.4 for one day, at which time the release media was replaced with a saturated Tr-A suspension in PBST pH 7.4. Microspheres were then separated and washed thoroughly on a 45 μm sieve with cold ddH_2O at 2, 4, 6, 8, 12, 24 and 48 h. Tr-A content in microspheres following uptake was determined by extraction with acetonitrile followed by analysis by UPLC. The uptake of Tr-A was fit using DataFit software to Crank's solution using for uptake into or release from particles of spherical morphology to determine an effective solid state diffusion coefficient [22]. See Supplementary Information for more details.

3.10. Statistical and regression analysis

Statistical analyses and regressions were performed using Prism (Graphpad, San Diego, CA). Rate constants, t_{50} values, and diffusion coefficients in various media were compared to standard conditions (PBST pH 7.4) using unpaired student t-tests to determine two-tailed P-values. The level of significance was established at the 95% confidence interval ($\alpha < 0.05$).

4. Results and discussion

PLGA microspheres encapsulating Tr-A were successfully prepared using micronized API powder in two types of PLGAs: a low molecular weight free-acid terminated PLGA and a moderate molecular weight ester-end capped PLGA. As Tr-A is poorly soluble in methylene chloride, the drug was encapsulated as a solid during formulation. In an effort to optimize encapsulation and release, the drug powder was micronized to obtain a small particle size which can be efficiently encapsulated by the polymer during formation of the first emulsion. Initially, Tr-A was micronized by grinding with mortar and pestle and sieving through a 45 μm sieve. However, this process resulted in heterogeneous powder size (Fig. 1A) and the resulting microsphere formulations exhibited poor and variable drug loading and encapsulation efficiency (Table 1). Therefore, a cryo-mill (Retsch®, PA, USA) was used to obtain a homogenous powder size less than 10 μm (Fig. 1B). The encapsulation of this milled powder greatly improved encapsulation efficiency, decreased variability in drug loading, and lowered initial burst in PBST pH 7.4 (Table 1). The incorporation of homogenously sized Tr-A powder into microspheres resulted in encapsulation efficiencies slightly higher than 100%. This slight excess loading was likely caused by discarding the fine fraction below 63 μm sieve size, which are expected to contain a lower drug loading. Accordingly, microspheres formulated using the cryo-milled Tr-A were used for all studies discussed herein.

Following successful microsphere preparation and characterization, Tr-A release kinetics was determined in several types of release media to examine the effects of media composition. Given the complexity of the make-up of the interstitial fluid in the subcutaneous space [23–39], it was important for these studies to incorporate several media, which could capture various elements of the administration environment in order to demonstrate the importance of the selection of proper release media. In addition to a "standard" media widely used in the literature for *in vitro* release testing of parenteral controlled release formulations, we chose to study release in the same buffer at pH 6.5 to examine the effect of acidifying the pH (as may occur during inflammation [40–42]); a completely different buffering system to elucidate effects of buffering capacity and components; and a "standard" PBS buffer with the addition of a plasticizer (TC) to determine the potential effects of plasticizing lipidic molecules present *in vivo* [7,27,38,39]. With follow-on examination of *in vivo* mechanisms of release, these data may inform us on which release media variables can be manipulated to approximately represent the *in vivo* environment. Release in each media was compared to what was observed in PBST pH 7.4, as this condition is commonly used

