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RESEARCH PAPER

Lysozyme Release and Polymer Erosion Behavior of Injectable Implants Prepared from PLGA-PEG Block Copolymers and PLGA/PLGA-PEG Blends

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ABSTRACT

Purpose We evaluated the controlled release of lysozyme from various poly(D,L-lactic-co-glycolic acid) (PLGA) 50/50-polyethylene glycol (PEG) block copolymers relative to PLGA 50/50.

Methods Lysozyme was encapsulated in cylindrical implants (0.8 mm diameter) by a solvent extrusion method. Release studies were conducted in phosphate buffered saline +0.02% Tween 80 (PBST) at 37°C. Lysozyme activity was measured by a fluorescence-based assay. Implant erosion was evaluated by kinetics of polymer molecular weight decline, water uptake, and mass loss.

Results Lysozyme release from an AB15 di-block copolymer (15% 5 kDa PEG, PLGA 28 kDa) was very fast, whereas an AB10 di-block copolymer (with 10% 5 kDa PEG, PLGA 45 kDa) and ABA10 tri-block copolymer (with 10% 6 kDa PEG, PLGA 27 kDa) showed release profiles similar to PLGA. We achieved continuous lysozyme release for up to 4 weeks from AB10 and ABA10 by lysozyme co-encapsulation with the pore-forming and acid-neutralizing $MgCO_3$, and from AB15 by co-encapsulation of $MgCO_3$ and blending AB15 with PLGA. Lysozyme activity was mostly recovered during 4 weeks.

Conclusions These block co-polymers may have utility either alone or as PLGA blends for the controlled release of proteins.

KEY WORDS controlled release · implants · lysozyme · PLGA-PEG block co-polymers

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INTRODUCTION

Biodegradable polymers have been investigated extensively as delivery systems owing to their well-established safety and ability to provide continuous long-term controlled drug release. Among those studied, copolymers of lactic and glycolic acids (PLGA) are still the most common synthetic biodegradable polymers for preclinical, clinical, and scientific evaluation (1,2). Although PLGA has been commercially available for controlled peptide release for >30 years in products such as the Lupron Depot® and Zoladex®, and very recently Bydureon®, its utility for sustained protein delivery is still a challenge. A principal obstacle is protein instability in the polymer during encapsulation and release, for example owing to an acidic microclimate pH commonly developed in PLGA delivery systems during its degradation that can severely compromise protein stability (3). Several additional key stresses on the protein leading to instability in PLGA delivery systems have been reviewed in detail (4–6).

For example, previous work in our lab showed that an acidic microclimate pH, generated during polyester hydrolysis is the major cause for instability of bovine serum albumin (BSA) encapsulated in PLGA (50/50) cylindrical implants (7). By co-encapsulating poorly water-soluble basic additive such as $Mg(OH)_2$, the acidic microclimate in PLGA was neutralized and encapsulated BSA was stabilized for over a month (7,8). Another strategy to avoid acid-induced protein destabilization demonstrated by our lab and others is to prevent acid build-up in the polymer device by blending slowly degrading poly(D, L-lactide) (PLA) and water-soluble poly(ethylene glycol) (PEG) (9,10). This strategy not only prevents acid build-up but it also increases water uptake, which may help prevent protein damage induced by deleterious intermediate water levels in the protein (11).

Poly(ethylene glycol) (PEG) is a water-soluble polymer with well documented biocompatibility. Owing to its rapid clearance from the body and low-immunogenicity, PEG has a wide

range of biomedical applications (12). Since PEG has excellent ability to reduce or eliminate protein adsorption, it has been largely studied for parenteral protein delivery.

Incorporation of hydrophilic PEG blocks modifies the degradation rate and permeability of PLGA affecting both protein stability and release behavior. During the release, due to the presence of hydrophilic PEG blocks, the swollen structures with high water content in the polymer are formed, allowing increased exchange of polymer degradation products with the surrounding medium, thus minimizing acid build-up and consequent protein degradation (10). Also, water uptake and aqueous pore formation induced by liberation of PEG from block copolymer backbone are expected to increase release of encapsulated protein before the excessive PLGA degradation occurs, providing more continuous protein release (10). Therefore, release from PLGA-PEG delivery systems should be controlled not only by the polymer erosion but also by PEG/water-uptake mediated pore formation in the polymer, providing more continuous protein release profile compared to moderate MW PLGA only (13,14).

In this study, we evaluated the potential of millicylindrical implants prepared with two different PLGA-PEG di-block copolymers and one PLGA-PEG-PLGA tri-block copolymer to control the release of lysozyme as a model protein relative to PLGA. We also investigated the effect of formulation parameters such as PEG content (10% *vs.* 15%), PEG block number (di-block *vs.* tri-block) and the presence of poorly soluble salt, $MgCO_3$, on lysozyme release profile, water uptake, and polymer erosion characteristics from injectable millicylindrical implants.

MATERIALS AND METHODS

Materials

Lysozyme, $MgCO_3$, urea, DTT (DL-Dithiothreitol), EDTA (ethylenediamine-tetraacetic acid), Na_2HPO_4, NaH_2PO_4, NaCl, and KCl were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). Tween 80 (10%), acetone, silicone rubber tubing, and Coomassie plus reagent assay kit were purchased from Fisher Scientific (Hanover Park, IL). PLGA 50:50 (i.v.=0.60 dL/g and MW=54.4 kDa ester terminated) was purchased from Durect bioabsorbable polymers (Birmingham, AL). Di-block PLGA-PEG copolymers (RGP d 50105 and RGP d 50155) and tri-block PLGA-PEG-PLGA copolymer (RGP t 50106) were purchased from Boehringer Ingelheim (Germany). RGP d 50105 di-block copolymer (AB10) contains 10% 5 kDa PEG and PLGA 45 kDa, RGP d 50155 di-block copolymer (AB15) contains 15% 5 kDa PEG and PLGA 28 kDa, and RGP t 50106 tri-block copolymer (ABA10) contains 10% 6 kDa PEG and PLGA 27 kDa.

Preparation of Injectable Millicylindrical Implants with Lysozyme

Lysozyme was encapsulated into PLGA, PLGA-PEG, or PLGA-PEG-PLGA implants by a solvent extrusion method, similarly as previously described (15). Briefly, the lyophilized lysozyme powder as received was ground and sieved through 90-μm screen (Newark Wize Wearing, Newark, NJ). Protein powder of size ≤90 μm was suspended into 50% (w/w) polymer acetone solution, with or without $MgCO_3$. The suspension was then extruded into silicone rubber tubing (I.D. = 0.8 mm) with a 3 ml syringe. The tubing was then dried at room temperature for 24 h followed by vacuum drying at 40°C and −23 in. Hg vacuum for an additional 48 h. The final millicylindrical implants were obtained by removal of silicone tubing and were cut into 1 cm long segments for future use.

Scanning Electron Microscopy (SEM)

The surface morphology of implants was examined by Hitachi S3200N scanning electron microscope (Hitachi, Tokyo, Japan). The implants were first fixed on a brass stub using double sided adhesive tape and then were made electrically conductive by coating with a thin layer of gold (3–5 nm) for 120 s at 40 W. The images of implants were taken at an excitation voltage of 15 or 20 kV.

Measurement of Protein Loading

About 2 mg of implants were dissolved in 1.5 ml of acetone. After centrifugation at 8,000 rpm for 10 min, the supernatant polymer solution was removed and the precipitated pellet was washed with acetone twice. The pellet was then air dried, reconstituted in 1 ml of PBST, incubated at 37°C and lysozyme content was measured by using a Coomassie Plus protein assay kit and lysozyme for standard solutions. The protein loading was calculated as the percentage of lysozyme amount *versus* the total weight of the mixture (i.e., protein, polymer, and salt).

