Stabilization of Proteins Encapsulated in Cylindrical Poly(lactide-*co*-glycolide) Implants: Mechanism of Stabilization by Basic Additives

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Purpose. A previous study from our group has shown that in the acidic microclimate of poly(lactide-*co*-glycolide) (PLGA) implants, encapsulated BSA forms insoluble noncovalent aggregates and is hydrolyzed during *in vitro* release. Incorporation of $Mg(OH)_2$ strongly inhibits these mechanisms of instability and facilitates continuous protein release. The purpose of this study was to determine the protein stabilization mechanism in the presence of basic additives.

Methods. BSA, as a model protein, was encapsulated in PLGA millicylinders by a solvent extrusion method. The release of BSA from the PLGA millicylinders with and without basic additives ($Mg(OH)_2$, $Ca(OH)_2$, $ZnCO_3$ and $Ca_3(PO_4)_2$) in a physiological buffer was carried out at 37°C and quantified by a modified Bradford assay. The insoluble aggregates extracted from the polymer with acetone were reconstituted in a denaturing (6 M urea) or denaturing/reducing solvent (6 M urea/10 mM DTT) to determine the type of aggregation.

Results. Aggregation of encapsulated BSA was inhibited with increasing amount of base co-encapsulated in the polymer, irrespective of the type of base used. The pH drop in the release medium and extent of acid-catalyzed PLGA degradation were both inhibited in the presence of base. The resultant effect was also reflected in an increase in water uptake and porosity of the devices. The inhibition and mechanism of BSA aggregation was correlated with the basicity of the additive. For $Ca(OH)_2$, at 3% loading, covalent BSA aggregation due to thiol-disulfide interchange was observed (indicative of ionization of albumin's free thiol at high pH), whereas at 3% $ZnCO_3$ or $Ca_3(PO_4)_2$, a higher percentage of non-covalent aggregates was observed compared to $Mg(OH)_2$. Decreasing the loading of BSA at constant $Mg(OH)_2$ content caused an increase in BSA aggregation.

Conclusions. The mechanism by which $Mg(OH)_2$ stabilizes encapsulated BSA in PLGA implants is through neutralizing the acidic microclimate pH in the polymer. The successful neutralization afforded by the basic additives requires a percolating network of pores connecting both base and protein. The microclimate pH inside PLGA implants can be controlled by selecting the type of basic salt, which suggests a potential approach to optimize the stability of encapsulated pharmaceuticals in PLGA including therapeutic proteins.

KEY WORDS: PLGA devices; basic additives; microclimate pH; protein stability; BSA aggregation.

INTRODUCTION

Controlled-release systems for proteins and peptides using poly(lactide-*co*-glycolide) (PLGA) have been studied for more than one decade. Although this type of biodegradable polymer has been successful in delivery of small peptides such as LHRH analogues (1), the delivery of large globular proteins in PLGA has been limited because of the irreversible inactivation of these therapeutic agents prior to their release *in vivo* (2,3). Previous work from our group has shown that encapsulated bovine serum albumin (BSA) in PLGA systems forms insoluble non-covalent aggregates and is hydrolyzed after incubation in a physiological buffer at 37°C for 28 days (4). The acidic pH and intermediate water content existing in the polymer were implicated as two major factors causing instability of the encapsulated protein, and the BSA was stabilized by co-encapsulating poorly water-soluble inorganic bases such as $Mg(OH)_2$ (4). The incorporation of the basic additive in the formulation was also successful in stabilizing therapeutic proteins such as recombinant human basic fibroblast growth factor and bone morphogenetic protein-2 (4).

In this study, to further characterize the protein stabilization mechanism in PLGA, the effect of: a) basicity of the salt additive, b) base salt content, and c) protein content, on protein stability and release kinetics was studied. Since acid-induced inactivation pathways (e.g., at pH $<$ 3) are common for most proteins, BSA was selected as a model protein. BSA undergoes unfolding from its *F* to *E* form at pH 2.7, and forms non-covalent aggregates in PLGA presumably due to this unfolding (4). The influence of $Mg(OH)_2$ on the delivery system such as pH change in the release medium, polymer degradation and water uptake kinetics was also examined.

Our results confirm that below a critical loading of either base or protein, both acidic and neutral pH regions in the nonporous polymer are present. Homogeneous acid-neutralization by the base requires: a) selection of the appropriate base, and b) manipulation of the polymer microstructure (e.g., via appropriate combination of base and protein loading), to facilitate diffusion of the base to all the acidic pores in the polymer.

MATERIALS AND METHODS

Chemicals

Poly(DL-lactide-*co*-glycolide) 50/50 with inherent viscosity of 0.23, 0.41, and 0.63 dl/g in hexafluoroisopropanol were purchased from Birmingham Polymers, Inc. (Birmingham, AL). Bovine serum albumin (A-3059, Lot 32H0463) was purchased from Sigma Chemical Co. (St. Louis, MO). Poly(vinyl alcohol) (80% hydrolyzed with Mw range of 8,000–9,000), $Mg(OH)_2$, $Ca(OH)_2$, and $Ca_3(PO_4)_2$ were obtained from Aldrich Chemical Co. (Milwaukee, WI). $ZnCO_3$ was from ICN Biopharmaceuticals Inc. (Aurora, OH). All bases were fine powders (<5 μm) and were used as received.