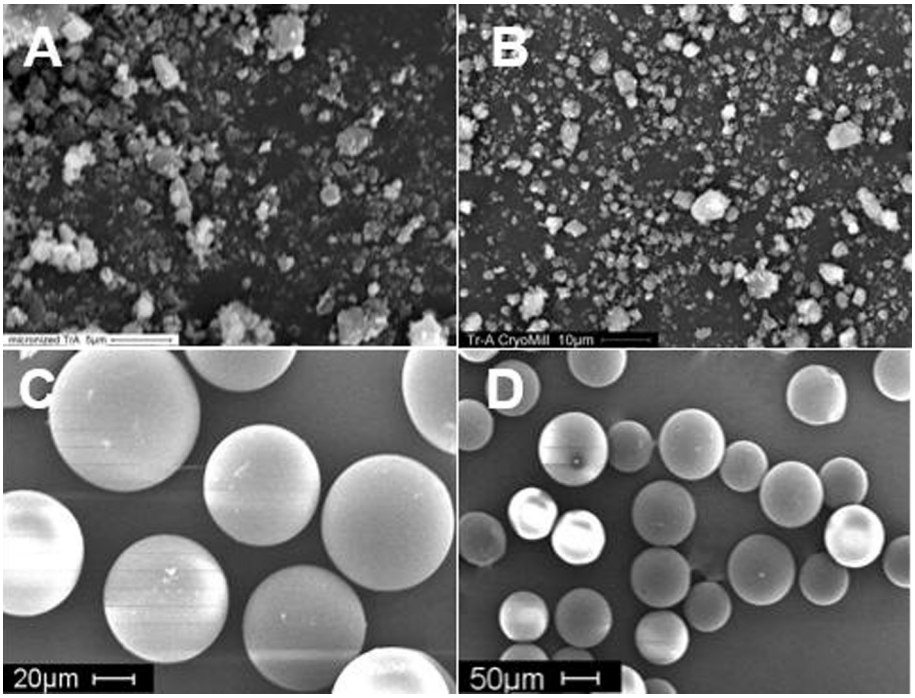

Fig. 1. SEM micrographs of Tr-A micronized by mortar and pestle (A) and after by Retsch® cryo-mill (B). Micronized Tr-A powder was then encapsulated in Tr-A_1 (C) and Tr-A_2 (D) microspheres.

Table 1
Characterization of microsphere formulations prepared using unmilled and milled Tr-A.

	Unmilled Tr-A		Milled Tr-A	
	Tr-A_1	Tr-A_2	Tr-A_1	Tr-A_2
Tr-A loading (w/w%)	3.2 ± 0.3 ‡	4.2 ± 0.1	5.4 ± 0.2	5.2 ± 0.1
Encapsulation efficiency (%)	64 ± 6	84 ± 3	108 ± 4	104 ± 1
Initial burst in PBST pH 7.4 (%)	9.8 ± 2.2	6.6 ± 0.4	1.9 ± 0.3	2.8 ± 0.4
Particle size (µm)	ND	ND	73 ± 2	71 ± 3

‡ All values are reported as mean ± SEM, n = 3. ND: not determined.

throughout the literature for conducting *in vitro* release tests. Release from Tr-A_1 was generally continuous over 35 days, with a slight lag observed in the first 7 days in 3 of the 4 media. Release from Tr-A_2 was slower and could be described as tri-phasic. A low initial burst was observed in the first day, followed by a lag phase in all four media. Following the lag phase, drug release was continuous in the third phase and lasted approximately 63 days in 3 of the 4 media. As seen in Fig. 2A, release from Tr-A_1 microspheres was accelerated in two media: PBST pH 6.5 and PBS + 1.0% TC. Release from this formulation in PBST pH 7.4 and 6.5 and in HBST pH 7.4 followed a typical tri-phasic profile with low initial burst on day one followed by a short lag phase corresponding to polymer degradation. Following the lag phase, which lasted approximately 7 days, a secondary apparent zero order release occurred faster at the slightly lower pH with complete release occurring after one month. This result is expected, as a more acidic pH may catalyze hydrolysis of the polymer to cause accelerated release [11,43,44]. In the presence of the plasticizer triethyl citrate (TC) a slightly higher initial burst (%) was followed by no apparent lag phase and complete release in just 3 weeks. The presence of TC also resulted in accelerated release from Tr-A_2 (Fig. 2B). Release from this microsphere formulation prepared from a higher MW ester end-capped PLGA (54 kDa) showed slower overall release than Tr-A_1 and a lag phase was noticeable in all four media, though it lasted approximately just 14 days in PBS + 1.0% TC versus roughly 21 days in the other three media.

After the initial burst, drug release of poorly soluble small molecules such as Tr-A from low molecular weight PLGAs is expected to be dominated by bulk erosion, while release from moderate molecular weight PLGAs is expected to occur via bulk erosion and/or diffusion through the polymer matrix, though erosion is expected to be the predominant mechanism [2,45]. Erosion-controlled release from PLGA microspheres typically exhibits a tri-phasic release. An initial burst of drug (usually due to surface-associated drug particles and/or diffusion through existing and swelling-induced pores before healing [46]) is followed by a lag phase, during which the polymer chains degrade by hydrolysis until a critical chain length is reached. Following the lag phase, the duration of which is dependent on polymer characteristics (*e.g.* MW, lactic acid: glycolic acid ratio, etc.) and formulation size/geometry [47], a bulk erosion controlled, continuous release phase continues until all encapsulated drug has been released. Additionally, polymers with a free acid end group, such as that used in Tr-A_1, are known to take up much more water in the polymer phase which can lead to faster erosion and release as compared to polymers which have been ester end-capped, such as that used in Tr-A_2 microspheres [2,3,45,46,48–51]. In addition, as the acid-capped polymer has very low molecular weight, drug release is expected to occur continuously