Evaluation of Protein Release Kinetics and Residual Protein

The *in vitro* protein release from implants was carried out in 10 mM PBST pH 7.4 (7.74 mM Na_2HPO4, 2.26 mM NaH_2PO4, 137 mM NaCl, 3 mM KCl, and 0.02% Tween® 80) as a release medium, at 37°C under mild agitation. Two 1 cm implants (about 15 mg) were placed into 1.5 ml polypropylene tubes with 1 ml PBST, incubated at 37°C and the medium was completely replaced with fresh medium at each predetermined time point. Lysozyme content in the release samples was measured by Coomassie plus

protein assay. The pH of the release medium was measured with an Orion 290A pH meter (Orion Research Inc., Boston, MA). All measurements were performed in triplicate ($n=3$).

At the end of release study, the remaining lysozyme was extracted from the polymer by the same procedure used to measure protein loading after freeze-drying the incubated implants. The protein pellet was then reconstituted in PBST and incubated at 37°C for 1 h to determine the soluble fraction of the protein remained in the polymer. Remaining insoluble precipitates were collected by centrifugation, brought up in a series of solvents, both denaturing (PBST/6 M urea/1 mM EDTA) and denaturing/reducing solvent (PBST/6 M urea/1 mM EDTA/10 mM DL-Dithiothreitol) to dissolve all protein aggregates, as described previously (7,8,15). Concentration of lysozyme aggregates was estimated by Coomassie plus protein assay and residual protein is reported as all aggregates dissolved at the end of the measurement. All measurements were performed in triplicate ($n=3$) and lysozyme standards were dissolved in the same solvent used for analysis.

Measurement of Lysozyme Activity

Lysozyme activity was measured by fluorescence-based EnzChek® Lysozyme Assay Kit (Molecular Probes, Inc., Eugene, OR). Lysozyme concentration in loading and release samples was measured by Coomassie plus protein assay. Samples were then diluted and incubated with DQ™ lysozyme substrate (*Micrococcus lysodeikticus*—fluorescein conjugate) for 30 min at 37°C. The fluorescence increase induced by free fluorescein released by lysozyme, was measured in a microplate reader using excitation/emission of ~485/530 nm. A background fluorescence of ~20 fluorescence units was subtracted from each value. Lysozyme activity, proportional to measured fluorescence, was then calculated from standard curve and fractional lysozyme activity was determined by the ratio of the lysozyme concentration from the activity assay to the enzyme concentration from the Commassie Plus assay.

Measurement of Water Uptake in Implants

After incubation in PBST at 37°C in different intervals, the implants were collected and blotted with tissue paper and weighed immediately. Then, the polymers were freeze-dried and weighed again. The water uptake was calculated by:

$$Water\ \ uptake(\%) = \frac{W_1 - W_2}{W_2} 100\%$$

where W_1 and W_2 are the weights of the fully hydrated implants and the dried implants, respectively.

Measurement of Polymer Degradation in Implants

After incubation in PBST at 37°C in different intervals, the implants were freeze-dried for analysis of weight averaged molecular weight (Mw) by gel permeation chromatography (GPC). The Waters 1525 GPC system (Waters, Milford, MA) consisted of two Styragel columns (HR 1 and HR-5E columns, 4.6×300 mm each, Waters, Milford, MA) connected in series, a binary HPLC pump, Waters 717 plus autosampler, Waters 2414 refractive index detector and Breeze™ software to compute molecular weight distribution. Sample solutions in tetrahydrofuran (THF) at a concentration of ~3 mg/mL were filtered through a 0.45 μm hydrophobic fluoropore (PTFE) filter (Millipore Corporation, Bedford, USA) before injection into the GPC system and were eluted with THF at 0.3 ml/min. The weight average molecular weight of each sample was calculated using monodisperse polystyrene standards, Mw 2,330–110,000 Da. The initial rate constant of Mw decline was determined by fitting a least squares linear regression to the ln(Mw) *vs* t data over the first 14 days of incubation.

Measurement of Kinetics of Polymer Mass Loss

After incubation in PBST at 37°C in different intervals, the implants were collected, freeze-dried and weighed to yield the eroded dry mass. The mass loss of each implant ($n=3$) was calculated as shown:

$$Mass\ \ loss(\%) = \frac{initial\ dry\ mass - eroded\ dry\ mass - protein\ mass\ released}{initial\ dry\ weight} 100\%$$

Statistical Analysis

All data are reported as mean ± standard error mean (SEM). Statistical analysis was performed on Tables III, IV and V and Fig. 3 to determine degree of similarity or difference between the mean values. We conducted a 2-*t* test to compare values in Tables III, IV and V and we used paired-*t* test (to preserve time line dependency) to compare values in Fig. 3 ($a=0.95$). We compared only the samples at the same days of the study and if the sample was not present in one set, we discarded the sample from the other set. Differences with the p values <0.05 were considered statistically significant. All data are summarized and presented as Supplementary Tables.

Table I Characteristics of PLGA-PEG Block Copolymers and PLGA (50/50) Polymer

Polymer Name	Polymer Type	PEG (%)	i.v. (dL/g)	Polymer MW (kDa)	PLGA MW (kDa)	PEG MW (kDa)
AB10	diblock	10	0.72	50	45	5
AB15	diblock	15	0.55	33	28	5
ABA10	triblock	10	0.61	60	27	6
PLGA	50/50	0	0.60	54.4	54.4	0

RESULTS AND DISCUSSION

Lysozyme Loading in Millicylindrical Implants

Some of the potential advantages of injectable PLGA millicylindrical implants for controlled drug release and treatment include simple preparation, good control of drug loading, and minimal migration of the implants from the injection site upon administration (5). Here, we evaluated two different PLGA-PEG di-block copolymers and one PLGA-PEG-PLGA tri-block copolymer for encapsulation and sustained release of lysozyme as a model protein. Block copolymers and a reference random moderate MW PLGA polymer used in this study are listed in Table I for comparison. Millicylindrical implants were prepared by the solvent extrusion method and measured lysozyme loading in all formulations was close to the theoretical value of 15% (w/w) (Table II).

Evaluation of Lysozyme Release from PLGA-PEG Implants

To evaluate the effect of PEG on lysozyme release, PLGA-PEG block copolymers and PLGA were used for implant preparation and lysozyme release was carried out in PBST at 37°C. In contrast to findings of Bittner *et al.* that initial protein burst from PLGA-PEG block copolymers was independent of PEG content (16), we measured much higher burst from di-block copolymer with 15% PEG (AB15) compared to di-block (AB10) and tri-block (ABA10) copolymers with 10% PEG. As seen in Fig. 1a, implants made with di-block copolymer AB15 showed very high initial burst with ~85% of lysozyme released within the first 24 h. The release of lysozyme encapsulated in these implants was almost completed by day 5 with ~98% of encapsulated lysozyme released (Fig. 1a).

The initial burst measured from implants made with di-block copolymer AB10 was significantly lower (~25%) and it was followed by continuous lysozyme release, without any lag time characteristic for the release from PLGA implants (Fig. 1a). However, after day 21 there was a considerable amount of lysozyme released during the fourth week of the study (Fig. 1a) similar to the release after the induction phase of the pure PLGA (see below). After this phase, lysozyme release slowed down and reached a plateau on day 35 with a total of ~85% enzyme release (Fig. 1a).

Implants prepared from tri-block copolymer ABA10 displayed a very low initial burst (~6%) followed by a slow and continuous lysozyme release for the first 10 days of the study. Similarly to the implants made with AB10, lysozyme release from ABA10 also had a rapid release phase after day 10. At the end of the third week, the release stopped with almost 88% of encapsulated lysozyme released (Fig. 1a).

