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Stability of proteins encapsulated in injectable and biodegradable poly(lactide-*co*-glycolide)-glucose millicylinders

Jichao Kang [a,1], Oliver Lambert [b,2], Michael Ausborn [b,3], Steven P. Schwendeman [a,*]

[a] *Department of Pharmaceutical Sciences, University of Michigan, 428 Church St., Ann Arbor, MI 48109-1065, USA*
[b] *Pharmaceutical R&D, Novartis Pharma AG, CH-4056 Basel, Switzerland*

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Abstract

Purpose: To characterize protein stability in poly(lactide-*co*-glycolide) 50/50-glucose star (PLGA-Glu) injectable millicylinders and to compare results with linear PLGA 50/50.
Methods: Bovine serum albumin (BSA), a model protein, was encapsulated in PLGA-Glu and linear PLGA millicylinders by solvent-extrusion and incubated under physiological conditions. Important system properties were characterized, including: polymer molecular weight distribution, soluble acidic residues, polymer morphology, polymer water uptake, microclimate pH, protein content and release, and protein aggregation. The polymer microclimate late in the release incubation was simulated and protein recovery was analyzed by UV_{280}, size exclusion chromatography, amino acid analysis, and a modified Bradford assay.
Results: PLGA-Glu contained higher levels of low molecular weight oligomers, more rapidly biodegraded, and exhibited a lower microclimate pH than the linear 50/50 PLGA, which is the most acidic type in the PLGA family. BSA, when encapsulated in PLGA-Glu millicylinders, underwent extensive noncovalent insoluble aggregation over 2 weeks *in vitro* release, which was almost completely inhibited upon co-encapsulation of $Mg(OH)_2$. However, by 5 weeks release for base-containing formulations, although insoluble aggregation was still suppressed, the soluble fraction of protein in the polymer was unrecoverable by the modified Bradford assay. Polymer microclimate simulations with extensive protein analysis strongly suggested that the low recovery was mostly caused by base-catalyzed hydrolysis of the oligomeric fraction of BSA.
Conclusions: In PLGA-Glu, the acidic microclimate was similarly responsible for insoluble aggregation of encapsulated BSA. BSA aggregation was inhibited in millicylinders by co-incorporation into the polymer an insoluble base, but over a shorter release interval than linear PLGA likely because of a more acidic microclimate in the star polymer.
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1. Introduction

Poly(DL-lactide-*co*-glycolide) (PLGA) has been used successfully to deliver therapeutic peptides and proteins in a controlled-release manner (Chung et al., 2006; Cleland and Jones, 1996; Dong et al., 2006; Kang and Schwendeman, 2007; Kissel et al., 1991; Okada, 1997; Okada et al., 1988; Schwendeman et al., 1997; Tracy et al., 1999). Its biocompatibility and biodegradability are primary driving forces behind the intensive research with this polymer (Anderson and Shive, 1997; Wise et al., 1979). Efforts have also been made to physically (Singh et al., 2000; Cui and Schwendeman, 2001; Cui et al., 2007) and chemically (Barrera et al., 1993; Dailey et al., 2006; Kissel et al., 2002; Kumar et al., 2001) modify PLGA to achieve more desirable properties, such as modified surface property, molecular weight, water uptake, hydrophilicity, solubility, degradation and erosion rate. By monomer placement and inter-segment linkage site, PLGA copolymers can be classified into graft, star, block, and random copolymers (Kumar et al., 2001). Several polymers and small molecules have been grafted to, or copolymerized with, PLGA, such as PVA (Jung et al.,

* Corresponding author. Tel.: +1 734 615 6574; fax: +1 734 615 6162.
E-mail address: schwende@umich.edu (S.P. Schwendeman).
[1] Current address: Department of Formulation Development, MedImmune Inc., 319 N. Bernardo Avenue, Mountain View, CA 94043, USA.
[2] Current address: Drug Product Operations, Actelion Pharmaceuticals Ltd., Gewerbestrasse 16, CH-4123 Allschwil, Switzerland.
[3] Current address: Pharmaceutical & Analytical R&D, F. Hoffmann-La Roche Ltd., Building 72/322A, CH-4070 Basel, Switzerland.

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2001; Westedt et al., 2007), PEG/PEO (Kissel et al., 2002), dextran (Zhang and Chu, 2000), and glucose (Kissel et al., 1991). For example, PLGA-PEG copolymers have been synthesized to explore the excellent flexibility, hydrophilicity and safety of PEG (Gref et al., 1994; Jeong et al., 2000; Mallarde et al., 2003; Mosqueira et al., 2001). It is found that covalently attached PEG on the surface of PLGA nanoparticles can substantially reduce their clearance from the blood compartment and alter their biodistribution (Gref et al., 1994; Mosqueira et al., 2001). Kissel et al. synthesized and characterized ABA triblock copolymers from polyester hard segments and poly(ethylene oxide) soft segments, which exhibited properties of hydrogels and was used to deliver proteins in the form of implants, micro- and nanospheres.