Preparation of PLGA Cylindrical Implants

A solvent extrusion method similar to that used previously by our group for intraocular implants (5) was used to prepare the PLGA cylinders with a diameter on the millimeter scale, which we term *millicylinders*. Briefly, a uniform suspension of sieved protein powder ($<$90 μm) with or without base in 50% (w/w) acetone-PLGA 50/50 solution was loaded in a syringe and extruded into a silicone tubing (I.D. = 0.8 mm) at about

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0.1 ml/min. The solvent extruded suspension was dried at room temperature for 24 h and then in a vacuum oven at 45°C for another 24 h before testing. The protein loading was calculated as the percentage of amount of BSA versus the total weight of mixture (i.e., protein, polymer, and base).

Evaluation of BSA Release from PLGA Implants

Release of protein was carried out in PBST (which consists of PBS (7.74 mM Na_2HPO_4, 2.26 mM NaH_2PO_4, 137 mM NaCl, and 3 mM KCl, pH 7.4), and 0.02% w/v Tween® 80) at 37°C under perfect sink conditions. Millicylinders (10 × 0.8 mm, 5–10 mg) were placed in 0.5–1.0 ml of the release medium, which was replaced at each time point. The protein content was determined by using Coomassie plus protein assay reagent, which is also compatible with denaturing agents (e.g., 6 M urea) and reducing agents (e.g., 10 mM DTT).

Evaluation of BSA Stability Within PLGA Implants

Protein stability was assessed by the percentage of water-insoluble non-covalent BSA aggregates generated within the implants versus the initial encapsulated protein. Protein stability within PLGA implants was analyzed in the presence of moisture and during *in vitro* release: First, millicylinders with a length of 1 cm were incubated under 80% and 96% relative humidity (RH) at 37°C for 21 days. Then, the polymer was dissolved in acetone and centrifuged. The remaining protein pellet was washed three times with acetone and then air-dried. The final protein pellet was analyzed as in *Analysis of the Protein Exacted from PLGA Implants*. The protein remaining in PLGA implants after release in PBST at 37°C was also extracted and analyzed by the same procedure.

Analysis of the Protein Extracted from PLGA Implants

The BSA pellet extracted from PLGA implants was first reconstituted in PBST and incubated at 37°C overnight and the soluble protein fraction remaining in the polymer was determined by protein assay. Any remaining aggregate was collected by centrifugation again, and brought up in the denaturing solvent (PBST/6 M urea/1 mM EDTA) and incubated at 37°C for 30 min; assayed protein gave a measurement of non-covalent bonded BSA aggregates. Then, any final undissolved BSA aggregate was collected again, dissolved in the reducing solvent (the denaturing solvent plus 10 mM DTT), and assayed to determine the amount of disulfide-bonded aggregates (7).

Protein Assay

For quantitation of soluble BSA, a modified Bradford assay was used as follows: 10 μl of standard or sample in PBST was added to 250 μl of Coomassie reagent/well on a 96-well plate and then the plate was read at 595 nm using a Dynex MRX microplate reader (Dynex Technology, Inc., Chantilly, VA). The concentration range of the standard curve was 50 to 1000 μg/ml. For quantitation of non-covalent and covalent BSA aggregates, the solvents used for preparation of standards and samples were 6 M urea/PBST and 6 M urea/10 mM DTT/PBST, respectively.

Measurement of Water Uptake in PLGA Millicylinders

After incubation either in PBST or under relative humidity at 37°C, the millicylinders were blotted with tissue paper, weighed immediately, and then freeze-dried. The water uptake of millicylinders was calculated by:

$$\text{Water uptake (\%)} = (W_1 - W_2)/W_2 \times 100\%$$

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

Measurement of Molecular Weight of PLGA

Weight-averaged molecular weight (M_W) of the degraded polymers was measured by gel permeation chromatography (GPC) on a Styragel™ HR 5E column (7.8 × 300 mm, Waters, Milford, MA), which was performed on a HPLC system (Waters, Milford, MA) equipped with a refractive index detector (Hewlett Packard). The mobile phase was tetrahydrofuran with a flow rate of 1 ml/min. M_W was calculated based on polystyrene standards (Polysciences Inc., PA) using Millenium Software Version 2.10.

SEM Image Analysis of PLGA Implants

Images of PLGA millicylinders were obtained by using a Philips XL30 field emission gun scanning electron microscope (SEM). Samples were coated with conductive gold palladium prior to the analysis.

pH Measurement of Saturated Basic Additives in Water

Basic additives (i.e., $Mg(OH)_2$, $Ca(OH)_2$, $ZnCO_3$ and $Ca_3(PO_4)_2$) in excess of their solubility were added to 5 ml of distilled water. The suspension was then incubated at 37°C for 7 days. The pH of the supernatant following centrifugation was determined with a Corning 430 pH meter (Corning Inc., NY).