As expected, release kinetics from the implants made with PLGA displayed a characteristic sigmoidal release profile with a low initial burst (~1%), followed by a lag phase during which a very low amount of lysozyme was released, and a rapid release phase initiated on day 14, which was completed on day 35 with total of 80% lysozyme released (Fig. 1a). Cumulative lysozyme release at the end of the release study was significantly different between all formulations with exception of the AB10 and ABA10 formulations ($p=0.070$, Supplementary Table 1).

Whereas lysozyme was released very rapidly from the implants with the highest PEG content (AB15), the release from implants made with AB10 and ABA10 copolymers was considerably slower and showed three phases: an initial burst, a phase of low continuous lysozyme release, and a terminal rapid release phase (Fig. 1a). Although the initial burst has been commonly thought to primarily occur from the lysozyme molecules released from the external surface and/or adjacent to the surface of the implants (13,17,18), data suggesting peptide and protein release from deep within the polymer from pores originating from preparation and/or initial implant hydration has also been reported (19,20).

Table II Loading of Millicylindrical Implants Prepared from PLGA-PEG Block Copolymers and PLGA (50/50) Polymer

Polymer Name	Lysozyme (%)	$MgCO_3$ (%)	Measured loading (%)	Loading efficiency (%)
AB10	15	0	16.2 ± 0.4	108 ± 3
AB10	15	3	16.0 ± 0.1	106 ± 1
AB15	15	0	16.8 ± 0.2	112 ± 1
AB15	15	3	15.7 ± 0.3	104 ± 2
ABA10	15	0	16 ± 1	105 ± 9
ABA10	15	3	18 ± 1	120 ± 9
PLGA	15	0	15.3 ± 0.9	102 ± 6
PLGA	15	3	14 ± 2	94 ± 14
AB10-PLGA	15	0	15.3 ± 0.1	102 ± 1
AB10-PLGA	15	3	14.8 ± 0.4	99 ± 2
AB15-PLGA	15	0	16.9 ± 0.1	113 ± 1
AB15-PLGA	15	3	15.0 ± 0.5	100 ± 3

At the end of the second lysozyme burst from ABA10 on day 21, a considerable pH drop from the initial pH 7.4 to of ~3.4 was measured in the release medium (Fig. 2a) indicating the strong release of acidic degradation products from the implant to overcome the buffer capacity of the PBST. A similar pH drop was measured a week later in the release medium incubated with implants made with PLGA-PEG di-block copolymers and with PLGA (Fig. 2a).

The residual lysozyme remaining in the devices after the 6-week release study was analyzed and listed in Table III and Supplementary Table 1. The total recovered lysozyme was from 93.8%–100.7% (Table III). We found that implants prepared with block copolymers released more lysozyme and had lower fraction of insoluble aggregates compared to implants made with PLGA (Table III).

Evaluation of the $MgCO_3$ Effect on Lysozyme Release from PLGA-PEG Implants

One of the central issues with proteins encapsulated in PLGA or any polymer for controlled release is protein damage, e.g., aggregation and hydrolysis caused by the acid build-up during the erosion phase (7). We have previously shown that PLGA compatibility with proteins could be improved by addition of poorly soluble basic additives for certain formulations (5,21). Co-encapsulation of $Mg(OH)_2$ or $MgCO_3$ was shown to stabilize the protein and to provide more continuous protein release from PLGA polymer (7,8,15,22,23). To examine the effect of $MgCO_3$ co-encapsulation in PLGA-PEG block co-polymers, we added 3% of this poorly soluble salt to the formulations with 15% lysozyme and carried out the release study in PBST at 37°C.

The presence of $MgCO_3$ did not significantly affect the lysozyme release profile from the implants prepared from the AB15. Without the base, these implants already displayed a very high initial burst and rapid lysozyme release within 5 days. Addition of $MgCO_3$ slightly increased the initial burst and almost all encapsulated lysozyme was released within 3 days (Fig. 1b). However, co-encapsulation of $MgCO_3$ significantly altered the lysozyme release profile of implants prepared from both di-block and tri-block copolymers with lower PEG content (10% PEG) (Fig. 1b). We measured sustained lysozyme release for 14 days from AB10 and for 28 days from ABA10 (Fig. 1b). Cumulative lysozyme release at the end of the release study was significantly different between all formulations with the exceptions of AB10 and AB15 ($p=0.090$, Supplementary Table 1). Addition of $MgCO_3$ also postponed the pH drop in the release medium for 1 week; the significant pH drop in the release medium (pH<4) incubated with ABA10 was detected on day 28 and with PLGA-PEG di-block copolymers and PLGA on day 35 (Fig. 2b).

The increased permeability of implants with co-encapsulated $MgCO_3$ is consistent with SEM images that show more porous and swollen matrix compared to the implants made with the same polymers without $MgCO_3$

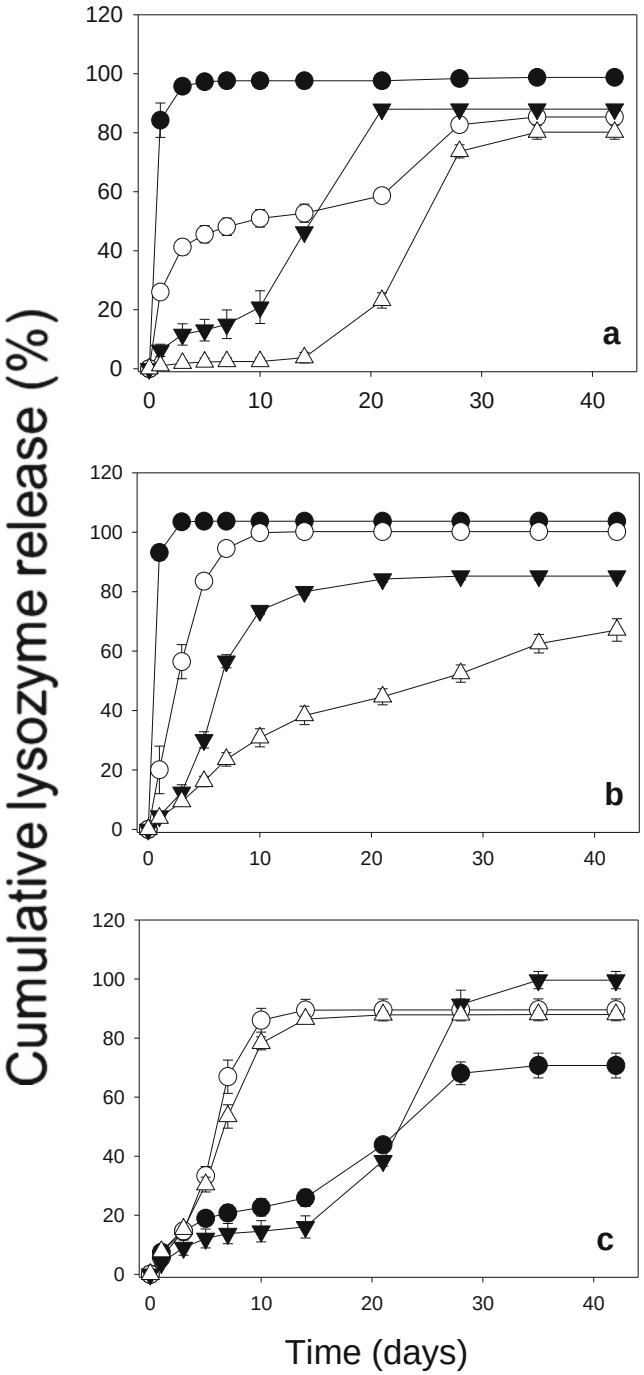

Fig. 1 (**a**) Release kinetics of lysozyme from implants prepared from polymers AB10 (*white circle*), AB15 (*black circle*), ABA10 (*black down-pointing triangle*), and PLGA (50/50) (*white triangle*). (**b**) Release kinetics of lysozyme from implants prepared from polymers AB10 (*white circle*), AB15 (*black circle*), ABA10 (*black down-pointing triangle*), and PLGA (50/50) (*white triangle*) co-encapsulated with 3% $MgCO_3$. (**c**) Release kinetics of lysozyme from implants prepared from PLGA (50/50) blended with: AB10 − $MgCO_3$ (*black down-pointing triangle*), AB10 + 3% $MgCO_3$ (*white triangle*), AB15 − $MgCO_3$ (*black circle*) and AB15 + 3% $MgCO_3$ (*white circle*). All studies were performed in PBST pH 7.4 at 37°C. *Symbols* represent mean ± SEM, $n=3$.

