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Effect of protein molecular weight on release from micron-sized PLGA microspheres

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Abstract

This study investigates the effect of protein molecular weight on release kinetics from polymeric microspheres (1–3 μm). Proteins were encapsulated at high and low loadings in poly(lactic-co-glycolic acid) (PLGA) by a phase inversion technique. Mechanism of release from this type of microsphere appeared to be dependent on protein molecular weight for microspheres with low loadings (0.5–1.6%), while independent of protein molecular weight for microspheres with high loadings (4.8–6.9%). At low loadings, release of larger proteins was dependent on diffusion through pores for the duration of the study, while smaller proteins seemed to depend on diffusion through pores initially and on degradation at later times. Following an initial diffusion phase from low loaded microspheres, lysozyme and carbonic anhydrase, the two smallest proteins, exhibited lag phases with curtailed protein release followed by a phase of increased protein release between 4 and 8 weeks, a phenomenon not evident for larger proteins. It appears that by 8 weeks, PLGA had degraded enough to allow additional release of smaller proteins which were entrapped efficiently within the microspheres. Higher loaded microspheres, which have more interconnecting channels, did not exhibit the pronounced shift from diffusion-based to polymer degradation-based release seen with the lower loaded microspheres. Interestingly, microspheres encapsulating large proteins maintained sustained release rates for 56 days.

Keywords: Pore size; PLGA degradation; Protein molecular weight; Erosion mechanism; Microsphere

1. Introduction

In the past several decades, polymeric delivery systems have been a major focus of pharmaceutical companies due to their many advantages over therapeutic bolus or repetitive administrations. Patient compliance, drug protection, and sustained release are some of the many benefits to encapsulating and releasing a therapeutic agent from a polymer matrix. Microspheres, in particular, are a very accommodating vehicle since their small size can often enable administration via injection or the oral route, and fabrication from a biodegradable polymer eliminates the need for surgical removal. Poly(lactic-co-glycolic acid) (PLGA) has been widely used in this vein for the encapsulation and delivery of a wide variety of agents in the past several years because it is biodegradable, nontoxic, and has been approved for several products. PLGA microspheres are made by many different techniques and have been used to

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encapsulate, release, and deliver drugs that are both hydrophobic [1–4] and hydrophilic [5,6]. One very diverse family of drugs that has been encapsulated in PLGA is proteins, a group with a wide span of molecular weights and an even wider variety of chemical properties. Therapeutic agents as small as oligopeptides [7,8] and insulin [9,10] have been encapsulated and released from PLGA microspheres as well as larger proteins such as BDNF [11], BMP-2 [12], gp120 [13], NGF [14], albumin [15], and TRH [16], just to name a few. Protein molecular weight plays a large role in release from PLGA microspheres, with the smallest proteins, such as insulin, exhibiting a very short, quick release [9,10] and large proteins having a much more prolonged release. Previous researchers have studied the effect of different molecular weight proteins in various types of PLGA microspheres [17,18]. In addition to the aforementioned drug size parameter, microspheres themselves contribute an added variable affecting protein release, their overall size. Very small delivery systems with large surface area to volume ratios such as small microspheres have traditionally been shown to release drugs more quickly than larger microspheres, rods, slabs, discs, or films period. Proteins in these small microspheres can not only diffuse from the interior to the surface more quickly but experience degradation-based protein release much more rapidly as well. This current study focuses on development of nano and microspheres in the size range of 0.2–4 μm and comparison of the size of proteins encapsulated, drug particle size within the matrix, and the degradation and release rates of these systems. One of the main questions that remains is to determine whether protein delivery from microspheres which are 0.5–5.0 μm in size can exhibit a prolonged, controlled release profile.

2. Materials and methods

2.1. Materials

All proteins used in this study were obtained from Sigma. PLGA was obtained from Boehringer Ingelheim. The micro BCA protein assay reagent kit was purchased from Pierce. Pre-cast polyacrylamide electrophoresis gels and molecular weight marker were obtained from Novex.