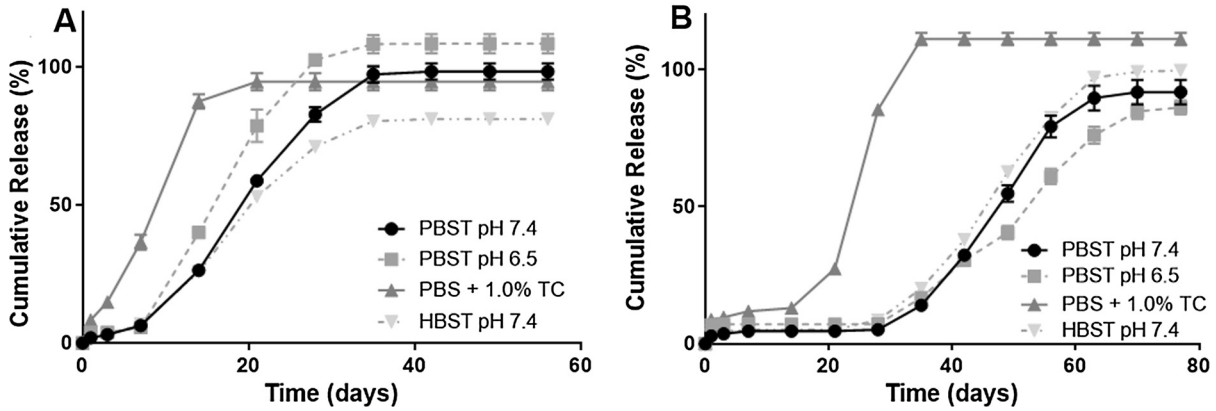

Fig. 2. *In vitro* release from Tr-A_1 (A) and Tr-A_2 (B) microspheres in various media. Data represent mean ± SEM, n = 3. Note: in some cases, average release was slightly greater or lower than 100% due to slight error associated with measurement.

without a lag phase, as water soluble oligomers either exist at time 0, or very soon thereafter during release incubation.

In order to understand the underlying factors, which cause accelerated release in some *in vitro* conditions, mechanisms of drug release from each formulation in all four media were studied by gathering physical-chemical evidence to support their respective contributions. Kinetics of PLGA hydrolysis during *in vitro* release is shown in Fig. 3 and the associated initial first order rate constants over 14 d are reported in Table 2. As expected, the slightly acidic pH in the media (pH 6.5) caused significantly faster degradation than standard conditions (pH 7.4), *i.e.*, 0.163 ± 0.003 versus 0.125 ± 0.018 day^{-1} for PLGA the 502H formulation (Tr-A_1). Degradation of the higher molecular weight, ester terminated polymer (Tr-A_2) was significantly faster in PBS + 1.0% TC than in other media (0.121 ± 0.012 vs. 0.034 ± 0.001 day^{-1}). PLGA hydrolysis results in the formation of water-soluble low molecular weight monomers and oligomers, which diffuse out of the polymer matrix into the bulk media and the bulk polymer porosity increases characteristic of bulk erosion. The result of this process is overall erosion of the solid microspheres which can be measured by mass loss. As expected due to the accelerated hydrolysis, Tr-A_2 microsphere mass loss was accelerated in PBS + 1.0% TC (Fig. 4). The uptake of the plasticizer into the polymer phase can increase the mobility of the polymer chains, allowing for increased mobility of water and polymer chains, which can lead to accelerated hydrolysis. The increased chain mobility can also cause increased diffusion of the low molecular weight degradation products out of the microspheres, causing the observed increase in overall mass loss [7]. It is also important to note that the diffusion of small organic molecules in PLGA 50/50 microspheres from the external solution can be rapid at 37 °C (*e.g.*, bodipy diffusion reaching equilibrium after just 3 days).