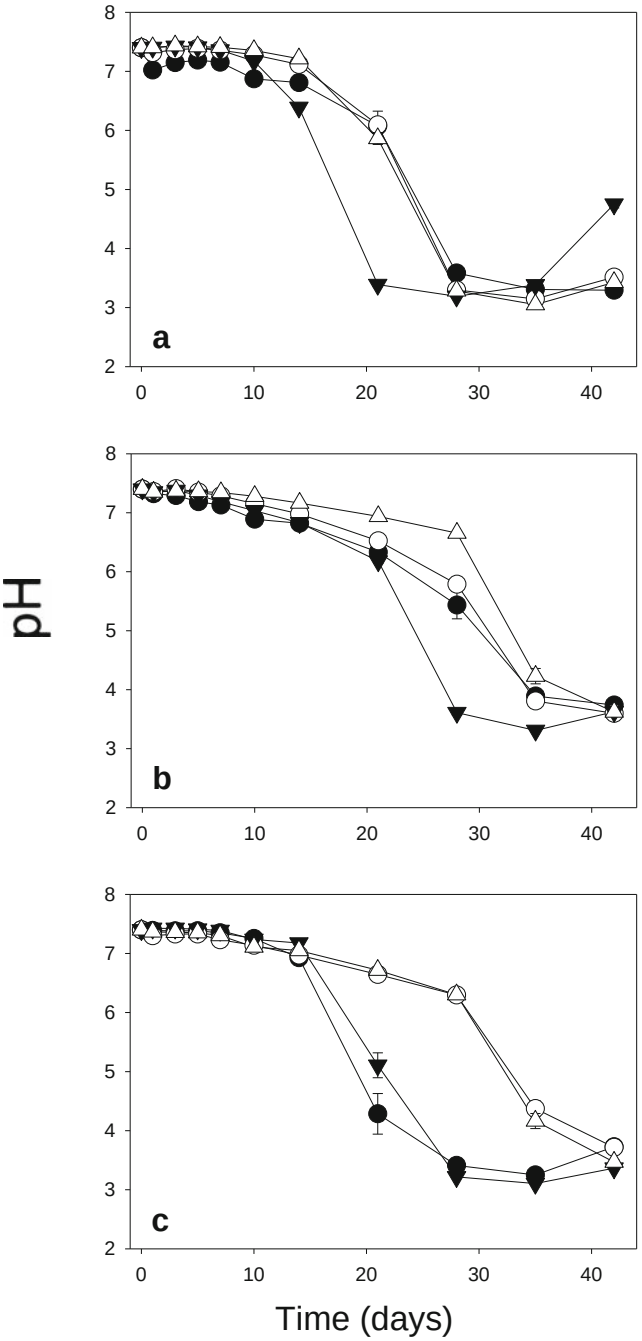

Fig. 2 Effect of co-encapsulated $MgCO_3$ on the pH change in the release medium incubated with (**a**) implants prepared from polymers AB10 (*white circle*), AB15 (*black circle*), ABA10 (*black down-pointing triangle*), and PLGA (50/50) (*white triangle*); (**b**) implants prepared from polymers AB10 (*white circle*), AB15 (*black circle*), ABA10 (*black down-pointing triangle*), and PLGA (50/50) (*white triangle*) co-encapsulated with 3% $MgCO_3$; and (**c**) implants prepared from PLGA (50/50) blended with: AB10 − $MgCO_3$ (*black down-pointing triangle*), AB10 + with 3% $MgCO_3$ (*white triangle*), AB15 − $MgCO_3$ (*black circle*) and AB15 + 3% $MgCO_3$ (*white circle*) (**c**). All studies were performed in PBST pH 7.4 at 37°C. *Symbols* represent mean ± SEM, $n = 3$.

(Supplementary Figure 1B), which is a well-established phenomenon in PLGAs (8,24).

The residual lysozyme remaining in the devices after the 6-week release study was analyzed and listed in Table III and Supplementary Table 1. The total recovered lysozyme was from 88.1%–105.8% (Table III). We found that implants prepared from the block copolymers released significantly more lysozyme and had a lower fraction of unreleased insoluble aggregates compared to PLGA implants (Table III, Supplementary Table 1). It is noteworthy that the low pH in the release medium by the end of the release study may have influenced amount of insoluble aggregation in the residual protein.

During encapsulation and release, full lysozyme activity was retained in nearly all samples (Fig. 3). However, some activity was lost in samples released in the fourth week from implants prepared from tri-block copolymers, most likely due to the significant pH drop (Fig. 2) caused by rapid polymer degradation in these implants, which might have affected enzymatic activity. No statistically significant difference in lysozymal activity was found between these groups (Supplementary Table 2).

Evaluation of Lysozyme Release from Implants Made with PLGA/PLGA-PEG Blends

Since both implants made with di-block copolymers, and particularly the one with 15% PEG, had high initial burst release, we sought to reduce this effect by modifying their composition and PEG content via blending the block copolymers with moderate MW PLGA 50/50 at a 1:1 wt. ratio. Blending resulted in significantly lower initial burst but did not provide more continuous and longer lysozyme release (Fig. 1c). The release profile was similar to the release profile measured in release media from tri-block copolymer implants and was accompanied by a similar pH drop to 4.3 (AB15/PLGA blend) and 5.1 (AB10/PLGA blend) detected on day 21 (Fig. 2c).

However, by adding 3% $MgCO_3$ to these formulations, a continuous release for 14 days (AB15/PLGA blend) and for 21 days (AB10/PLGA blend) (Fig. 1c) were achieved with only a slight pH drop (pH 6.3) in the release medium at the end of the forth week, when the lysozyme release had already completed (Fig. 2c).

The residual lysozyme remaining in the devices after the 6-week release study was analyzed and listed in Table IV. The total recovered lysozyme was from 85.9%–105.8%. We found that by adjusting the polymer composition and $MgCO_3$ co-encapsulation more continuous lysozyme release was achieved and significantly less amount of aggregates was generated, particularly in the PLGA blend with AB15 (Table IV, Supplementary Table 3).

Evaluation of Water Uptake by PLGA-PEG Implants

Due to the hydrophobic nature of PLGA, water penetration in large PLGA devices may be reduced leading to transient

Table III Summary of Cumulative 42-day Release and Aggregation Behavior of Lysozyme from Implants Prepared from Various Polymers With or Without 3% $MgCO_3$

Polymer Name	Lysozyme (%)	$MgCO_3$ (%)	Cumulative Release (%)	Soluble Residue (%)	Insoluble Aggregates (%)	Recovery (%)
AB10	15	0	85 ± 1	0.5 ± 0.1	7.9 ± 0.4	94 ± 1
AB10	15	3	100 ± 1	0.1 ± 0.1	4.8 ± 0.6	105 ± 1
AB15	15	0	99 ± 2	0	1.9 ± 0.5	101 ± 2
AB15	15	3	104 ± 2	0	2.0 ± 0.3	106 ± 3
ABA10	15	0	88 ± 1	0	6.8 ± 0.2	95 ± 1
ABA10	15	3	85 ± 1	0	2.8 ± 0.1	88 ± 1
PLGA	15	0	80 ± 2	0.5 ± 0.1	13.2 ± 0.2	94 ± 2
PLGA	15	3	67 ± 4	12 ± 3	20.3 ± 0.9	100 ± 4

intermediate water contents that could cause protein aggregation (7,13,16). As mentioned earlier, one way to increase water uptake capacity is to introduce water-soluble PEG segments in the hydrophobic PLGA chains.