Among modified PLGA copolymers, poly(DL-lactide-*co*-glycolide)-glucose (PLGA-Glu), a star polymer, with PLGA domains extending from hydroxyl groups of a D-glucose core has been used successfully to control the release of an octapeptide somatostatin analog, octreotide acetate. This dosage form is marketed by Novartis Pharma AG for treatment of acromegaly. However, there are only limited reports about the protein stability in and controlled-release from PLGA-Glu (Bodmer et al., 1992; Kissel et al., 1991). The purpose of this study was to characterize the protein release from, and stability in, PLGA-Glu injectable delivery systems, with a focus on protein degradation by acidic microenvironment in the polymer and protein stabilization.

Bovine serum albumin was encapsulated in PLGA-Glu millicylinders as a model acid-labile protein, and several important properties of this delivery system were characterized with respect to protein stability and release. These results were analyzed and compared with those obtained with linear PLGA 50/50 polymers with similar molecular weight.

2. Materials and methods

2.1. Materials

Poly(DL-lactide-*co*-glycolide) 50/50, end-group capped, with an inherent viscosity of 0.61 dl/g in HFIP at 30 °C was obtained from Birmingham polymers, Inc. (Birmingham, AL). Poly(DL-lactide-*co*-glycolide)-glucose 50/50 was a generous gift from Novartis Pharma AG (Basel, Switzerland). Poly(vinyl alcohol) (PVA) (80% hydrolyzed, MW 9–10 kDa) was from Aldrich Chemical Company, Inc. (Milwaukee, WI). Bovine serum albumin (BSA), magnesium hydroxide, urea and DL-dithiothreitol (DTT) were from Sigma Chemical Company (Louis, MO). NaOH solution, acetone, methylene chloride and tetrahydrofuran were from Fisher Scientific (Hampton, NH).

2.2. Analysis of PLGA-Glu by GPC and potentiometric titration

A column bank (Styragel® HR 5E-HR 1, 7.8 mm × 300 mm, Waters Corporation, Milford, MA) was used to analyze the molecular weight distribution of PLGA-Glu. Tetrahydrofuran was delivered as the mobile phase at 0.8 ml/min. The HPLC system consists of a Waters 1525 binary pump, a 717 plus autosampler and is controlled by Breeze® software with GPC package. Detection was by a Hewlett Packard 1737A Refractive index detector. The molecular weights were calculated from a relative calibration curve produced with polystyrene narrow standards (Polysciences, Inc., Warrington, PA).

The water-soluble acid content in the polymer was measured by potentiometric titration (Ding and Schwendeman, 2004). One hundred and fifty milligrams accurately weighed polymer was dissolved in 1 ml CH_2Cl_2. Five milliliters of water was added to the polymer solution to extract the water-soluble monomer/oligomers by vortex and centrifugation. The extraction was repeated for 4 more times and all the aqueous solutions were mixed together and were potentiometrically titrated to the equivalence point by 1.00 mN NaOH solution.

2.3. Encapsulation of BSA in PLGA-Glu millicylindrical implants

A solvent-extrusion method was used to encapsulate BSA in PLGA millicylinders with a diameter of 0.8 mm, as previously described (Kang and Schwendeman, 2002; Zhu et al., 2000). Briefly, a uniform suspension of sieved protein powder and excipients (<90 μm) in 50% (w/w) polymer/acetone solution was loaded into a 5 ml syringe and extruded into silicone rubber tubing (0.8 mm I.D.). The extruded polymer was air-dried at room temperature for 24 h and vacuum-dried at 45 °C for 48 h before the tubing was cut and the cylinders taken out for *in vitro* examinations.

2.4. Morphology characterization of PLGA-Glu millicylinders by scanning electron microscopy (SEM)

PLGA millicylinders were first coated with gold for 200 s by a Vacuum Coater (Desk II, Denton Vacuum, Inc., Hill, NJ). The microsphere morphology was then observed by a scanning electron microscope (S3200N variable pressure SEM, Hitachi). The voltage was set at 15 keV.

2.5. Protein concentration measurement

Protein release was determined by a Coomassie Plus (Pierce, Rockford, IL) protein assay. SEC, UV_{280} absorption and amino acid analysis were also used to quantify the protein content. For SEC, a TSK 2000 SWxl (Toso Biosep LLC, Montgomeryville, PA) column with a guard cartridge was used. The mobile phase consisted of 50 mM sodium phosphate and 150 mM sodium chloride and was delivered at 1 ml/min. A Waters 2487 dual wavelength detector was used to monitor the elution at 280 nm. The amino acid analysis was performed in the Protein Structure Facility of University of Michigan using a standard procedure.

2.6. Evaluation of protein release from PLGA-Glu

In vitro release studies were carried out at 37 °C under mild agitation conditions. Millicylinders (length = 10 mm, diameter = 0.8 mm, 5–8 mg) were placed in 1.5 ml microcentrifuge tubes with 1 ml PBST (PBS, pH 7.4 with 0.02% Tween-80) and

incubated at 37 °C. At pre-determined time intervals, release medium was replaced and protein content determined. The pH of each release medium was also measured.