RESULTS AND DISCUSSION

Stabilization of BSA Encapsulated in PLGA Implants During *In Vitro* Release

Our previous studies have demonstrated that after an initial burst on the first day, BSA release from 15%BSA/millicylinders (0.63 dl/g PLGA 50/50) during 4 weeks incubation in PBST at 37°C is insignificant, and the remaining protein mostly consists of water-insoluble non-covalent aggregates (4). The cause of the BSA aggregation was mainly an acidic microclimate pH generated by polymer degradation and water uptake by the polymer during incubation in PBST (4). We found that incorporation of 3% $Mg(OH)_2$ into 15%BSA/PLGA50/50 millicylinders can increase BSA release from PLGA cylindrical implants and reduce BSA aggregation. Structural characterization by using SDS-PAGE, IEF, CD, and fluorescence spectroscopy have confirmed that the structure of BSA from 3%$Mg(OH)_2$/15%BSA devices was mostly retained in a native form (4).

It should be noted that BSA is known to undergo dehydration-induced structure changes when dried in the absence of excipients (16). In the absence of basic additive, this initial partial unfolding in the implant may accelerate BSA aggregation and hydrolysis in the acidic environment. In the presence of

basic additive, we have confirmed that the protein extracted from the fresh preparation as well as the released and residual protein fractions are all > 90% native for over a month of release (4). This data indicate that successful refolding of encapsulated BSA occurs in the presence of the basic additive.

To examine the effect of $Mg(OH)_2$ content, the base was co-encapsulated in 15%BSA/PLGA millicylinders as a function of base loading and the BSA release study was carried out in PBST at 37°C. As seen in Fig. 1A, with the increasing $Mg(OH)_2$ content from 0.5% to 6%, both BSA release rate and total releasable amount of protein increased. The residual BSA remaining in these devices after the 4-week release interval was analyzed and listed in Table I. In the absence of $Mg(OH)_2$, most of the remaining protein became water-insoluble aggregates, which were nearly completely soluble in the denaturing solvent (i.e., non-covalent aggregates were formed). As the content of $Mg(OH)_2$ was increased, the amount of water-insoluble aggregates decreased. As $Mg(OH)_2$ content was raised to 6%, almost no aggregates were formed within the device. For all the aggregates, an insignificant amount of covalent aggregates was observed in each polymer specimen. These results indicate that an increase in $Mg(OH)_2$ content even up to 6% does not generate an alkaline microclimate in the polymer during release, which suggests that $Mg(OH)_2$ may be a good neutralizing substance for protein delivery from PLGA implants. However, the compromise of this neutralization effect is the shorter release duration due to faster protein release from the devices in the presence of $Mg(OH)_2$.

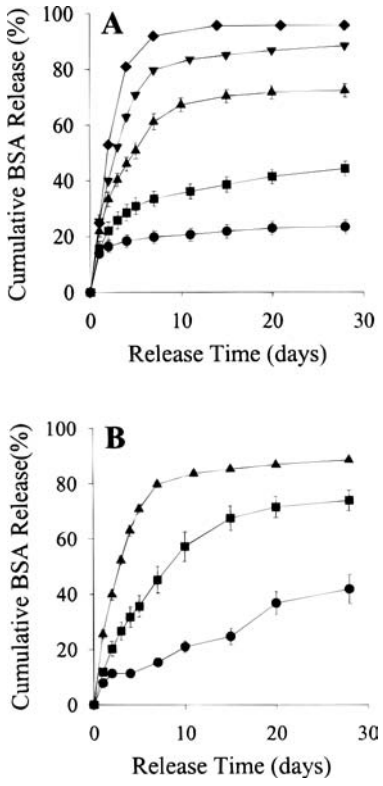

Fig. 1. A. Effect of $Mg(OH)_2$ content on BSA release from 15% BSA/PLGA 50/50 (i.v. = 0.63 dl/g) millicylinders. $Mg(OH)_2$ content: 0% (●), 0.5% (■), 1.5% (▲), 3% (▼), and 6% (♦); B. Effect of BSA loading on BSA release from 3%$Mg(OH)_2$/PLGA50/50 (0.63 dl/g) millicylinders. BSA loading: 5% (●), 10%(■), and 15%(▲). (Average ± SEM, n = 3).