Erosion (*i.e.* polymer mass loss), one of the major mechanisms of drug release from PLGA microspheres, can be further described by determining the time-scale over which mass loss of the polymer matrix occurs. Mass loss curves shown in Fig. 4 were fit using four-parameter logistic nonlinear or linear regressions (Figs. S6 and S7), and the time to 50% mass loss ($t_{50,erosion}$) was determined in each media. The same process was also applied to release curves to determine the 50% release time ($t_{50,release}$) (Figs. S4 and S5). The contribution of this mechanism to overall drug release can be evaluated by comparing these two defining parameters of the time scales of erosion and release. For example, if $t_{50,release}/t_{50,erosion} \approx 1$, one can reasonably surmise that erosion is the dominant mechanism responsible for drug release in a given condition, as erosion and release happen over the same time scale. These values are reported in Table 3, and the same trends are shown in Fig. 5, where release in each media was plotted vs. mass loss and compared to the line plotted to represent release = mass loss. In the standard conditions (PBST pH 7.4), erosion-controlled drug release was observed in both formulations. In the case of Tr-A_1, release occurred much faster than erosion in PBS + 1.0% TC ($t_{50,release}/t_{50,erosion} = 0.52$), indicating that another mechanism was contributing to, and likely controlled, the accelerated Tr-A release in that media. Although release was slightly accelerated in PBST pH 6.5, the release increased appeared to be due to increased erosion and the predominant mechanism of release in this instance was unchanged. As discussed, the only media which caused a significant increase in the rate of Tr-A release from Tr-A_2 microspheres was PBS + 1.0% TC and erosion was increased in this media as well ($t_{50,erosion} = 8 \pm 2$ days). It should be noted that in most cases the release was even slower than the erosion (*i.e.*, data falling below the release = erosion line) at the beginning of release. This effect is characteristic of the non-uniformity in the drug particle distribution in the s/o/w microspheres and the necessity to develop initial porosity before release begins. Put another way, if no drug particles were located in regions of the microsphere near the surface, which begin to erode first, the mass loss could easily precede drug release in the initial stages. In other cases later in the release (*e.g.*, Tr-A_2 in PBS + TC and HBST), release was also slower than erosion. Other factors may have contributed such as rapid polymer healing [52,53], causing newly formed pores during erosion to close before the poorly soluble drug has had a chance to diffuse out of the polymer matrix.

Further investigation into the mechanisms of release of Tr-A from PLGA microspheres included measurement of water uptake into the polymer matrix (Fig. 6). HBST pH 7.4 caused noticeably more water uptake than the other three media in Tr-A_1 and Tr-A_2 at later time points. This may be due to the potential interaction between the cationic species of the zwitterionic HEPES molecule interacting with free carboxylic acids present in Tr-A_1 at all times during release, and in Tr-A_2 as degradation causes production of acidic oligomers. The electrostatic interaction would presumably increase osmotic pressure, resulting in increased water uptake and microsphere swelling. However, given no clear trend in these data as related to the release kinetics of either formulation strongly suggests that water-mediated processes likely do not play a significant role in Tr-A release.

Finally, degrading particle morphology and diffusion through the polymer matrix, controlled primarily by the solid polymer phase, was studied using the fluorescent probe, bodipy. This small, pH insensitive dye partitions preferentially into the polymer phase

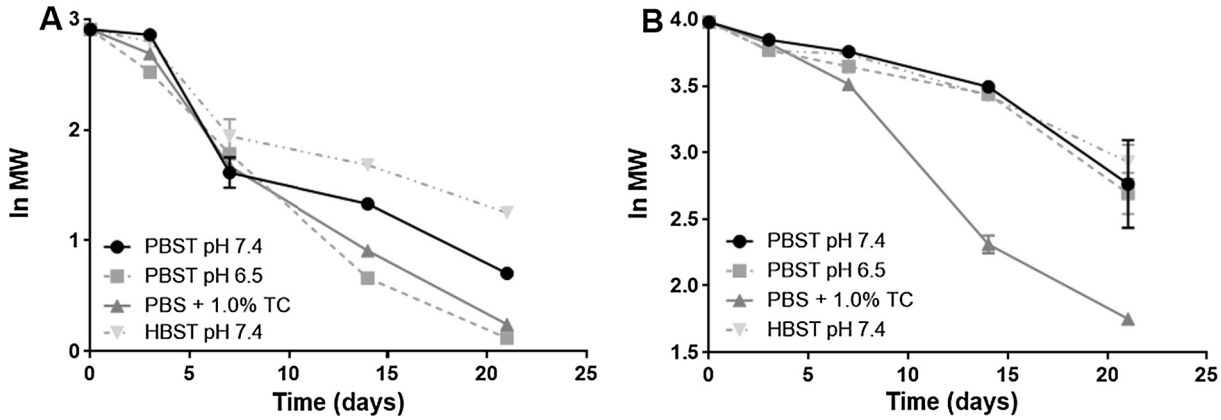

Fig. 3. Decline of molecular weight of PLGA in Tr-A_1 (A) and Tr-A_2 (B) microspheres in various *in vitro* release media. Data represent mean ± SEM, n = 3.