2.7. Analysis of protein stability inside PLGA-Glu millicylinders

At the end of release, protein residue in PLGA-Glu millicylinders/microspheres was characterized by a previously reported method (Costantino et al., 1994; Kang and Schwendeman, 2002; Zhu et al., 2000). Briefly, polymers were dissolved in acetone and centrifuged. The supernatant polymer solution was removed and the precipitated protein pellet was washed twice with acetone. The pellet was then air-dried and reconstituted in 1 ml release medium at 37 °C for 1 h to determine the soluble residue in polymer. Any remaining precipitate was reconstituted in 1 ml denaturing solution (PBST/6 M urea/1 mM EDTA) and incubated at 37 °C for 30 min to determine the content of any physical aggregate. Then, any final insoluble BSA precipitate was collected again and incubated at 37 °C for 30 min in the reducing solution (the denaturing solution plus 10 mM DL-dithiothereitol) to determine any disulfide-bonded aggregate.

2.8. BSA stability in saturated $Mg(OH)_2$ solution

To investigate the reason for the incomplete recovery of BSA in $Mg(OH)_2$-containing millicylinders, 20 mg/ml BSA was incubated for 14 days in saturated $Mg(OH)_2$ solution together with 40 mM oligomers/monomers of either PLGA-Glu or linear PLGA. The polymer monomers/oligomers were prepared by hydrolysis of the polymer in water at 37° for 21 days, following removal of the insoluble residue by filtration. The mole concentration of the hydrolysate was estimated by potentiometric titration with standard NaOH solution. This condition was used to simulate the surrounding environment of BSA in polymer millicylinders. Twenty milligrams per milliliters blank microspheres were added in some samples to imitate the polymer surface. The blank microspheres were prepared by a previously reported double emulsion-solvent-evaporation method (Kang and Schwendeman, 2002; Zhu et al., 2000), and pre-incubated in PBST at 37 °C and 14 days before use. Four milligrams per milliliters free L-lysine was added in some samples to test the potential for reaction between polymer and lysine residues of protein molecules. After incubation, excess amount of base and blank microspheres were removed by filtration and BSA content was measured by Coomassie protein assay, UV absorption, SEC and amino acid analysis.

2.9. Measurement of water content of PLGA-Glu millicylinders

After incubation, millicylinders were blotted with tissue paper, weighed immediately, and then vacuum-dried. The water content of millicylinders was calculated as:

$$\text{Water content}\,(\%) = \frac{W_1 - W_2}{W_1 \times 100\%}$$

where W_1 and W_2 are the weights of the wet and dry millicylinders, respectively.

2.10. Monitoring microclimate pH in PLGA-Glu films coating glass electrodes

pH electrodes were coated with an acetone polymer solution, 500 mg/ml for linear PLGA 50/50 and 750 mg/ml for PLGA-Glu. Because of the lower viscosity of the polymer solution at a given concentration for the PLGA-Glu, a higher polymer concentration was used. The thickness of the dried coating film for both samples was roughly 200–250 μm. Coated electrodes were incubated in 5 ml PBST at 37 °C and the medium was replaced every week. At pre-determined times, the microclimate pH (μpH) was measured at room temperature in PBST against a calomel reference electrode, without correction for residual interfacial potentials, as previously described (Shenderova et al., 2004).

3. Results

3.1. Insoluble oligomers and soluble free acids in polymers

In Fig. 1, the gel permeation chromatograms (GPC) of PLGA-Glu and linear PLGA (50/50, 0.61 dl/g) are displayed. PLGA-Glu had a polydispersity of 3.7. The small and broad peak after the main one indicates the existence of oligomers/monomers in the PLGA-Glu. The soluble acid in PLGA-Glu before incubation was determined by potentiometric titration to be 2.8 nmol/mg. By comparison, the linear PLGA had a polydispersity of 1.8 and no significant oligomer/monomer peak was observed. On the other hand, linear PLGA had more than 10-fold higher water-soluble acid content (35 nmol/mg) than did PLGA-Glu. These results suggest that PLGA-Glu had a higher oligomer/monomer ratio than did linear PLGA, and that most of the low molecular weight species in PLGA-Glu detected by GPC were water-insoluble oligomers.

3.2. Effect of solvent-extrusion process on BSA stability

We monitored the protein stability by SEC after every step in the solvent-extrusion process. As shown in Table 1, BSA

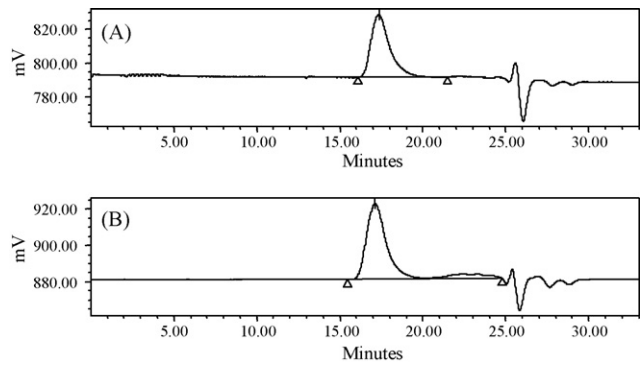

Fig. 1. GPC chromatograms of regular PLGA (A) and PLGA-glucose polymer (B).