2.2. Microsphere fabrication

Microspheres were fabricated from PLGA 50:50 (MW 12 068) by a novel phase inversion technique [19]. Briefly, two solutions, 1 ml of protein in water (8 or 29 mg/ml) and 19 ml polymer in dichloromethane (20.6 or 19.9 mg/ml), were added together for a final volume ratio of 1:20. This two-phase system was then probe sonicated at an amplitude setting of 20% for 30 s with a Cole Parmer ultrasonic homogenizer at room temperature. Following sonication, the resulting water in oil emulsion was frozen in liquid nitrogen followed by lyophilization on a Labconco Lyph-Lock 6 at −50°C and 30 μmHg for 48 h. The dried polymer product was then resuspended with agitation in dichloromethane at a concentration of 2% or 20 mg/ml solvent. This suspension of solubilized polymer and insoluble protein particles was then quickly introduced into a nonsolvent bath of petroleum ether at a solvent:nonsolvent ratio of 1:50. The resulting microspheres were collected with a Millipore high pressure ultrafiltration cylinder system and stored at 4°C. Microspheres were loaded with either 2 or 7.3% (w/w) (theoretical loadings) of one of five proteins: lysozyme (LZ, 13.4 kDa), carbonic anhydrase (CA, 29 kDa), bovine serum albumin (BSA, 66 kDa), alcohol dehydrogenase (AD, 150 kDa), or thyroglobulin (TG, 669 kDa). Unloaded microspheres were also fabricated and used as a control for polymer MW and mass loss.

2.3. Microsphere loading

To determine microsphere loading, the same microsphere samples analyzed for protein release for 8 weeks ($n=3$) were lyophilized for 48 h, weighed, and dissolved in 500 μl of dichloromethane. PBS (500 μl) was then added to the dissolved microspheres to extract any remaining protein that had been encapsulated but not released during the 8-week protein release experiment. The two-phase system was vortexed, followed by centrifugation for 5 min. The aqueous layer was removed and saved for analysis, while the organic layer was extracted again until no protein could be detected in successive

aqueous fractions (three extractions). The amounts obtained from the extractions in this method were added to the total amount released by 8 weeks, and this total sum was taken as the actual protein loading of the microspheres.

2.4. Protein release analysis

Microspheres were divided into three, 30-mg aliquots and placed in glass scintillation vials. A 3-ml aliquot of pH 7.0 HPLC-grade water, with 0.03% sodium azide to deter bacterial growth, was added to each vial. The vials were capped and positioned on their sides so that the maximum surface area of each sample would be available for release into water. Vials were agitated after the addition of each new aliquot of water. Samples were incubated at 37°C for a total of 8 weeks and were assayed at various time points: 0.5, 1, 2, 4, 8, 24, and 72 h and 1, 2, 4, and 8 weeks. At each timepoint, the samples were centrifuged at 2000×*g* for 5 min, and the supernatants were removed and saved for further analysis. Fresh water was replaced in each vial at each timepoint to allow further protein release. A 50-μl aliquot of each supernatant was assayed with a micro BCA reagent kit (sensitivity range 1–20 μg/ml) and samples read with a Beckman DU-65 spectrophotometer at 562 nm. Calibration curves were made for each protein and run against a BSA standard supplied with the assay kit. Calibration curves had different slopes for each protein tested and were 0.0061 OD/(μg/ml) for the supplied BSA standard, 0.0058 OD/(μg/ml) for LZ, 0.0037 OD/(μg/ml) for CA, 0.0040 OD/(μg/ml) for BSA, and 0.0043 OD/(μg/ml) for AD. Values were summed to obtain cumulative release and are reported as per cent release of loaded protein. Supernatants were subjected to SDS–PAGE and were run on a 4–20% gradient Tris–glycine gel for 90 min at 125 V, 35 mA, and 5.0 W to determine the apparent molecular weight of released proteins. Gels were stained with a Coomassie blue (0.04% G-250, 3.5% perchloric acid) solution. One SDS–PAGE was run for each sample tested.