Table 2
Initial first order rate constants (day^{-1}) of PLGA hydrolysis in Tr-A_1 and Tr-A_2 microspheres as determined by linear regression analysis of data shown in Fig. 3. Values were taken from regression over the first 14 days.

	PBST pH 7.4	PBST pH 6.5	PBS + 1.0% TC	HBST pH 7.4
Tr-A_1	0.125 ± 0.018 ‡	0.163 ± 0.003*	0.151 ± 0.009	0.095 ± 0.013
Tr-A_2	0.034 ± 0.001	0.037 ± 0.003	0.121 ± 0.012*	0.037 ± 0.004

‡ All values are reported as mean ± SEM, n = 3.
* $p < 0.05$ compared to PBST pH 7.4.

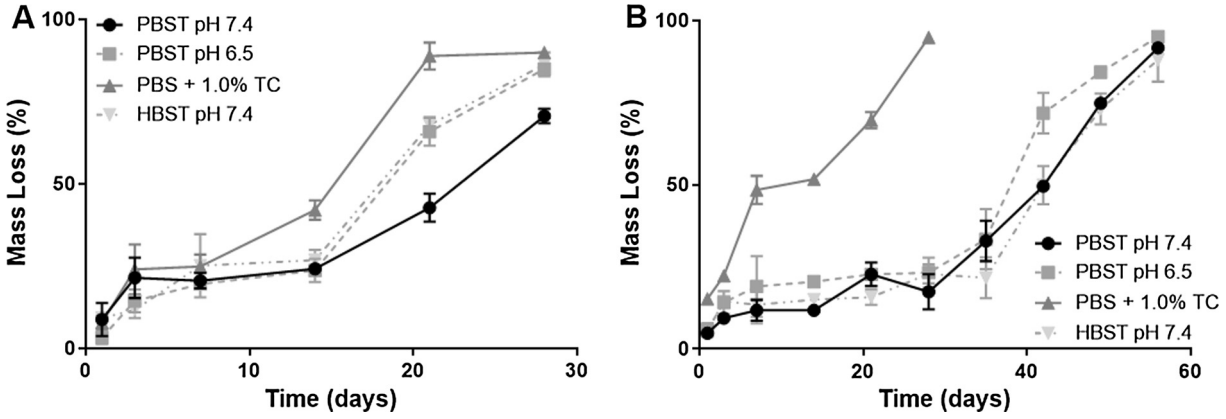

Fig. 4. Mass loss of Tr-A_1 (A) and Tr-A_2 (B) microspheres in various *in vitro* release media. Data represent mean ± SEM, n = 3.

Table 3
Characteristic times (in days) of release and erosion from Tr-A_1 and Tr-A_2 microspheres. Values represent mean ± SEM, n = 3.T_{50} ratios were calculated from mean values of t_{50}release and t_{50}mass loss in each media.

		PBST pH 7.4	PBST pH 6.5	PBS + 1.0% TC	HBST pH 7.4
Tr-A_1	$t_{50,release}$	19.0 ± 0.4	16.6 ± 0.4	8.0 ± 0.4*	17.6 ± 0.2
	$t_{50,erosion}$	25 ± 8	18.6 ± 0.8	15 ± 1	18 ± 2
	$t_{50,release}/t_{50,erosion}$	**0.77**	**0.89**	**0.52**	**0.96**
Tr-A_2	$t_{50,release}$	46.8 ± 0.6	50.1 ± 0.8	25.0 ± 0.3*	46.1 ± 0.3
	$t_{50,erosion}$	46 ± 3	39 ± 2	18 ± 2*†	43 ± 2
	$t_{50,release}/t_{50,erosion}$	**1.02**	**1.28**	**1.43**	**1.06**

* $p < 0.05$ compared to PBST pH 7.4.
† linear regression was used.

and thus is a suitable marker for solid state diffusion of low molecular weight unionized organic solutes [21]. As the probe is also water soluble, it can also be used to view the larger pore structure in the polymer matrix. The dye diffuses rapidly through the aqueous pores back into the polymer, so that following incubation for a short time, the polymer phase is labelled by the dye and the aqueous pores are marked by an absence of fluorescence by LSCM. Representative images of Tr-A_1 and Tr-A_2 particles during release can be seen in Fig. 7 (additional images are shown in Figs. S8 and S9). These confocal micrographs show the morphology of degrading microspheres and provide visual evidence of the previously discussed mechanisms of drug release. Micrographs of particles