Table 1
Effect of cylinder preparation process on BSA stability

Process	BSA from bottle	Sieving to <90 μm	A	B	C	D	E	F
Monomer content (%)	74	74	72	70	73	69	37[a]	73

Potential deleterious stresses were: (A) mixing polymer/acetone solution, BSA and base and then drying at RT for 48 h. (B) Mixing polymer/acetone solution, BSA and base and then drying at 45 °C for 48 h. (C) Mixing polymer/acetone solution, BSA and base and then extruding and drying at RT for 48 h. (D) Mixing polymer/acetone solution, BSA and base and then extruding and drying at 45 °C for 48 h. (E) Grinding BSA for 3 min and sieving to <90 μm. (F) Grinding a mixture of BSA and 20% sucrose for 3 min and sieving to <90 μm.

[a] Insoluble aggregate was observed.

directly from the bottle had a monomer content of 74%, and the remainder oligomer fraction of 26% is presumably covalent aggregates (Costantino et al., 1994). No insoluble aggregate was observed in the millicylinders. Acetone did not affect the stability of BSA. A slight increase in soluble oligomer was observed by SEC after the drying process. Three minutes grinding at RT decreased the monomer content by ~50%. Insoluble aggregates were also observed after grinding. Adding sucrose (1/5 sucrose was added to 100 mg/ml BSA in water solution and was lyophilized) completely inhibited protein aggregation during grinding. All the millicylinders further characterized for protein stability and release were prepared with sieved BSA powder without grinding.

3.3. BSA loading in millicylinders and polymer morphology

BSA loading in millicylinders with and without 3% $Mg(OH)_2$ was 16.1 ± 1.4% and 15.7 ± 2.1% (Mean ± S.D.), respectively, which was close to the theoretical loading of 15%. As shown in Fig. 2, solvent extruded millicylinders had a diameter of 0.8 mm, which is the inner diameter of the silicone tubing. They had a dense surface and cross-section, with protein and excipient particles uniformly suspended in the polymer matrix.

3.4. Protein stability in, and release from, millicylinders

As seen in Fig. 3, the release kinetics of BSA from PLGA-Glu millicylinders without excipients was continuous over the first 2 weeks and then flattened out. To find out the reason of no-release after 2 weeks, BSA was recovered from the polymer and analyzed for aggregation after removal of the polymer with acetone (Zhu et al., 2000). As shown in Table 2, at 2 weeks, 48% BSA was released. Among the unreleased protein, only 4% was soluble in PBST, indicative of insoluble aggregation. The remaining insoluble aggregate was 55% urea-soluble indicative of physical aggregation. The remaining insoluble portion after urea dissolution was very low and only 3% additional protein could be dissolved in urea + DTT, indicative of disulfide-bonded aggregate (Costantino et al., 1994; Zhu et al., 2000). Adding 3% $Mg(OH)_2$ did not largely affect the protein release in week 1, as shown in Fig. 3. In week 2 and 3, the base-containing polymer released slightly more protein. However, adding 3%

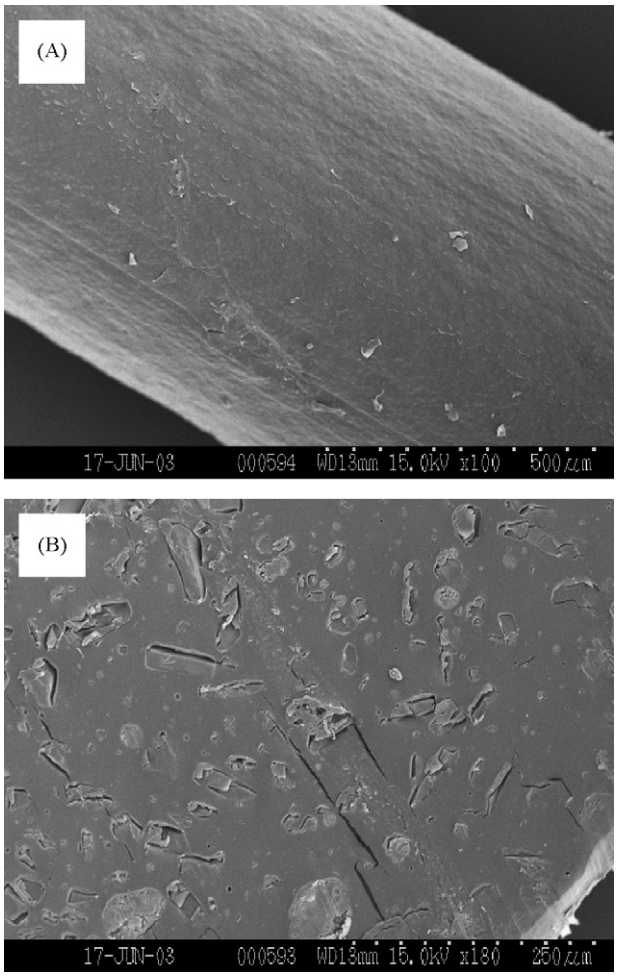

Fig. 2. Scanning electron micrographs of PLGA-glucose millicylinders containing both 15% BSA and 3% base. (A) Surface; (B) cross-section.