2.5. Polymer degradation analysis

Additional 2% loaded and unloaded microsphere aliquots of either 10, 30, 50, 60, 70, or 90 mg were placed in microcentrifuge or 15 ml conical tubes and allowed to degrade at 37°C in HPLC-grade water at concentrations of 10 mg/ml for various amounts of time: 1, 2, 3, 4, 5, 6 days, and 1, 2, 3, 4, 5, 6, 7, and 8 weeks. As in the protein release experiments, vials were agitated only when the water was changed. At each timepoint, all of the tubes were centrifuged and the supernatant was discarded to remove any acidic degradation products which would contribute to auto-catalytic degradation of PLGA. If the samples were to continue degradation, fresh water was added back to the tube. If not, the remaining pellet was frozen and lyophilized, and the final dry weight of the pellet was compared to the original sample weight. For this study, each timepoint was terminal, rather than cumulative, and was run only once. Dried PLGA pellets from the weight loss study were also used to determine the molecular weight of degrading microspheres. For each sample, a 5% solution was made in chloroform and analyzed on a Perkin-Elmer LC pump model 250 gel permeation chromatography system composed of isocratic LC pump model 250, an LC column oven model 101, and LC-30 RI detector, and a 900 series interface computer. Samples were eluted through a PL gel 5 μm mixed column and a 5 μm/50 Å column connected in series at a flow-rate of 1.0 ml/min and a temperature of 40°C. The system was calibrated with a series of monodisperse polystyrene standards (MW: 600–200 000).

2.6. Microsphere sizing and imaging

For SEM, samples were mounted and coated for 2.5 min with a gold and palladium mixture and examined for morphology and size with a Hitachi S-2700 scanning electron microscope. Thirty microspheres were then randomly selected and measured for diameter using Adobe Photoshop software and results averaged according to microsphere type. For TEM, samples were dehydrated in 100% ethanol, osmicated with OsO_4, embedded in LR White embedding media in gelatin capsules, and cured in a 30°C oven for 3 days. Sections were then cut to a thickness of 95 nm with a diamond knife on a Reichert-Jung Ultracut E microtome. A Philips EM 410 transmission electron microscope was used to

examine sections. Diameter averages were taken from measurements of 14–28 microspheres from a single section and analyzed with Adobe Photoshop. Cross-sections were assumed to be representative of the microsphere diameter although there is the possibility that microspheres may not be cut in the center. For pore size analysis, a sample of one to three porous microspheres was selected for each type. Between 40 and 80 pores were measured per microsphere. To determine protein particle size within the microspheres, 2-mg aliquots of microspheres were dissolved in dichloromethane on a glass coverslip. The resulting polymer film with dispersed solid protein particles was observed by SEM, and particle size analysis performed with Adobe Photoshop. Sizes were averaged among 40–50 particles per microsphere type. For particle size distribution, a 3-mg aliquot of unloaded control microspheres was measured in dry mode with a Sympatec Helos model H0849 particle size analyzer in order to determine the entire population size distribution.

3. Results

3.1. Microencapsulation efficiencies

Encapsulation efficiencies were found to be between 25.0 and 80.0% for the 2% (low loading) theoretically loaded microspheres and between 65.8 and 94.5% for the 7.3% (high loading) theoretically loaded microspheres. Protein losses during the process of micronization were assumed to be due to adsorption of protein to the preparation vessel or due to spattering of solution during the sonication step. Actual loadings and efficiencies are listed in Table 1. The decreased efficiency of the lower loadings compared to the higher loadings was assumed to be