It has been previously shown that PEG segments of PLGA-PEG di-block copolymers are surface oriented (12) and that PEG moieties anchored to PLGA in PLGA-PEG-PLGA tri-block copolymers are extended out into the aqueous environment, exhibiting chain flexibility and mobility similar to those of PEG molecules dissolved in water (25). These PEG chains allow significant water uptake that is expected to dilute the acidic degradation products formed inside the polymer matrix. The dissolution of PEG in the release medium may also create more water channels between pores to allow acidic species to diffuse out from the polymer and potentially allow buffering species enter the polymer matrix from the surrounding medium (10). Moreover, PEG chain location toward the aqueous solution should enhance polymer surface wettability

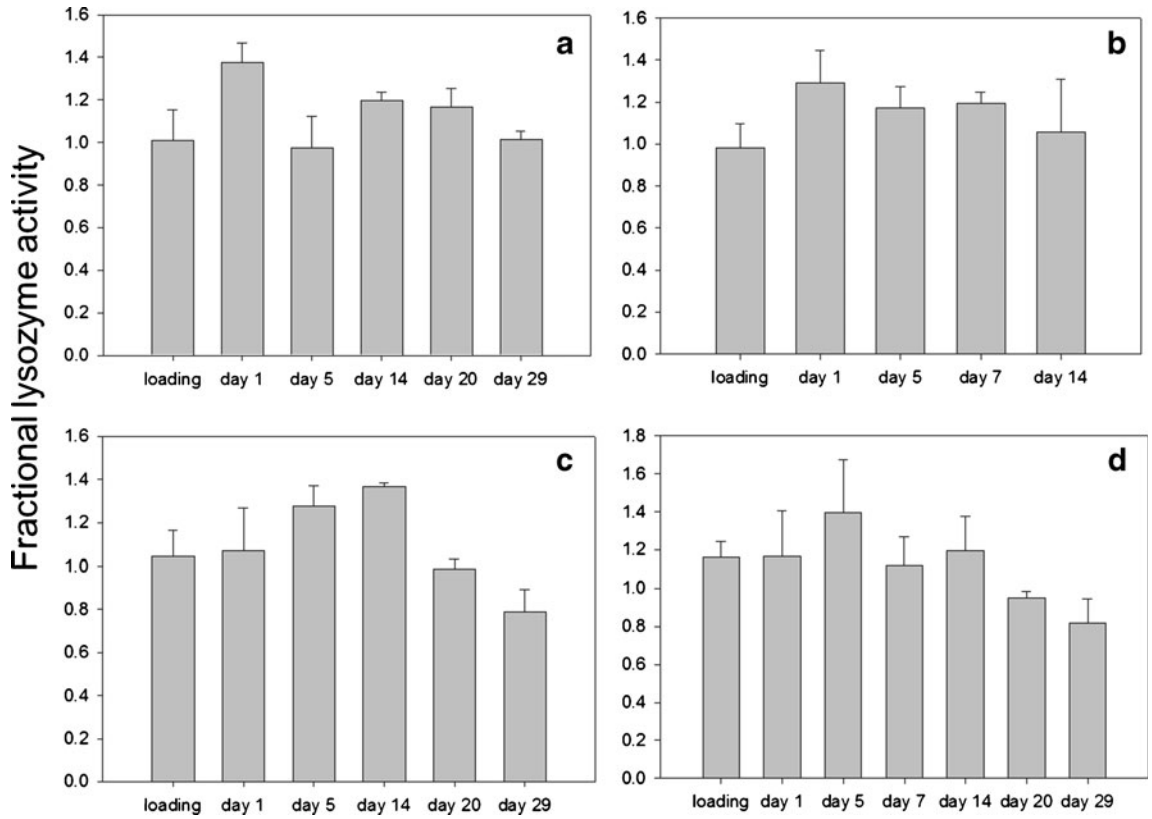

Fig. 3 Fractional activity of lysozyme released from implants prepared from AB10 di-block copolymer with (**b**) or without co-encapsulated 3% $MgCO_3$ (**a**), and ABA10 tri-block copolymer with (**d**) or without co-encapsulated 3% $MgCO_3$ (**c**). Lysozyme activity was not evaluated after 2 weeks from implants in (**b**) because release was complete by the two-week time point. *Bars* represent mean ± SEM, $n = 3$.

Table IV Summary of Cumulative 42-day Release and Aggregation Behavior of Lysozyme from Implants Prepared from di-Block Copolymers Blended with PLGA (50/50) at 1:1 Weight Ratio with or Without 3% $MgCO_3$

Polymer Name	Lysozyme (%)	$MgCO_3$ (%)	Cumulative Release (%)	Soluble Residue (%)	Insoluble Aggregates (%)	Recovery (%)
AB10-PLGA	15	0	100 ± 3	0.3 ± 0.1	5.9 ± 0.4	106 ± 3
AB10-PLGA	15	3	88 ± 1	0.6 ± 0.1	6.5 ± 0.5	95 ± 1
AB15-PLGA	15	0	71 ± 4	0.7 ± 0.2	14 ± 2	86 ± 4
AB15-PLGA	15	3	90 ± 4	0	2.9 ± 0.7	92 ± 4

and reduce protein adsorption, preventing possible protein damage (12).

To evaluate the effect of PEG on water content in PLGA-PEG block copolymers evaluated here, we measured water uptake kinetics in all the implants. Water uptake in blank implants (without protein) was strongly affected by the PEG content. For example, AB15 implants had higher water uptake than those prepared from AB10. Water uptake reached its peak on day 21 when ~260% of the dry implant weight was measured in both AB15 and ABA10 and ~180% in AB10 (Fig. 4a). On the same day, as expected, the pure PLGA implant water uptake was low (only ~20%) (Fig. 4a).

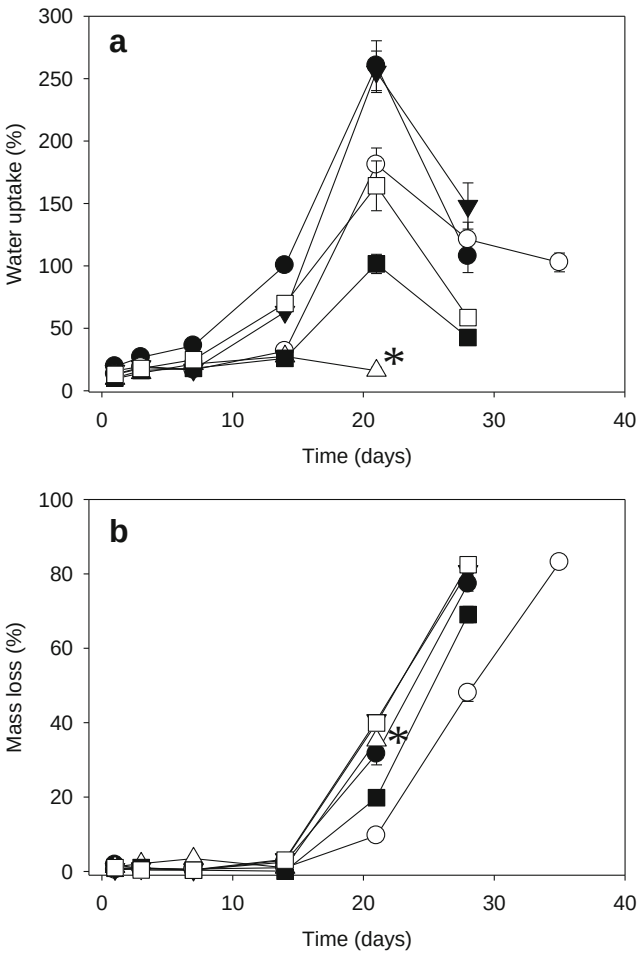

Fig. 4 Kinetics of water uptake (**a**) and mass loss (**b**) of blank implants prepared from polymers AB10 (*white circle*), AB15 (*black circle*), ABA10 (*black down-pointing triangle*), PLGA (50/50) (*white triangle*), and PLGA (50/50) blends with AB10 (*black square*) or with AB15 (*white square*). *Symbols* represent mean ± SEM, $n = 3$. * PLGA samples after 21 days were unrecoverable due to implant disintegration.