$Mg(OH)_2$ significantly improved the stability of BSA in PLGA-Glu during incubation. As seen in Table 2, in the presence of $Mg(OH)_2$, >90% of BSA was either released or soluble in the encapsulated fraction (54% released; 37% PBST soluble; 8%

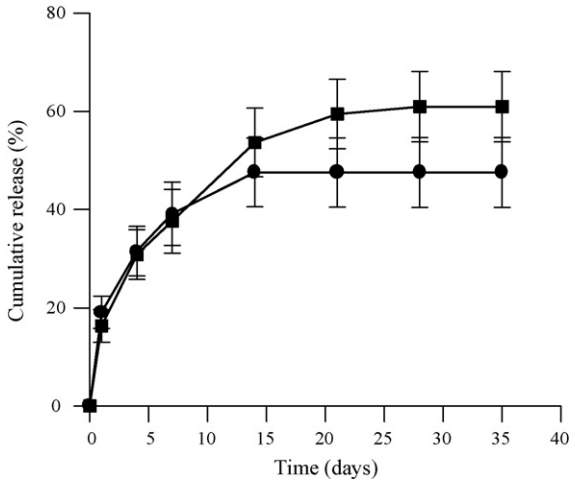

Fig. 3. Cumulative release of BSA from PLGA-glucose millicylinders with (■) or without (●) 3% $Mg(OH)_2$ (Mean ± S.E.M., $n = 3$).

Table 2
BSA stability in PLGA-Glu millicylinders during incubation

Formulation	Percentage of total encapsulated BSA[a]				
	Released part	Soluble residue	Physical aggregate	Covalent aggregate	Total recovery
BSA only after 2 weeks	48 ± 7	4.0 ± 0.4	55 ± 2	3.3 ± 0.2	109 ± 3
BSA + 3% Base after 2 weeks	54 ± 5	37 ± 2	7.6 ± 1	1.3 ± 0.1	99 ± 0.7
BSA only after 5 weeks	48 ± 7	0.5 ± 0.3	49 ± 7	2.4 ± 0.9	103 ± 7
BSA + 3% Base after 5 weeks	61 ± 4	0.7 ± 0.4	8.0 ± 3	0.2 ± 0.1	70 ± 4

[a] Mean ± S.E.M., $n = 3$.

urea-soluble; 1% urea + DTT soluble) after 2 weeks of incubation. Hence, over 14 days, BSA underwent the same form of aggregation observed in linear PLGA 50/50, and 3% $Mg(OH)_2$ conferred excellent stabilization of BSA by neutralization of the acidic microenvironment (Costantino et al., 1994; Kang and Schwendeman, 2002; Zhu et al., 2000; Zhu and Schwendeman, 2000).

After 5 weeks of incubation, all the protein remaining in the polymer aggregated even in the presence of $Mg(OH)_2$ (Table 2). The soluble portion of protein in the polymer recorded after 14 days of incubation also became unrecoverable in the sum of released and residue fractions. As seen in Table 2, while the insoluble portion of the encapsulated fraction in the base-containing specimens remained virtually constant between 14 and 35 days, the soluble portion dropped from 37% at day 14 to 0.7% at day 35 without significant release over the same period. The total recovery also dropped from 99% at day 14 to 70% at day 35. By contrast, BSA recovery in the non-base-containing polymer did not change significantly after 35 days.

3.5. BSA recovery after incubation with saturated $Mg(OH)_2$

To investigate the reason for incomplete recovery of protein in the base-containing PLGA-Glu, BSA was incubated for 14 days together with 40 mM oligomers/monomers of either PLGA-Glu or linear PLGA in saturated $Mg(OH)_2$ solution to mimic the microenvironment inside the polymer in the later stages of incubation. In certain instances, pre-eroded blank microspheres of the respective polymer were added to the suspension to create a polymer surface. BSA content was measured by Coomassie protein assay, UV_{280} absorption, SEC and amino acid analysis. BSA content changes determined by the several assays after 14 days of incubation with saturated $Mg(OH)_2$ solution are shown in Table 3. Saturated $Mg(OH)_2$ elevated the pH of BSA solution from ~7 to ~9. BSA content determined by amino acid analysis was essentially unchanged after 14 days incubation, indicating BSA and/or peptide fragments thereof remained in a soluble form after filtration of base (and in some cases polymer). However, BSA content determined by Coomassie protein assay decreased by some 10–20% after 14 days incubation with either PLGA or PLGA-Glu oligomer/monomer solution. The underestimation of BSA content by Coomassie protein assay is thus very likely a major source of incomplete recovery of BSA from base-containing millicylinders.

SEC analysis indicated that over 20% BSA was in oligomeric form before incubation. As seen in Table 3, after 14 days of incubation, BSA monomer content was maintained constant while the BSA oligomers strongly declined in the samples incubated with microspheres. At the same time, as shown in Fig. 4, protein fragments were also detected by SEC in all the base-containing samples.