Stabilization of Encapsulated BSA in PLGA Implants by $Mg(OH)_2$ in the Presence of Moisture (No Protein Release)

The increased release rate of BSA in the presence of $Mg(OH)_2$ brings up a potential artifact when considering BSA stabilization by the base. If the base only accelerates release, there may be insufficient time for BSA aggregates to form. To demonstrate the stabilization effect of $Mg(OH)_2$ in the implants with the same amount of encapsulated protein, 15%BSA/PLGA implants with and without 3% $Mg(OH)_2$ were exposed to a humid environment at 37°C. The unique feature of this experiment is that all the protein presumably remains inside the device during incubation and PLGA degradation occurs due to uptake of water vapor at 37°C. Two different humidities, 80% and 96% RH, were selected since the base may also affect water content of the protein, which also affects protein aggregation kinetics (13). After 3 weeks incubation, the remaining BSA was extracted from the polymer.

In the absence of the base, the same type of water-insoluble non-covalent BSA aggregates was observed among the remaining BSA, as previously shown during incubation in the release medium (Table II). With the increasing relative humidity, the water content of the device increased and the amount of the aggregates increased. Our previous results have shown that more than 60% of the initially encapsulated BSA formed non-covalent aggregates after 2 weeks release in PBST at 37°C (4). This indicates that during release the microclimate in the polymer may become more acidic due to the additional water uptake by the polymer compared to the humid conditions. These results further demonstrate that non-covalent BSA aggregation is caused by the acidic microclimate generated from PLGA degradation products.

In contrast, in the presence of 3% $Mg(OH)_2$ much less non-covalent aggregates were generated (Table II) under both 80% and 96% RH conditions. This result confirms that incorporation of $Mg(OH)_2$ in PLGA implants indeed can inhibit non-covalent BSA aggregation in the absence of protein release. Since the water uptake by the devices with or without the base was similar (Table II), the stabilization effect of $Mg(OH)_2$ is most likely through its neutralization of the acidic microclimate pH as the polymer degrades. We also found previously that the amount of BSA release from PLGA50/50 (0.64 dl/g) microspheres prepared with or without $Mg(OH)_2$ was almost identical after 28 days release, but the soluble protein remaining in the polymer was significantly greater in the presence of base (i.e., 65% versus 17%) (4). These results confirm the stabilization effect of the base and rule out the potential artifact due to the faster release of the protein in the presence of the salt.

Characterization of Neutralization Effect of $Mg(OH)_2$ in PLGA Implants

To examine the neutralization effect of $Mg(OH)_2$ in the acidic microclimate of PLGA millicylinders, changes in pH in the release medium and the PLGA degradation rate were monitored (Table III). During the release period from day 21 to 28, the pH of 500 μl release medium containing 5 mg of 15% BSA/PLGA millicylinder dropped to 3.5, while in the presence of 3%

Table I. The Effect of $Mg(OH)_2$ and BSA Content on BSA Aggregation in BSA/PLGA (0.63 dl/g) Millicylinders after 4-Week Release Study in PBST at 37°C

	$Mg(OH)_2$ Content (15% BSA Loading)					BSA Content (3% $Mg(OH)_2$ Loading)		
	0	0.5	1.5	3[c]	6	5	10	15[c]
Soluble BSA[a], %	2.0 ± 0.3	9.4 ± 0.4	1.9 ± 0.1	4.6 ± 0.2	3.4 ± 0.1	10 ± 1	5.6 ± 0.3	4.6 ± 0.2
Non-covalent aggregate[b],%	74 ± 2	45 ± 3	20 ± 3	6.8 ± 0.3	1.0 ± 0.1	48 ± 6	21 ± 3	6.8 ± 0.3

Note: Average ± SEM, n = 3.
[a] Soluble in PBST.
[b] Soluble in PBST containing 6 M urea and 1 mM EDTA. Less than 2% were covalent aggregates soluble in PBST/6 M urea/1 mM EDTA/ 10 mM DTT.
[c] Columns duplicated to show the trend.

$Mg(OH)_2$ the pH remained close to 7.0. The characterization of polymer MW by GPC also showed that degradation rate of the polymer in 15% BSA/PLGA millicylinders was faster than that in the presence of $Mg(OH)_2$, which suggests that fewer acidic species were generated during release in these $Mg(OH)_2$-containing millicylinders, consistent with the release medium pH data. Therefore, the $Mg(OH)_2$ inhibited the autocatalytic degradation mechanism of PLGA (8,9) and neutralized the acidic microclimate, which is consistent with the result reported by our group previously using a fluorescent probe (10).

To explain the faster release profiles in the presence of $Mg(OH)_2$, the water uptake kinetics of the millicylinders was characterized. As seen in Fig. 2, the presence of 3% $Mg(OH)_2$ significantly increased the water uptake rate of PLGA millicylinders. At 7 days, the total water content in 3%$Mg(OH)_2$/15% BSA/PLGA millicylinders was much higher than the polymer without $Mg(OH)_2$. This result suggests that the higher permeability is expected in the millicylinders with $Mg(OH)_2$, which was also consistent with the SEM images (Fig. 3). After 7 days release, the cross section of the device with $Mg(OH)_2$ (Fig. 3D) was more porous than that without $Mg(OH)_2$ (Fig. 3C). Similar results were also observed when $Mg(OH)_2$ was encapsulated in PLGA films (8, 9).