Water uptake decreased after day 21 suggesting a possible loss of PEG blocks as a result of physical disintegration of block copolymers. This decrease in water uptake is in contrast with previous findings of *Jeong et al.*, who showed lowered water uptake by PLGA-PEG/PLGA blends as a result of too much hydration of PEG blocks located between PLGA domains (12). As expected, water uptake by blank implants made with PLGA-PEG/PLGA blends was slightly lower compared to the corresponding di-block copolymers (Fig. 4a).

Water uptake in implants prepared from block copolymers with encapsulated lysozyme was also strongly affected by the presence of the protein. When the protein is incorporated into an ester end-capped PLGA matrix of moderate molecular weight, a system of hydrophobic matrix and hydrophilic protein is created and water uptake is governed mostly by the protein (16). However, when protein is encapsulated into PLGA-PEG block copolymers, water uptake is governed by hydrophilic moieties, protein and PEG. On day 1, water uptake by implants prepared from AB15 and AB10 was 66% and 44% of the dry implant weight, respectively (Fig. 5a). Although tri-block copolymer implants did not show much higher water uptake compared to PLGA implants within the first week, by the end of week 2, the water uptake in implants made with ABA10 reached almost 140% of the dry implant weight compared to ~54% for PLGA implants (Fig. 5a).

The incorporation of PEG responsible for rapid water uptake also facilitates dissolution of encapsulated protein and its release from the implants. As mentioned before, lysozyme release from block co-polymers with 10% PEG (AB10 and ABA10) follows tri-phasic kinetics. Lysozyme release after the initial burst appears to be governed by a combination of multiple mechanisms, with a likelihood of passive pore-diffusion in initial pores and pores created during swelling of the polymer matrix, followed by some degree of pore healing (i.e., closure). The second phase is likely governed by diffusion through the channels formed by osmotic forces, PEG

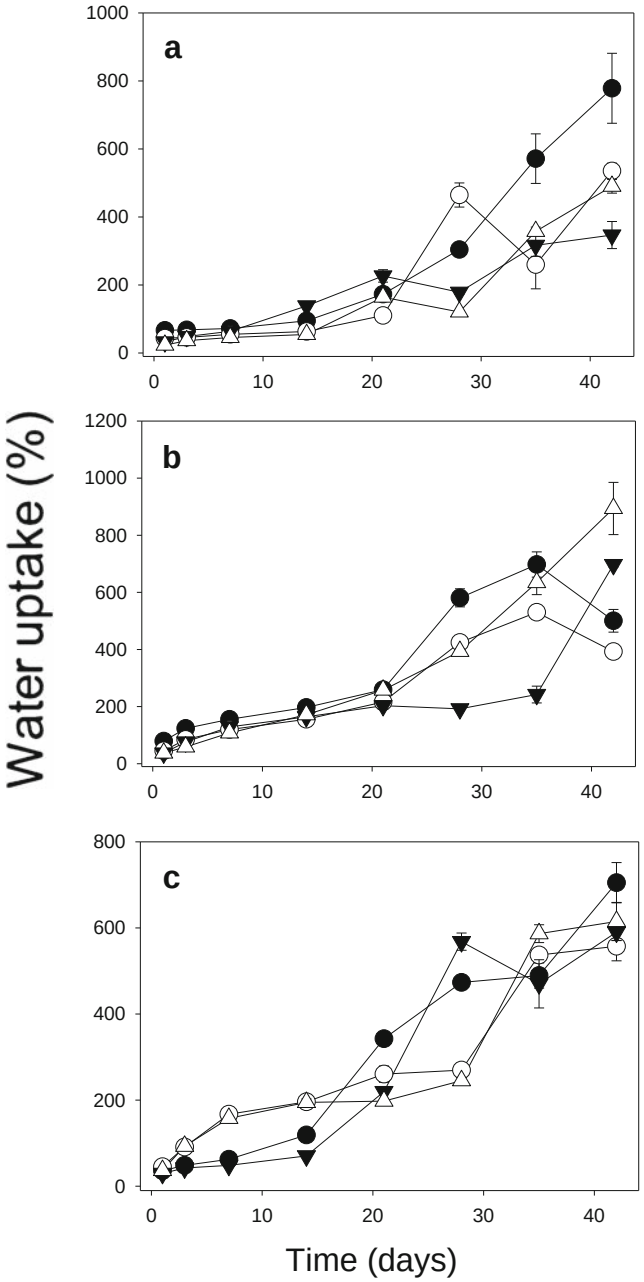

Fig. 5 The effect of lysozyme and $MgCO_3$ on water uptake by implants prepared from polymers AB10 (*white circle*), AB15 (*black circle*), ABA10 (*black down-pointing triangle*), and PLGA (50/50) (*white triangle*) without $MgCO_3$ (**a**) and with 3% $MgCO_3$ (**b**) as a function of incubation time in PBST pH 7.4 at 37°C. **c** Water uptake by implants prepared from PLGA (50/50) blended with AB10 without $MgCO_3$ (*black down-pointing triangle*), with AB10 and with 3% $MgCO_3$ (*white triangle*), with AB15 without $MgCO_3$ (*black circle*), and with AB15 and with 3% $MgCO_3$ (*white circle*) incubated in PBST pH 7.4 at 37°C. *Symbols* represent mean ± SEM, $n = 3$.

leaching, and/or by matrix swelling, while the later rapid release phase seems to be mostly affected by the polymer erosion. Formation of nearly inter-connected pores/channels and swollen matrix could be clearly observed in SEM images of the implants made with PLGA-PEG block copolymers during the release (Supplementary Figure 1). The highest porosity was found in implants made with AB15 with the highest PEG content.

As seen in Figs. 1a and 5a, AB15 implants displayed higher water uptake and released more lysozyme in the first 5 days compared to those from AB10, ABA10, or PLGA only. After the first week, water uptake by AB15 implants was mostly governed by hydrophilic PEG segments of the polymer and only partly by the unreleased aggregated protein remained in the implants (Tables III and IV). This protein aggregation could be a result of protein adsorption/absorption into a hydrogel-like swollen matrix created by PEG-induced water uptake. Further studies would be necessary to test this hypothesis.

In general, higher water content in the polymer device should provide a more stabilizing environment for the protein by creating an aqueous protein solution in the PEG network and swollen pore structures, which are expected to allow rapid ion exchange leading to neutral pH inside the device and preventing protein damage (13,26). By increasing molecular density and solvent viscosity, PEG can lower protein aggregation, thus acting as an osmolytic stabilizer (13,27) and it can also potentially stabilize proteins by minimizing their adsorption to PLGA (28). On the other hand, higher PEG content in PLGA-PEG block copolymers could induce matrix swelling that is also potentially damaging to the protein. In order to get the optimal protein-stabilizing effect, both PEG content and molecular weight of PLGA and PEG presumably need to be carefully optimized by adding right amount of PEG that will not induce extensive matrix swelling but will stabilize the protein. By contrast, it should be noted that PEG is not necessarily stabilizing to the structure of certain proteins (22).