Table 1
Summary of protein release data from protein-loaded PLGA microspheres

Protein	Molecular weight (kDa)	Actual loading (%)	Encapsulation efficiency (%)	First phase duration and release	Second (lag) phase duration and release	Third phase duration and release	Total released (%)
(A) Low loading							
LZ	14.3	1.6	80.0	30 min 12.1%	30 min–4 weeks 6.9%	4–8 weeks 47.3%	66.3
CA	29	1.2	60.0	72 h 33.6%	72 h–4 weeks 12.7%	4–8 weeks 44.7%	91.0
BSA	66	0.9	45.0	1 week 89.1%	1–8 weeks 5.4%	None	94.5
AD	150	1.1	55.0	8 weeks 85.4%	None	None	85.4
TG	669	0.5	25.0	8 weeks 89.8%	None	None	89.8
(B) High loading							
LZ	14.3	6.9	94.5	30 min 64.1%	30 min–4 weeks 4.7%	4–8 weeks 13.0%	81.8
CA	29	6.0	82.2	8 weeks 99.6%	None	None	99.6
BSA	66	5.1	69.9	4 h 92.3%	4 h–8 weeks 7.0%	None	99.3
AD	150	6.9	94.5	8 weeks 99.6%	None	None	99.6
TG	669	4.8	65.8	8 weeks 99.4%	None	None	99.4

due to the higher relative percentage of the total protein lost at these steps of the fabrication process. Protein loss could be reduced if the batch size were to be scaled up.

3.2. Protein release analysis

Five proteins commonly used as molecular weight markers, lysozyme, carbonic anhydrase, bovine serum albumin, alcohol dehydrogenase, and thyroglobulin, were encapsulated in PLGA in order to determine how diffusion, polymer degradation, and protein molecular weight affect release from microspheres. Proteins were loaded at theoretical levels of 2 and 7.3% (w/w) and were released at sink conditions (10 mg microspheres/ml). These results are shown in Fig. 1A–E.

Three phases were observed during release from these formulations (see Table 1). During the first 30 min of the first phase, protein release was fastest for LZ, followed by BSA, AD, CA, and TG from microspheres with low loadings (Fig. 1A–E). Similar patterns were observed for higher loaded microspheres, with the largest release from LZ during the first 30 min, followed by BSA, CA, AD, and TG (Fig. 1A–E).

The duration of the first phase, characterized by a typical diffusion profile, was somewhat dependent on the protein molecular weight for microspheres with low loadings, but independent of protein molecular weight for microspheres with high loadings. For lower loaded microspheres, this phase lasted 30 min for LZ, 72 h for CA, 1 week for BSA, and 8 weeks each for AD and TG. Microspheres with higher loadings exhibited first phases that lasted 30 min for LZ, 4 h for BSA, and 8 weeks each for CA, AD, and TG. A second phase, or lag phase, followed by additional release was only present for the two smallest proteins released from the lower loaded microspheres and lasted approximately 4 weeks for LZ and 3 weeks for CA. A lag phase was also present for low loaded BSA and lasted approximately 7 weeks, but was not followed by an additional phase of protein release since 89.1% of the protein was released in the first phase. A lag phase followed by additional protein release was only present for higher loaded microspheres releasing LZ and lasted 4 weeks. BSA also exhibited a lag phase lasting almost 8 weeks but was not followed by a phase of additional release. The third phase, a quick additional spurt of protein following the second phase, occurred between 4 and 8 weeks and was assumed to begin at 4 weeks based on the available data and since samples were not analyzed at times between 4 and 8 weeks. The existence of this third phase did seem to be somewhat dependent on protein molecular weight, occurring only for microspheres encapsulating proteins less than or equal to 29 kDa in molecular weight, LZ and CA. Third phases appeared to be exhibited only by those microspheres which had not released most of their contents during the first phase (greater than ~80%). Release of proteins from microspheres with high loadings was assumed to be very efficient due to the high density of interconnecting channels as opposed to that of the low loaded microspheres. Overall, it is believed that release from higher loaded microspheres was predominantly due to diffusion in pores, while for lower loaded microspheres, diffusion in pores was the main means of release only for the larger proteins. The two smallest proteins at lower loadings were released according to the typical diffusion/degradation profiles seen in PLGA polymeric delivery systems and were, therefore, dependent on diffusion in pores initially followed by a combination of diffusion in pores and degradation [20]. For the intermediate molecular weight protein, BSA, at both high and low loadings, it is assumed that the very high percentage of release in the rapid initial phase may relate to diffusion in pores but also to some properties related to the protein itself. The inset plots of percent release (Fig. 1A–E) versus square-root of time provide support for the diffusive nature of the protein release in the initial phase [21]. For both low and high loaded microspheres, the percent released increases linearly with square-root of time during the initial portions of release.