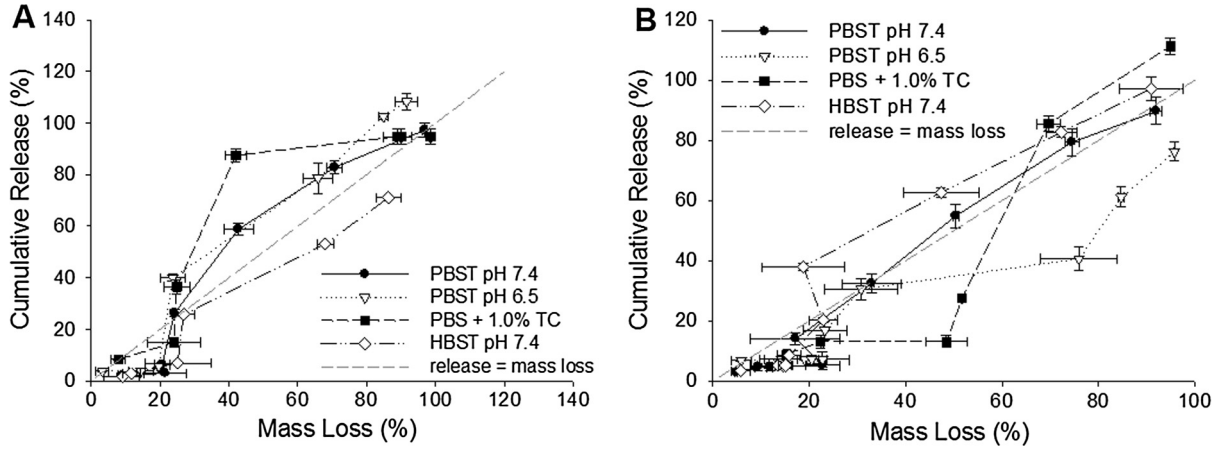

Fig. 5. Release vs. mass loss of Tr-A_1 (A) and Tr-A_2 (B) microspheres. Dashed line represents release = mass loss, indicating pure erosion controlled release. X and Y data represent mean ± SEM, n = 3.

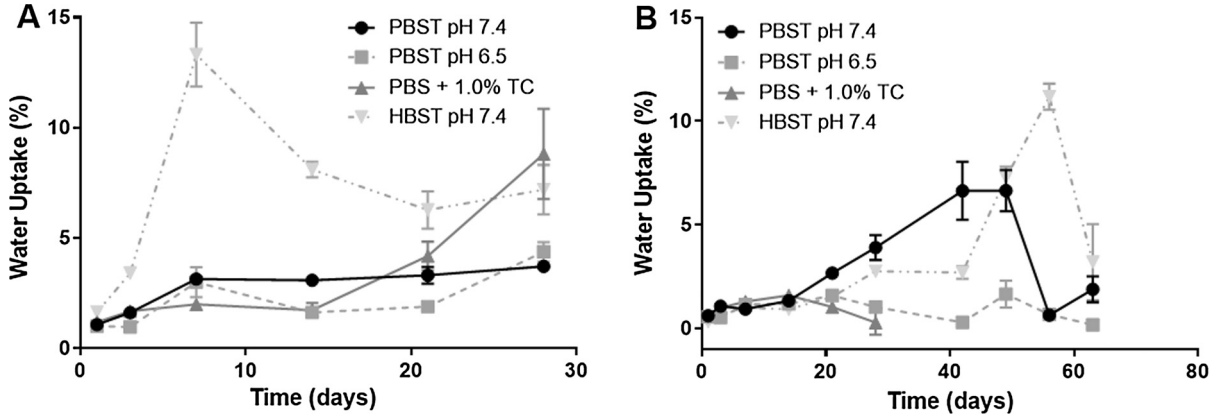

Fig. 6. Water uptake Tr-A_1 (A) and Tr-A_2 (B) microspheres in various *in vitro* release media. Data represent mean ± SEM, n = 3.

incubated in PBST pH 6.5, PBS + 1.0% TC, and HBST pH 7.4 were compared to those incubated in PBST pH 7.4 to determine the effects of these non-standard conditions on particle morphology. Incubation in PBST pH 6.5 caused a noticeable increase in the penetration of the dye from the surface of Tr-A_1 microspheres. This effect can be explained by the slightly reduced pH of the buffer and thus a reduced pH at the surface of the microspheres. As previously mentioned, this increases the hydrolysis of PLGA and thus allows for increased penetration of molecules such as bodipy into the regions of degraded polymer. The presence of TC increased mobility of the polymer in Tr-A_1 microspheres, resulting in a loss of spherical morphology and formation of larger pores on the surface at early time points and swelling and dye saturation at later time points. This increase in polymer chain mobility resulted in a significant increase in the effective diffusion coefficient in the polymer matrix (Fig. 8A) following 3 days in the release media. It should be noted that this was the latest release time point diffusion coefficient could be measured at the dye uptake time point selected in three of the four *in vitro* conditions due to rapid polymer degradation in this low molecular weight PLGA. Tr-A_1 microspheres swelled dramatically in HBST pH 7.4 as early as three days.