When $MgCO_3$ was co-encapsulated with lysozyme, the water content was further increased in all the polymers, including PLGA (Fig. 5b), suggesting that the driving force for water uptake governed by $MgCO_3$ is stronger than the one governed by PEG and lysozyme. The effects of $MgCO_3$ co-encapsulation can therefore be summarized as follows: (1) increased continuous protein release, (2) raised microclimate pH, (3) increased in water uptake, and (4) decreased acid-catalyzed hydrolysis of PLGA. Implants with co-encapsulated 3% $MgCO_3$ showed higher water uptake (Fig. 5b) and slower mass loss (Fig. 6b) compared to those without $MgCO_3$. This higher water uptake and slower degradation pattern in implants with co-encapsulated $MgCO_3$ have been attributed to: (1) formation of Mg-carboxylate salts by reaction between $MgCO_3$ and water-soluble PLGA degradation products (that disrupts PLGA autocatalysis), (2) osmotic pressure created by the newly formed salts in the polymer pores, which creates new pores in polymer matrix, and (3) interactions between Mg ions and polymer functional groups such as carboxylic or ester groups to decrease ester bond hydrolysis (3,7,8,15,29).

Erosion of PLGA-PEG Block Copolymers

The influence of PEG on the polymer erosion was monitored by polymer MW decline, pH change in the buffered release medium, and kinetics of mass loss. We first evaluated polymer degradation in blank implants (with no encapsulated protein) prepared from block copolymers and PLGA and we found that both di-block copolymers showed different degradation kinetics, as measured by the initial degradation rate constant (Table V, Supplementary Table 4). However, the initial rate constants for ABA10 tri-block copolymer and PLGA were higher (Table V), indicating faster degradation of these polymers. The initial rate constants for ABA10 and PLGA did not show a statistically significant difference ($p = 0.089$, Supplementary Table 4). Despite the polymer degradation, blank implants did not start losing mass until after first 2 weeks of incubation. During the third week, a significant mass loss of 40% was measured for ABA10, 35% for PLGA and 31% for AB15, and only 9% for AB10 (Fig. 4b). Implants from ABA10 and AB15 both lost ~80% of the mass by day 28, and the similar mass loss of ~83% was measured for AB10 on day 35 (Fig. 4b). Both polymer degradation and mass loss kinetics are a result of balance of PLGA molecular weight and PEG content—polymers with shorter PLGA chains and lower PEG content are expected to degrade and lose mass faster than those with longer PLGA chains and higher PEG content. In the block copolymers evaluated here, the presence of PEG, which facilitates removal of acidic degradation products thereby slowing auto-catalytic polymer degradation and mass loss, was compensated by shorter PLGA chains in ABA10 and AB15 (27 kDa and 28 kDa compared to 54.4 kDa PLGA that we used for comparison). As a result, these block copolymers showed very similar mass loss kinetics to PLGA (Fig. 4b). On the other hand, although AB10 has lower PEG content than AB15, AB10 lost mass slower, probably because of the much longer PLGA chains required longer to degrade (45 kDa compared to 28 kDa, Table I). Implants prepared from di-block PLGA-PEG/PLGA blends showed higher initial polymer degradation rate constant and exhibited faster mass loss compared to corresponding di-block co-polymers likely due to the lower PEG content in the blends (Fig. 4b, Table V, Supplementary Table 4).

Encapsulation of lysozyme caused significantly slower degradation of all polymers and mass loss in all the implants, particularly those prepared from AB15 and PLGA (Table V, Supplementary Table 4). Tri-block copolymer ABA10 still had the highest initial degradation rate constant (Table V) and implants prepared from this polymer lost mass faster than all the others (Fig. 6). Significant mass loss of 33.2% was measured in implants from ABA10 on day 21 (Fig. 6a) together with a considerable pH drop to 3.3 (Fig. 2a). This mass loss overlapped with the end of the lysozyme rapid release phase measured between days 10 and 21 (Fig. 1a) and a water uptake of 203.2% (Fig. 5a). These data suggest that during the first 2 weeks lysozyme was released mainly through diffusion through pores formed via water uptake (with water uptake rate-controlling), while the release during the third week was primarily governed by polymer erosion. Co-encapsulation of $MgCO_3$ slowed polymer degradation (Table V) and postponed mass loss (Fig. 6) and pH drop (Fig. 2) for 1 week.

Di-block copolymer AB10 had a lower initial degradation rate constant than ABA10 (Table V) and implants prepared from the former started losing mass later, after 3 weeks of incubation (Fig. 6a). A considerable mass loss of 35.7% was detected in these implants at the end of fourth week (Fig. 6a), accompanied by a significant pH drop (pH 3.3) in release medium (Fig. 2a). During the fourth week of the release study water uptake of 464% was measured (Fig. 5a) and a large amount of lysozyme was released after the induction period (Fig. 1a) at the same time as a pH drop in the release medium. Co-encapsulation of $MgCO_3$ did not affect polymer degradation (Table V) but it did postpone mass loss and the pH drop for 1 week, when 49.4% mass of implant was lost (Fig. 6b) causing a pH to drop to 3.8 (Fig. 2b). However, lysozyme release from these implants was already completed by the day 10.

Although the AB15 di-block copolymer degraded slower than all the polymers, AB15 implants started losing mass during the third week of incubation, similarly to the implants prepared with ABA10. On day 28, a significant mass loss of 36.2% (Fig. 6a) accompanied by a pH drop to 3.5 was observed (Fig. 2a). Polymer erosion did not affect lysozyme release from this polymer formulation, which was completed by the day 5 (Fig. 1a). Unlike other block copolymers, the initial degradation rate constant for AB15 significantly increased by co-encapsulation of $MgCO_3$ (Table V, Supplementary Table 4), and the implants prepared from this polymer with co-encapsulated $MgCO_3$ also lost mass somewhat faster (Fig. 6b). The pH drop was postponed for 1 week (Fig. 2b), but did not affect lysozyme release, which was already complete by day 3 (Fig. 1b).

We observed that molecular weight of PLGA in block copolymers affected degradation rate and mass loss significantly, as suggested by the fact that ABA10 eroded much faster than AB10 (Table V, Fig. 6, Supplementary Figures 2 and 3). Both block copolymers contained 10% PEG but the PLGA blocks in the ABA10 were 27 kDa each, whereas the molecular weight of PLGA in AB10 was much higher—45 kDa (Table I). Even though the AB15 had a higher PEG content than AB10, which should facilitate faster release of acidic degradation products and slower polymer degradation, the AB15 eroded faster during the first 3 weeks, most likely due to higher water uptake (Fig. 5) and smaller PLGA chains in AB15 (Table I). Both di-block polymers implant types showed identical mass loss at the end of week four, after which the mass loss of AB10 was much faster compared to AB15. The latter slowed down and was similar to the mass loss of PLGA at the end of week 6 (Fig. 6).