The reason that $Mg(OH)_2$ increases water uptake is likely due to changes in water activity within the PLGA millicylinders. $Mg(OH)_2$ increases the microclimate pH in the polymer, which will cause the dissociation of the end groups [i.e.,-COOH, with a pK_a of 3.83 for both glycolic- and lactic acids (11)] of PLGA and ionization of the monomers/oligomers. Therefore, ionization of the polymer end groups and the increased osmotic pressure will be the driving forces for water molecules to diffuse into the polymer matrix, resulting in higher water content.

Table II. The Effect of $Mg(OH)_2$ on BSA Aggregation Within PLGA50/50 (0.63 dl/g) Millicylinders After Incubation at 37°C Under Different Relative Humidity Conditions for 3 Weeks

Incubation condition	80% RH		96% RH	
$Mg(OH)_2$ content, %	0	3	0	3
Water content[a], %	2.1 ± 0.4	1.8 ± 0.9	20 ± 4	39 ± 7
Soluble protein[b], %	82 ± 2	98 ± 1	71 ± 5	95 ± 1
Insoluble protein[c], %	18 ± 2	1.8 ± 0.1	29 ± 5	4.8 ± 1.0

Note: BSA loading: 15%, average ± SEM, n = 3.
[a] Water uptake is the percentage of weight increase of the device after incubation relative to the weight of the device prior to incubation.
[b] Soluble in PBST.
[c] Insoluble protein refers to the non-covalent aggregates, which are soluble in 6 M urea.

Effect of Protein Loading on BSA Release and Stability

Confocal micrographs of fluorescein-loaded PLGA microspheres with co-encapsulated $Mg(OH)_2$ and no protein, which have been reported by our group (10), indicate a population of both acidic and neutral pH pores in the polymer matrix. This pH heterogeneity suggests that in order for BSA to be stabilized, the base must be able to diffuse to the BSA-containing pores. Moreover, from control studies we have observed that 15% protein loading is sufficient for BSA to percolate effectively throughout the polymer. For example, if the BSA loading without the base is increased to 20%, >90% of the protein is release in 1 day (data not shown). Therefore, a decrease in the percolation of BSA particles in the polymer with base would be expected to cause a rise in BSA aggregation, corresponding to increased exposure of BSA to acidic pores.

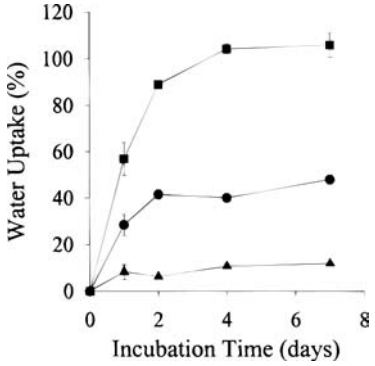

Fig. 2. Effect of $Mg(OH)_2$ on water uptake kinetics of PLGA 50/50 (i.v. = 0.63 dl/g) millicylinders without protein or base (▲), with 15% BSA (●), and with 15% BSA and 3% $Mg(OH)_2$ (■). (Average ± SEM, n = 3).