The PLGA used for formulation of Tr-A_2 microspheres was a higher molecular weight (54 kDa) with ester end capping and thus, these particles eroded at a slower rate than was observed in Tr-A_1 particles. Particles incubated in PBST pH 6.5 exhibited some preferential surface erosion similar to what was observed in Tr-A_1. The presence of triethyl citrate also created a visible bodipy diffusion front as a result of polymer chain flexibility caused by plasticization. This mobilization also had a significant impact on bodipy diffusion after 7 and 14 days, while PBST pH 6.5 and HBST pH 7.4 buffer solutions exhibited no effect on solid-state diffusion as compared to PBST pH 7.4 at any measurable time point (Fig. 8B).

Considering the evidence above, the release of Tr-A *in vitro* for both formulations in each media condition is consistent with erosion-controlled release but with one exception. The presence of triethyl citrate in the media during evaluation of the Tr-A_1 resulted in release rate that exceeded the rate of mass loss with over 80% release with only ~40% mass loss of the polymer (Fig. 5A). Similarly, the bodipy diffusion probe showed a dramatic increase in uptake rate (Fig. S9) and effective diffusion coefficient (Fig. 8), indicating a decrease in viscosity of the polymer for molecular diffusion. We verified that Tr-A does in fact diffuse in blank microspheres similar to bodipy, albeit at a very slow rate (effective diffusion coefficients in the range of $\sim 10^{-12}$–10^{-11} cm^2/s)(see Fig. S10). It is also known that steroids can diffuse through membranes of PLGA [54]. In addition, we ruled out any effect of solubility in the release media. The Tr-A solubility was measured in PBST and PBS + 1.0% TC and found to have the same solubility (see Supplementary information for detail, S). Hence, addition of TC to the media implicates diffusion control of the steroid from PLGA microspheres.