Because PLGA-Glu is a star polymer with a glucose core, we suspected that polymer degradation might produce free glucose, which is susceptible to Maillard reaction with proteins (Schwendeman et al., 1997; Takahasi, 1977). To test this hypothesis, lysine was added in some samples to inhibit the potential Millard reaction. As shown in Table 3, lysine did not aid in the

Table 3
BSA recovery in saturated $Mg(OH)_2$ solution after incubation at 37 °C for 14 days[a]

Sample	pH	Coomassie measurement (%)	UV_{280} measurement (%)	Monomer by SEC[b] (%)	Monomer + oligomer by SEC[b] (%)	Amino acid assay measurement (%)
Water (w/o $Mg(OH)_2$)	7.1	95	100	106	99	103
PLGA oligomer[c]	9.2	92	102	103	92	95
PLGA oligomer[c] + MS[d]	9.0	85	115	100	86	99
PLGA oligomer[c] + MS[d] + lysine[e]	9.1	82	142	107	93	*n.d.*
PLGA-Glu oligomer[c]	9.2	87	118	113	100	97
PLGA-Glu oligomer[c] + MS[d]	8.8	81	112	101	87	95
PLGA-Glu oligomer[b] + MS[d] + lysine[e]	8.8	82	130	103	89	*n.d.*

n.d.: Not determined.
[a] 20 mg/ml BSA.
[b] Peak areas relative to pre-incubation samples.
[c] 40 mmol/l oligomers/monomers.
[d] Pre-incubated microspheres with a concentration of 20 mg/ml.
[e] 4 mg/ml L-lysine.

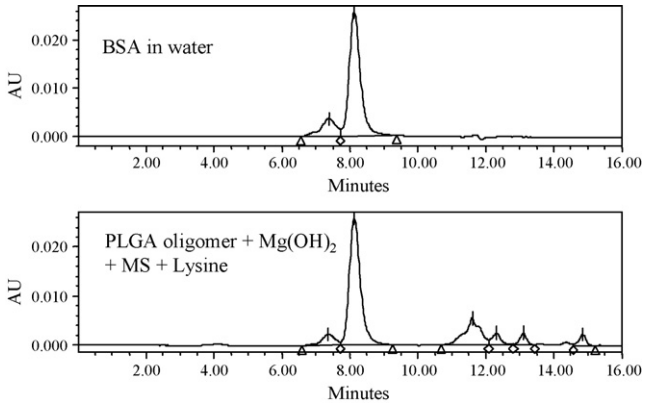

Fig. 4. Size exclusion chromatogram of BSA in saturated $Mg(OH)_2$ solution, monitored at 280 nm.

recovery of protein, strongly suggesting the absence of reactive aldehydes during bioerosion of the polymer.

3.6. pH change of the release medium

As shown in Fig. 5, the release medium of PLGA-Glu millicylinders maintained a neutral pH in all the samples over the first 2 weeks. After this, a rapid pH drop occurred in the release media containing BSA only millicylinders or blank millicylinders, whereas a pH > 6 was maintained in the release media containing the millicylinder with both $Mg(OH)_2$ and BSA.

3.7. Effect of protein and base on polymer water content

Moisture content is an important factor affecting protein release and stability in the polymer. It serves both as a catalyst and reactant in polymer degradation and protein degradation (Costantino et al., 1994). For example, BSA often undergoes thiol-disulfide interchange aggregation at neutral pH and ~20–50 g water/100 g protein moisture range, but forms primarily physical aggregate at very low pH and broad moisture window (~20–500 g water/100 g protein) (Schwendeman, 2002).

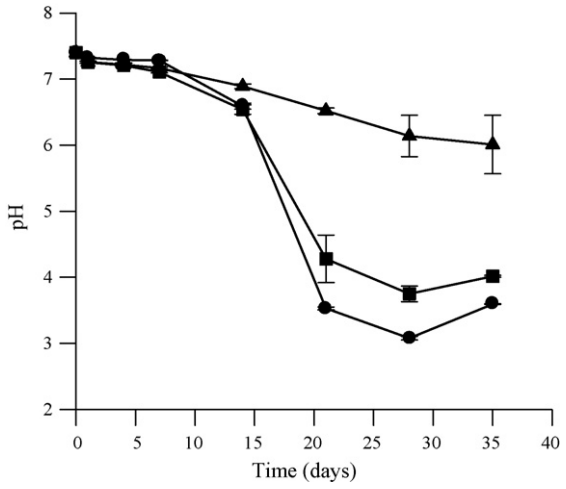

Fig. 5. pH change of the release medium of PLGA-glucose millicylinders (Mean ± S.E.M., $n = 3$). Polymer only (●); with 15% BSA (■); with 15% BSA + 3% $Mg(OH)_2$ (▲).

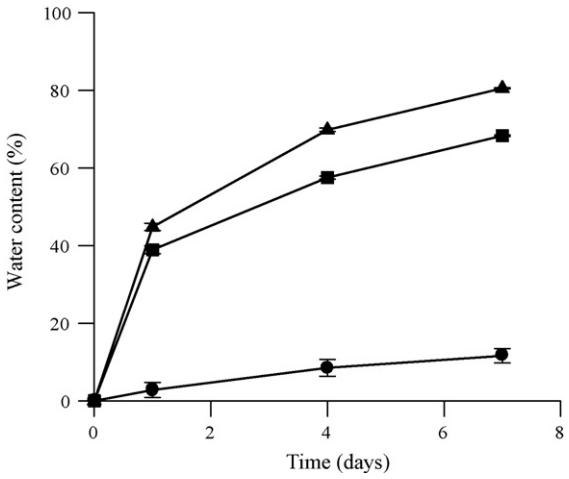

Fig. 6. Water uptake kinetics of PLGA-glucose millicylinders (Mean ± S.E.M., $n = 3$). Polymer only (●); with 15% BSA (■); with 15% BSA and 3% $Mg(OH)_2$ (▲).