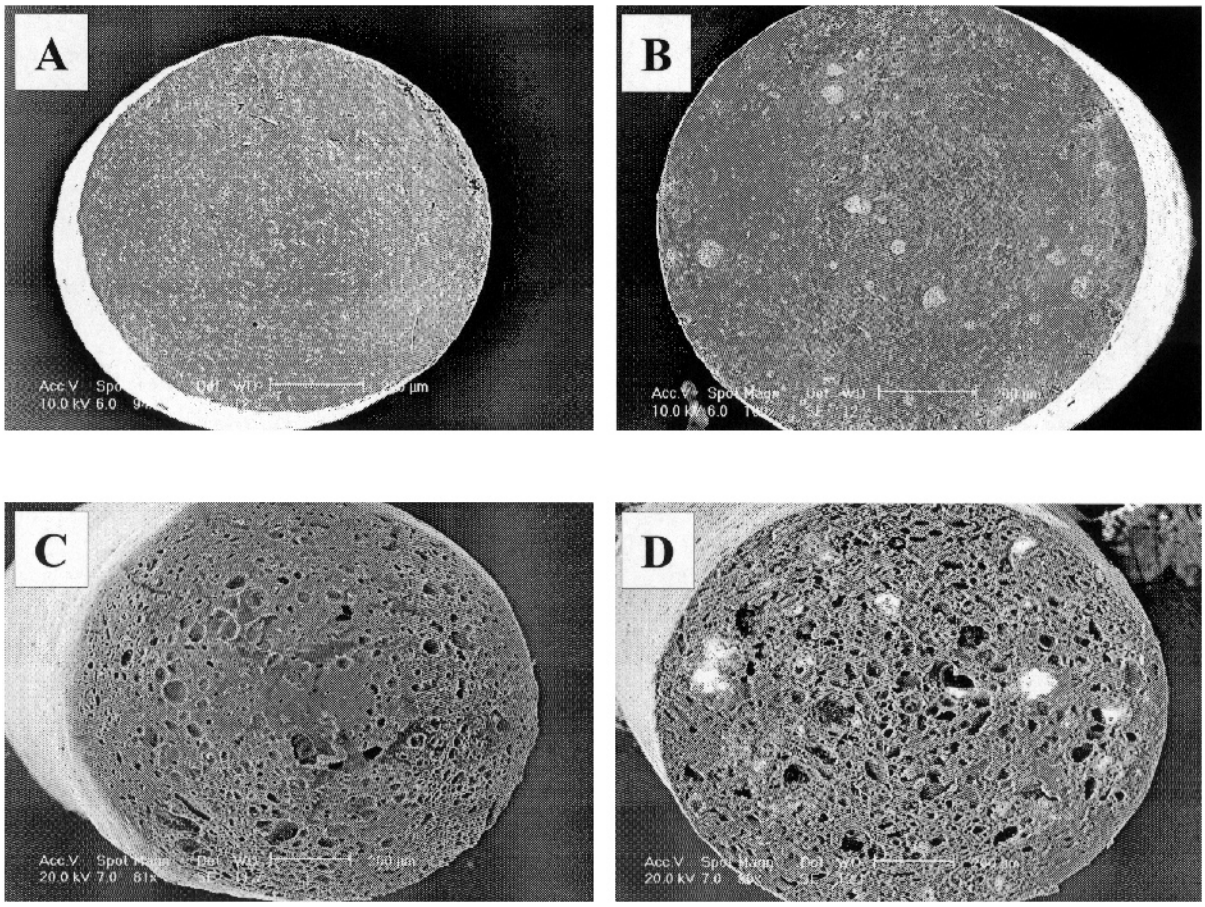

Fig. 3. The SEM images of 15% BSA/PLGA 50/50 (i.v. = 0.63 dl/g) millicylinders without bases (A, C) and with 3% $Mg(OH)_2$ (B, D). A, B: before incubation; C, D: after 7-day incubation in PBST at 37°C.

To test this hypothesis, we decreased BSA loading to reduce its percolation, and studied the BSA release. As expected, as the loading was decreased, an increase in BSA aggregation was observed (Table I). As seen in Fig. 1B, as BSA loading was decreased there was also a corresponding decrease in the release rate. A similar phenomenon was observed for the stabilization of BSA in PLGA50/50 microspheres at a similar BSA loading (4%) to the 5% BSA/PLGA millicylinders depicted here (Fig. 1B and Table I) (4). A higher soluble base, $MgCO_3$, which has ~10-fold greater driving force for diffusion, stabilized BSA much better than the $Mg(OH)_2$ even though both bases neutralize acidity in a saturated solution to the same extent (4).

Table III. Neutralization Effect of $Mg(OH)_2$ on the Acidic Microclimate in 15% BSA/PLGA (i.v. = 0.63 dl/g) Millicylinders

$Mg(OH)_2$ content	pH of the release medium[a]	M_w of PLGA[b]
0%	3.5	10000 ± 1000
0.5%	6.0	16800 ± 800
3.0%	7.0	28700 ± 200

[a] 500 μl of PBST containing 5 mg of millicylinders was incubated at 37°C for 4 weeks.

[b] Polymers were extracted from the millicylinders after 4-week release study in PBST at 37°C (average ± SEM, n = 3). Initial M_W of PLGA was 66700 ± 500.

Effect of Other Basic Additive on BSA Release and Aggregation in PLGA Implants

Besides $Mg(OH)_2$, other bases such as calcium carbonate, calcium orthophosphate, and sodium acetate have also been used as buffer salts to counteract the pH drop within PLGA microspheres during hydrolytic polymer degradation for tetanus toxoid delivery (12). To examine the effect of the basic additives with different alkalinity on the stability and release of BSA encapsulated in the polymer, we chose one relatively strong base, $Ca(OH)_2$, and two relatively weak bases, $ZnCO_3$ and $Ca_3(PO_4)_2$, and examined whether similar stability and release profiles can be achieved in 15% BSA/PLGA millicylinders as was demonstrated with the use of $Mg(OH)_2$.

As seen in Fig. 4, with the increasing content of $ZnCO_3$, $Ca(OH)_2$, or $Ca_3(PO_4)_2$, both the release rate and total releasable amount of BSA increase, which is quite similar to the effect of $Mg(OH)_2$ (Fig. 1A). Analysis of the residual BSA in these devices is listed in Table IV. For $ZnCO_3$ and $Ca_3(PO_4)_2$, similar to $Mg(OH)_2$, the total amount of water insoluble aggregates decreased with the increasing base content. For $Ca(OH)_2$, only