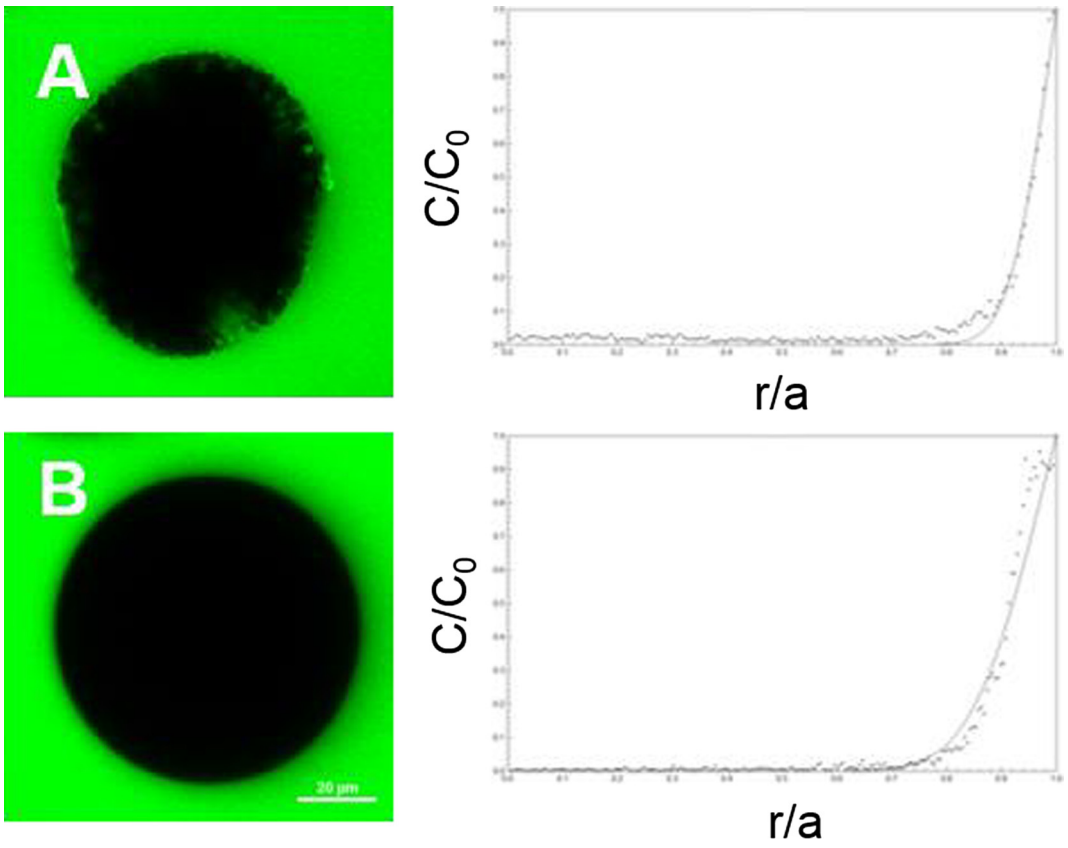

Fig. 7. Representative images of Tr-A_1 (A) and Tr-A_2 (B) microspheres following 3 days release in PBST pH 7.4 and 3 h in 5 μg/mL bodipy in PBST pH 7.4 and resulting bodipy concentration gradient plots shown at right.

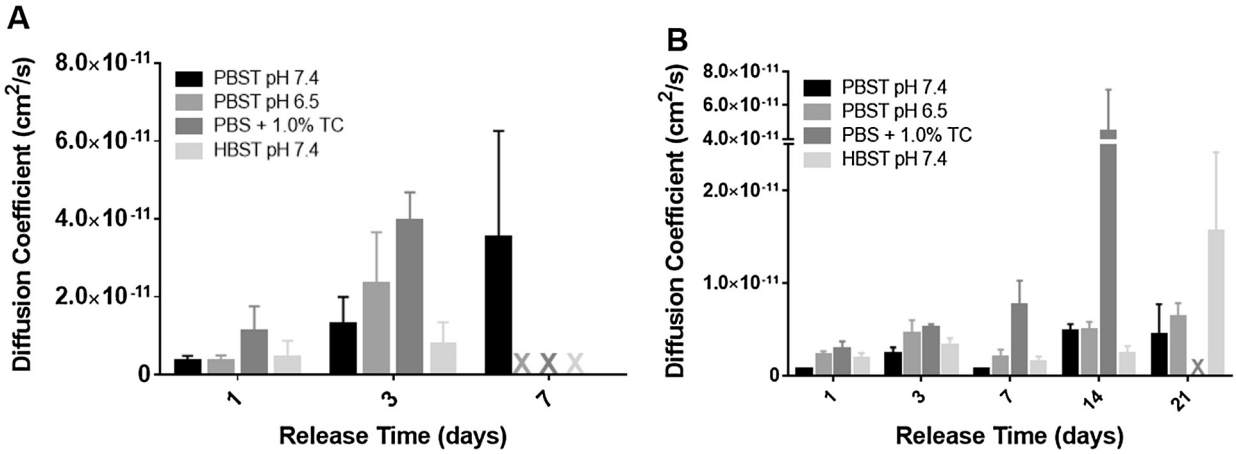

Fig. 8. Efffective BODIPY diffusion coefficients in degrading Tr-A_1 (A) and Tr-A_2 (B) microspheres in varying release media. Data represent mean ± SEM, n = 6. X indicates no diffusion coefficient could be determined due to agglomeration and/or saturation of the flourescent signal microspheres.

5. Conclusions

In closing, this study shows how *in vitro* release conditions affect not only the release rate of Tr-A, but also the mechanisms by which these microsphere formulations release the encapsulated drug. Release from low molecular weight acid-capped PLGA 50/50 microspheres (Tr-A_1) was accelerated at slightly acidic pH 6.5 and in media containing the plasticizer triethyl citrate. Drug release from this formulation was dominated by erosion in three of the four conditions studied, including PBST pH 6.5, where the rate of polymer hydrolysis was faster than in standard conditions. In the presence of TC, however, release was triggered not only by accelerated erosion, but also by increased diffusion in the polymer matrix with the latter as the principal controlling mechanism. Triethyl citrate also caused accelerated release from moderate molecular weight ester-capped PLGA 50/50 microspheres (Tr-A_2) by accelerating PLGA hydrolysis and causing accelerated erosion. These data further lay the foundation for comparing the release mechanism *in vivo* and developing mechanistic strategies for IVIVCs [55]. Future work to determine mechanisms of release *in vivo* will allow comparison to

the *in vitro* dataset presented here for the rational design of *in vitro* release tests to ultimately result in mechanism-based IVIVCs.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejpb.2016.11.008.

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