Fig. 6 displays the kinetics of moisture content of PLGA-Glu millicylindrical matrices. Blank millicylinders only absorbed 12% water after 1 week of incubation at 37 °C, which is comparable to the value we previously obtained for linear 50/50 PLGA (~10%) (Kang and Schwendeman, 2002). Incorporation of 15% BSA boosted the water content to 68% after 1 week, and with additional 3% $Mg(OH)_2$ water content further increased to 80%. The boosted water uptake by BSA and $Mg(OH)_2$ is consistent with, but significantly greater than, that observed for linear PLGA (Zhu and Schwendeman, 2000).

3.8. Microclimate pH in PLGA-Glu films

In order to characterize the microclimate pH (μpH) behavior of PLGA-Glu, we coated pH-glass electrodes with the star and linear PLGAs (Shenderova et al., 2004). When the polymer coated electrode was incubated in PBST at 37 °C, PLGA-Glu detached after 5 weeks whereas linear PLGA remained closely attached, suggesting a faster degradation of PLGA-Glu. As seen in Fig. 7, the measured μpH in PLGA-Glu films coating glass electrodes was similar to what was observed in the linear PLGA, though a couple of notable differences were found. The μpH in PLGA-Glu films began slightly above 3 and then dropped to just above 2 by 3 weeks before a rapid increase just before the film detached from the electrode. For the linear PLGA, there was a very mild increase in μpH over the first 2 weeks followed by sustained low value beyond 5 weeks. When 5% BSA was mixed in PLGA-Glu film, near neutral μpH was maintained in the first 2 weeks of incubation followed by a sharp drop of μpH to about 2 by 4 weeks.

4. Discussion

When PLGA is implanted in the body or immersed in an aqueous release medium, water will rapidly penetrate into the polymer matrix (Batycky et al., 1997; Kang and Schwendeman,

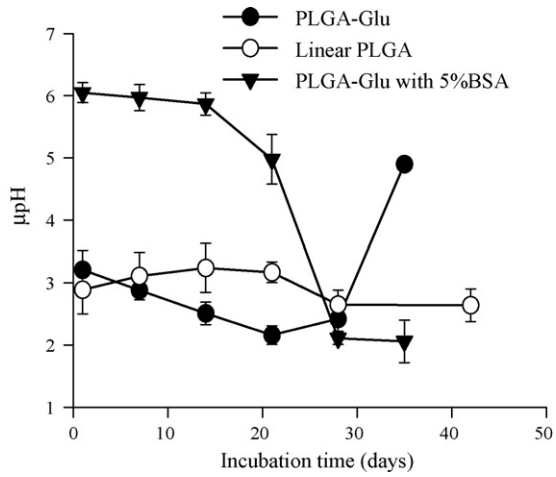

Fig. 7. Microclimate pH (μpH) in PLGA-glucose and PLGA measured by coating electrode method.

2003). The characteristic time required for penetration of water molecules to the core of the 800 μm millicylinders is expected to be ≈(400 μm)^2/(5 × 10^{-8} cm^2/s) ≈7 h, assuming no pores/channels in the polymer matrix. As water enters the polymer, soluble oligomers/monomers begin to partition between the polymer and aqueous pores. Water also hydrates any insoluble oligomer/monomer fraction (Ding et al., 2006). The soluble acid impurities pre-existing in the polymer have been implicated as the source of the acidic microenvironment in the initial stage of the polymer (Bittner et al., 1999; Schwendeman, 2002). This initial acidic microenvironment will further enhance the water uptake and catalyze polymer degradation, which subsequently results in more acidic microenvironment depending on the diffusion rate of the acidic species out of the polymer device and the extent to which the acids partition in the polymer phase (Ding and Schwendeman, 2004). Therefore, both molecular weight and molecular distribution, especially the amount of soluble oligomer/monomer in the polymer are important factors contributing to the polymer degradation and the formation of microenvironment in biodegradable polymers. GPC and potentiometric titration were used to characterize the total oligomers and water-soluble oligomer/monomer content in the polymer, respectively. It was found that although PLGA-Glu had less water-soluble oligomers/monomers, it contained more total oligomers/monomers, which might have contributed to the faster degradation rate of PLGA-Glu than linear PLGA.

Because of the importance of microclimate pH (μpH) on polymer degradation and protein stability, numerous methods have been developed to measure μpH. We have developed and validated a direct potentiometric method to quantitatively estimate the μpH by coating glass pH electrodes with thin polymer films (Shenderova et al., 2004). Moreover, we have demonstrated accurate prediction of the μpH from the potentiometric measurement using a mathematical model, which calculates the water-soluble acid concentration in the polymer pores and the relative contribution of each acid to the μpH value (Ding et al., 2006).