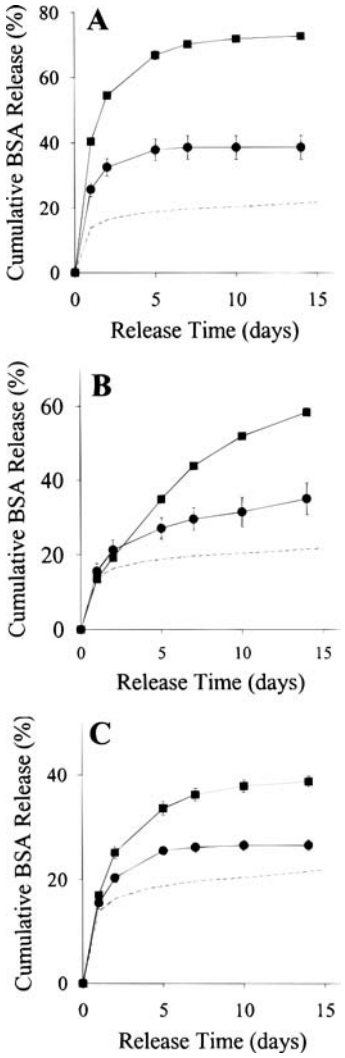

Fig. 4. Effect of bases on BSA release from 15% BSA/PLGA 50/50 (i.v. = 0.63 dl/g) millicylinders. A: $Ca(OH)_2$; B: $ZnCO_3$; C: $Ca_3(PO_4)_2$. Base content: 0.5% (●) and 3% (■). Dashed line: without base. (Average ± SEM, n = 3).

0.5% base loading was required to attain a similar inhibition of BSA aggregation as attained with 3% of the weak bases. However, when the loading was raised to 3%, a significant amount of covalent bonded aggregates of BSA formed, which suggests that the microclimate pH in the presence of $Ca(OH)_2$ becomes more alkaline than with the weaker bases. Compared to a pH of 9.97 for saturated $Mg(OH)_2$ solution, the pH of a saturated $Ca(OH)_2$ solution was found to be 12.4. As pH becomes alkaline, the free thiol group of Cys residues ionizes to become the more reactive thiolate and readily catalyzes disulfide bonded BSA aggregates via thiolate-disulfide interchange (13). For $ZnCO_3$ and $Ca_3(PO_4)_2$, the pH of their saturated solution was found to be 7.34 and 7.77, respectively, which indicates that both species are very weak bases. Therefore, compared to $Mg(OH)_2$, after only two weeks incubation, a larger amount of non-covalent aggregates in PLGA millicylinders with $ZnCO_3$ or $Ca_3(PO_4)_2$ were observed and no detectable amount of covalent aggregates were formed (Table IV).

The faster release of BSA from PLGA with $ZnCO_3$ than from $Ca_3(PO_4)_2$-loaded specimens may be explained as follows: Since $ZnCO_3$ and $Ca_3(PO_4)_2$ will react with protons generated from PLGA degradation to form a weak acid [H_2CO_3, pKa_1 = 6.35 (14)] and a strong acid [H_3PO_4, pKa_1 = 2.16 (14)], respectively, the microclimate in the millicylinders with $Ca_3(PO_4)_2$ is expected to be more acidic than that with $ZnCO_3$. This explanation was confirmed by the following experimental data simulating the reaction of acidic species and base occurring in the polymer: when 100 μl of 1 N HCl was added to a saturated $ZnCO_3$ solution containing excess of base, the pH dropped to 5.36, whereas the same amount of HCl added to a saturated $Ca_3(PO_4)_2$ solution caused the pH to drop to 3.71. The difference in the neutralization effect from the bases is also reflected in the different water contents of their PLGA devices. The 3% $ZnCO_3$/15% BSA/PLGA millicylinders had a water content of 168 ± 5% (n = 3) after 2-week release compared to 81 ± 1% (n = 3) in the 3% $Ca_3(PO_4)_2$/15% BSA/PLGA millicylinders, which suggests that $ZnCO_3$ should raise the microclimate pH in the polymer greater than does $Ca_3(PO_4)_2$.

In general, although addition of basic additives to BSA/PLGA devices can reduce the aggregation and enhance protein release, the use of high base content results in shorter release duration, while low base content cannot eliminate aggregation. Therefore, modulation of both stability and release kinetics by adding other excipients appears to be necessary. As an example, we found that when sucrose was added to 15%BSA/3% $Mg(OH)_2$/PLGA, BSA aggregation was still prevented but the release rate was substantially reduced (data not shown) (15). Therefore, in our future work, the effects of both bases and additional protein stabilizers will be evaluated with respect to protein stability and release kinetics.