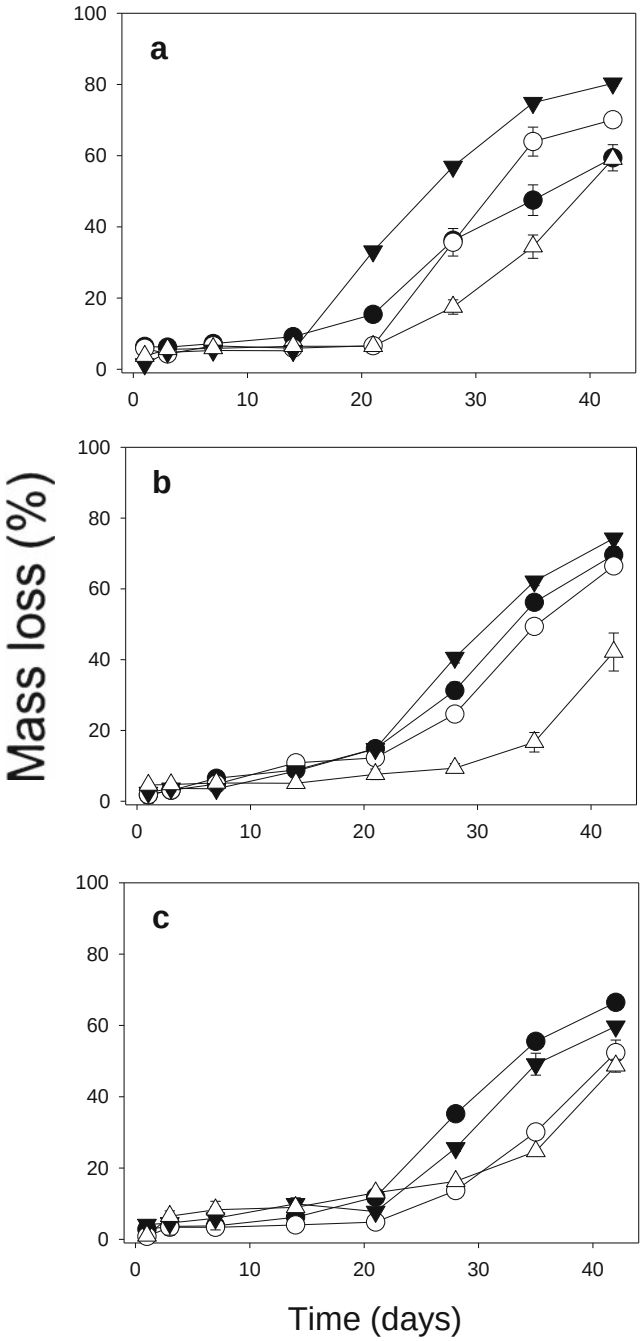

Fig. 6 The effect of lysozyme and $MgCO_3$ on mass loss of implants prepared from polymers AB10 (*white triangle*), AB15 (*black circle*), ABA10 (*black down-pointing triangle*), and PLGA (50/50) (*white triangle*) without $MgCO_3$ (**a**) and with 3% $MgCO_3$ (**b**) as a function of incubation time in PBST pH 7.4 at 37°C. (**c**) Mass loss of implants prepared from PLGA (50/50) blended with AB10 without $MgCO_3$ (*black down-pointing triangle*), with AB10 and with 3% $MgCO_3$ (*white triangle*), with AB15 without $MgCO_3$ (*black circle*), and with AB15 and with 3% $MgCO_3$ (*white circle*) incubated in PBST pH 7.4 at 37°C. *Symbols* represent mean ± SEM, $n = 3$.

Table V Summary of Initial Rate Constants for Polymer Degradation in Blank Implants and in Implants with Encapsulated Lysozyme, with or Without $MgCO_3$, Measured by GPC

Polymer Name	Lysozyme (%)	$MgCO_3$ (%)	Initial rate constant (day^{-1})
AB10	0	0	0.075 ± 0.003
AB15	0	0	0.104 ± 0.002
ABA10	0	0	0.116 ± 0.002
PLGA	0	0	0.120 ± 0.002
AB10-PLGA [a]	0	0	0.110 ± 0.002
AB15-PLGA [a]	0	0	0.117 ± 0.004
AB10	15	0	0.047 ± 0.002
AB10	15	3	0.050 ± 0.003
AB15	15	0	0.023 ± 0.001
AB15	15	3	0.039 ± 0.002
ABA10	15	0	0.094 ± 0.002
ABA10	15	3	0.053 ± 0.002
PLGA	15	0	0.034 ± 0.003
PLGA	15	3	0.015 ± 0.001
AB10-PLGA [a]	15	0	0.050 ± 0.006
AB10-PLGA [a]	15	3	0.029 ± 0.003
AB15-PLGA [a]	15	0	0.056 ± 0.003
AB15-PLGA [a]	15	3	0.028 ± 0.001

[a] Block copolymers were blended with PLGA 50/50 at a 1:1 w/w ratio

It has been shown before that during PLGA erosion mass loss is preceded by a substantial decrease in polymer molecular weight that is positively correlated to water influx (30). However, degradation and erosion of PLGA-PEG block copolymers, due to rapid water uptake, were shown not to be chronologically separated and to start immediately after their incubation (16).

Our data show that in all of the blank implants examined, polymer degradation starts immediately upon incubation (Supplementary Figure 2), but without substantial mass loss until the third week (Fig. 4b). Significant mass loss of all polymers, PLGA-PEG block copolymers and PLGA, is preceded with higher water uptake that reaches maximum on day 21, when a considerable mass loss was observed (Fig. 4).

Lysozyme encapsulation reduced the polymer degradation rate in all the implants, and most strongly in the implants prepared from AB15 (Table V, Supplementary Figure 3) and postponed mass loss, particularly from the implants prepared from AB15 (Fig. 6). The examined tri-block copolymer degraded faster than other polymers, although its degradation was slowed down by co-encapsulation of $MgCO_3$ (Table V, Supplementary Figure 3B).

We have previously shown that co-encapsulation of poorly soluble $MgCO_3$ considerably increased the water uptake profile and caused substantial decrease in the degradation rate and mass loss (30). Although we did observe the similar trend with tri-block copolymer ABA10 and with PLGA, we found that co-encapsulation of $MgCO_3$ did not have effect on degradation rate of AB10 and that it actually increased degradation rate and mass loss of AB15 (Fig. 6, Table V). It is possible that co-encapsulation of $MgCO_3$ in implants prepared from AB15

increased the concentration of carboxylate ions and water in the polymer phase, thereby stimulating base-catalyzed polymer degradation. In a related study, we found the similar trend with implants prepared from AB15 with encapsulated BSA, with or without $MgCO_3$ (unpublished data).

CONCLUSION

PLGA-PEG block copolymers and PLGA investigated here showed noticeable differences with regard to their degradation profile and lysozyme release. Higher PEG content (15%) caused very fast lysozyme release from AB15 but the release profile was much improved by blending AB15 with intermediate MW PLGA. Di-block copolymer with lower PEG content (10%) AB10 showed lower initial burst but the remaining release was discontinuous and showed a considerable second rapid release phase caused by polymer erosion. By blending AB10 with PLGA and co-encapsulation of $MgCO_3$ we managed to obtain sustained lysozyme release for 21 days with minimal protein aggregation or enzyme activity loss. However, obtaining lysozyme continuous release from AB15 required both co-encapsulation of $Mg(CO)_3$ and blending AB15 with PLGA. The tri-block copolymer ABA10 (10% PEG content) showed fastest degradation rate of all polymers tested and although with favorably low initial lysozyme burst, a large rapid release of lysozyme was observed between days 10 and 21 mostly caused by polymer erosion. By co-encapsulation of $MgCO_3$ we achieved continuous 1-month lysozyme release and improved lysozyme stability. All three block co-polymers used here were found to degrade faster than PLGA, with ABA10 made with lower molecular weight (27 kDa) PLGA blocks and moderate (10%) PEG content, being the fastest. Lysozyme activity was mostly recovered in the release media during 4 weeks. Therefore, these block copolymers have potential to be used for continuous protein controlled release.

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