The differences in microclimate pH kinetics of the two polymer specimens were likely caused by the different molecular distribution of water-soluble oligomers/monomers in linear PLGA and PLGA-Glu. Because there were less water-soluble oligomers/monomers in PLGA-Glu prior to polymer degradation, PLGA-Glu exhibited a slightly higher pH at the beginning. The μpH decrease in PLGA-Glu could have been caused by the faster degradation rate of PLGA-Glu, which is evidenced by the earlier detachment of polymer coating from electrode. In addition, it is also likely that the star and linear forms of the polymer result in different polymer/water partition coefficients of the low molecular weight acids, which dictate the μpH for a given water-soluble acid content in the polymer matrix (Ding et al., 2006). The slight increase of μpH in linear PLGA until 2 weeks was probably caused by the relatively slower polymer degradation and faster diffusion of water-soluble acidic species out of the polymer matrices.

When BSA was encapsulated in PLGA-Glu films, it facilitated the release of water-soluble acidic species (Ding et al., 2006) and provides some buffering capacity, resulting in a neutral μpH during the first 2 weeks of incubation. The measured microclimate pH is consistent with the pH drop in release medium: a slow pH drop as low molecular weight acids were slowly released until 2 weeks followed by a sharp drop in pH as acids were rapidly released.

We note in control studies (Ding, 2005) that BSA, when encapsulated in the linear PLGA 50/50 coated on electrodes as described here, does not aggregate during this more neutral pH phase, consistent with the acid-induced pH aggregation mechanism (Zhu et al., 2000). Therefore, the thin and porous BSA-encapsulated PLGA coated on electrodes typically possess a substantially higher pH than the dense PLGA millicylinders (Ding, 2005). The coating electrode techniques give a direct evidence of the acidic microclimate in PLGA-Glu polymer, both with and without BSA encapsulation, which accounts for the BSA aggregation in PLGA-Glu millicylinders during incubation.

A solvent-extrusion method was used to encapsulate protein in PLGA-Glu millicylindrical implants. The stresses that could lead to protein instability in this process include: (1) organic solvent: acetone. Trace amount of water is inevitable in both acetone and protein powder, so there is a potential of transient water/organic solvent interface and/or mobilized protein molecules in the solid state; (2) mechanic stress during sieving, mixing and extrusion; and (3) heat: the elevated temperature during organic solvent removal from the polymer. The results from this study showed that neither exposure to organic solvent, extrusion, nor mild heat significantly affected BSA stability according to the assays used herein. However, the mechanic stress during grinding caused substantial BSA aggregation and adding sucrose protected BSA from mechanic stress-induced aggregation. The protection effect of sucrose could be explained by water replacement theory and vitrification mechanism (Carpenter and Izutsu, 2004). Proteins often have a more native-like conformation when dried with disaccharide. The restriction of translational and relaxation processes, and the dilution of protein molecules

within the glass matrix may also help prevent protein aggregation.

We have shown that co-encapsulation of insoluble base in linear PLGA polymer would inhibit BSA aggregation caused by acidic microenvironment (Zhu et al., 2000). Similar results were observed for PLGA-Glu polymer. Co-encapsulation of insoluble base, $Mg(OH)_2$ stabilized BSA in PLGA-Glu millicylinders as well as increased greatly the water uptake by the polymer.

However, BSA recovery from $Mg(OH)_2$-contained PLGA-Glu millicylinder, when measured by Coomassie protein assay, was reduced. Comparing BSA recovery results obtained by Coomassie protein assay with that by amino acid analysis and SEC strongly suggested that the oligomers, which existed in BSA samples, were hydrolyzed by the base-containing solution. These data provide a plausible explanation for the underestimation of BSA content by Coomassie protein assay since protein molecules have to be above a certain molecular weight to be detected by Coomassie protein assay (Bradford, 1976; Compton and Jones, 1985). In the case of UV_{280} absorption measurement, the overestimation of BSA is also likely from the hydrolysis, as peptide fragments may allow increased exposure of the aromatic amino acids giving rise to increased UV absorption at 280 nm. The accelerated decomposition of BSA oligomers can be explained by their preferential hydrolysis since they commonly exist in a more unfolded state and are thus more susceptible to degradation. It can also be seen that the protein recovery by the Coomassie protein assay for PLGA-Glu was similar to that for linear PLGA, suggesting the incomplete recovery of BSA is not caused by a PLGA-Glu specific reaction.

5. Conclusions

A model protein, BSA, was encapsulated in PLGA-Glu millicylinders by a solvent-extrusion method. PLGA-Glu demonstrated a similar acidic microenvironment during the incubation as does linear PLGA. The acidic microenvironment was responsible for the significant amount of insoluble aggregates of BSA formed during the incubation. Addition of insoluble base confers excellent stabilization by neutralizing acidic microenvironment in the polymer, although this effect was restricted to 2 weeks for the faster degrading PLGA-Glu. Protein recovery, when measured by Coomassie protein assay, was lower in base-containing formulations, which was attributed in large part to base catalyzed protein hydrolysis.

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