Table IV. Effect of Basic Additives on BSA Aggregation in 15% BSA/PLGA(0.63 dl/g) Millicylinders after 2-Week Release Study in PBST at 37°C

		$Ca(OH)_2$		$ZnCO_3$		$Ca_3(PO_4)_2$	
Bases %	No base	0.5	3.0	0.5	3.0	0.5	3.0
Soluble BSA,[a] %	14 ± 7	51 ± 4	13 ± 1	36 ± 1	30 ± 1	44 ± 2	52 ± 1
Non-covalent aggregate,[b] %	65 ± 8	10 ± 1	3.9 ± 0.1	30 ± 4	10 ± 1	30 ± 2	8.4 ± 0.3
Covalent aggregate,[c] %	n.d.[d]	n.d.	11 ± 1	n.d.	1.8 ± 0.1	n.d.	1.0 ± 0.1

Note: Average ± SEM, n = 3.
[a] Soluble in PBST.
[b] Soluble in PBST containing 6 M urea and 1 mM EDTA.
[c] Soluble in PBST containing 6 M urea, 1 mM EDTA and 10 mM DTT.
[d] n.d. - not detectable.

CONCLUSIONS

The results indicate that incorporation of 3% $Mg(OH)_2$ in 15%BSA/PLGA50/50 (0.63 dl/g) cylindrical implants can inhibit the BSA aggregation under both *in vitro* release and humid (no protein release) conditions. The mechanism of BSA stabilization afforded by the basic additives is the neutralization of the acidic microclimate pH in PLGA implants, which inhibits the acid-induced BSA instability. The data also show that an appropriate selection of base type, base loading, and protein loading is the key to attaining a neutral microclimate pH homogeneously throughout the polymer matrix, which enhances both protein stability and controlled release.

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REFERENCES

1. F. G. Hutchinson and B. J. A. Furr. Biodegradable polymer systems for the sustained release of polypeptides. *J. Contr. Rel.* **13**:279–294 (1990).
2. S. P. Schwendeman, M. Cardamone, M. R. Brandon, A. Klibanov, and R. Langer. The stability of proteins and their delivery from biodegradable polymer microspheres. In S. Cohen and H. Bernstein (eds.), *Microparticulate Systems for the Delivery of Proteins and Vaccines*, Marcel Dekker, New York, 1996, pp. 1–49.
3. S. P. Schwendeman, H. R. Costantino, R. K. Gupta, and R. Langer. Peptides, proteins, and vaccine delivery from implantable polymeric systems. In K. Park (ed.), *Controlled Drug Delivery*, American Chemical Society, Washington, D.C., 1997, pp. 229–267.
4. G. Zhu, S. R. Mallery, and S. P. Schwendeman. Stabilization of proteins encapsulated in injectable poly(lactide-co-glycolide). *Nature Biotechnol.* **18**:52–57 (2000).
5. T. Zhou, H. Lewis, R. E. Foster, and S. P. Schwendeman. Development of a multiple-drug delivery implant for intraocular management of proliferative vitreoretinopathy. *J. Contr. Rel.* **55**: 281–295 (1998).
6. A. Shenderova, T. G. Burke, and S. P. Schwendeman. Stabilization of 10-hydroxycamptothecin in poly(lactide-co-glycolide) microsphere delivery vehicles. *Pharm. Res.* **14**:1406–1413 (1997).
7. S. P. Schwendeman, H. R. Costantino, R. Gupta, G. R. Siber, A. M. Klibanov, and R. Langer. Stabilization of tetanus and diphtheria toxoids against moisture-induced aggregation. *Proc. Natl. Acad. Sci. USA* **92**:11234–11238 (1995).
8. Y. Zhang, S. Zale, L. Sawyer, and H. Bernstein. Effects of metal salts on poly(DL-lactide-co-glycolide) polymer hydrolysis. *J. Biomed. Mater. Res.* **34**:531–538 (1997).
9. H. Bernstein, Y. Zhang, M. A. Khan, and M. A. Tracy, US Patent 5,656,297 (1997).
10. A. Shenderova, T. G. Burke, and S. P. Schwendeman, The acidic microclimate in poly(lactide-co-glycolide) microspheres stabilizes camptothecins. *Pharm. Res.* **16**:241–248 (1999).
11. The Merck Index, 8th edition, Merck & Co., Inc., New Jersey, 1968.
12. P. Johansen, Y. Men, R. Audran, G. Corradin, H. P. Merkle, and B. Gander. Improving stability and release kinetics of microencapsulated tetanus toxoid by co-encapsulation of additives. *Pharm. Res.* **15**:1103–1110 (1998).
13. H. R. Costantino, R. Langer, and A. M. Klibanov, Solid-phase aggregation of proteins under pharmaceutically relevant conditions. *J. Pharm. Sci.* **83**:1662–1669 (1994).
14. CRC Handbook of Chemistry and Physics, 78th edition, CRC Press LLC, Boca Raton, Florida, 1997.
15. G. Zhu, S. R. Mallery, and S. P. Schwendeman. Controlled release of basic fibroblast growth factor from cylindrical poly(lactide-co-glycolide) implants. *Pharm. Sci.* **1**:S-229 (1998).
16. K. Carrasquillo, R. A. Cordero, S. Jo, J. M. Franquiz, and K. Griebenow. Structure-guided encapsulation of bovine serum albumin in poly(lactide-co-glycolide) acid. *Pharm. Pharmacol. Commun.* **4**:563–571 (1998).