Analysis of released proteins by SDS–PAGE showed that LZ and BSA that had been released from PLGA microspheres for 30 min migrated similarly to and at the same apparent molecular weight as native proteins. LZ also migrated similarly after being released for 8 weeks. Released CA appeared somewhat denatured or cleaved by 30 min and completely denatured by 8 weeks, while released AD appeared completely denatured by 30 min. TG

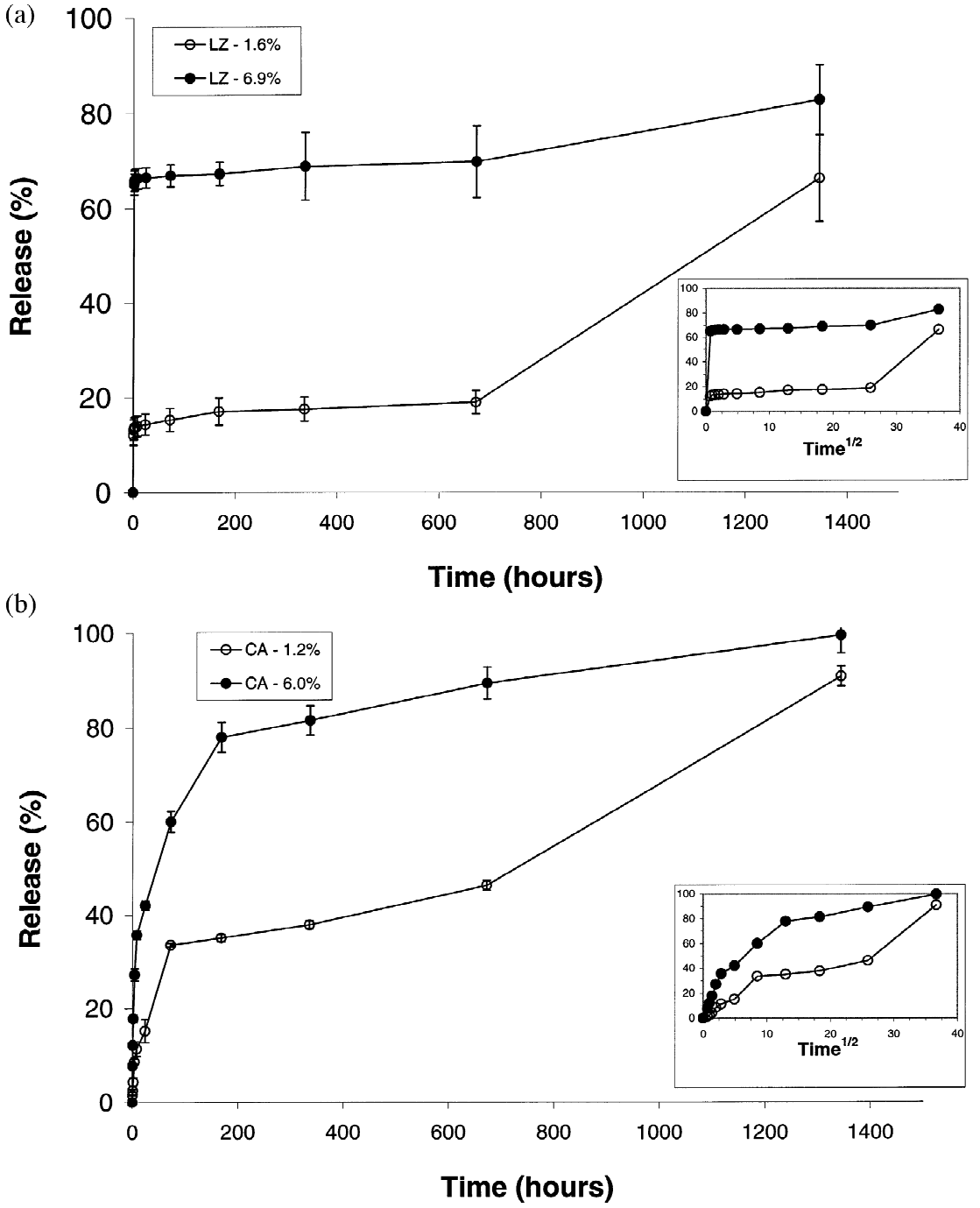
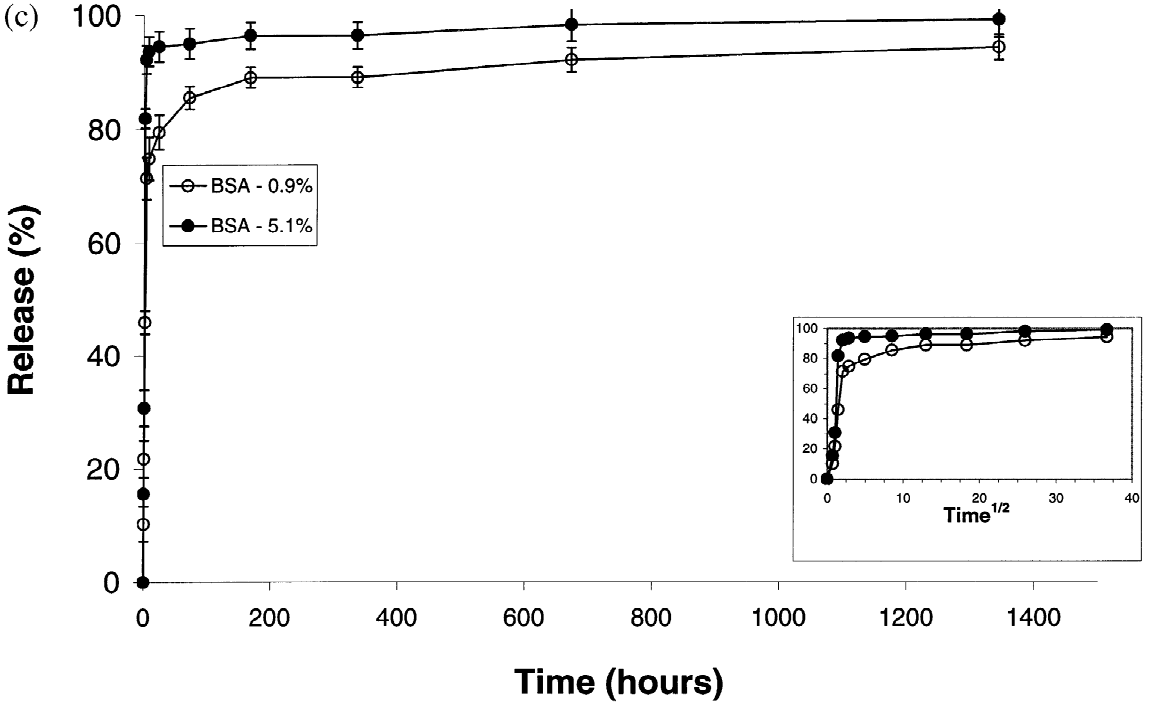


Fig. 1. Percent of total protein loading released over time from PLGA microspheres. Inset: Percent released compared to square root of time. (A) Lysozyme, (B) carbonic anhydrase, (C) bovine serum albumin, (D) alcohol dehydrogenase, (E) thyroglobulin. Low loading (○), high loading (●).



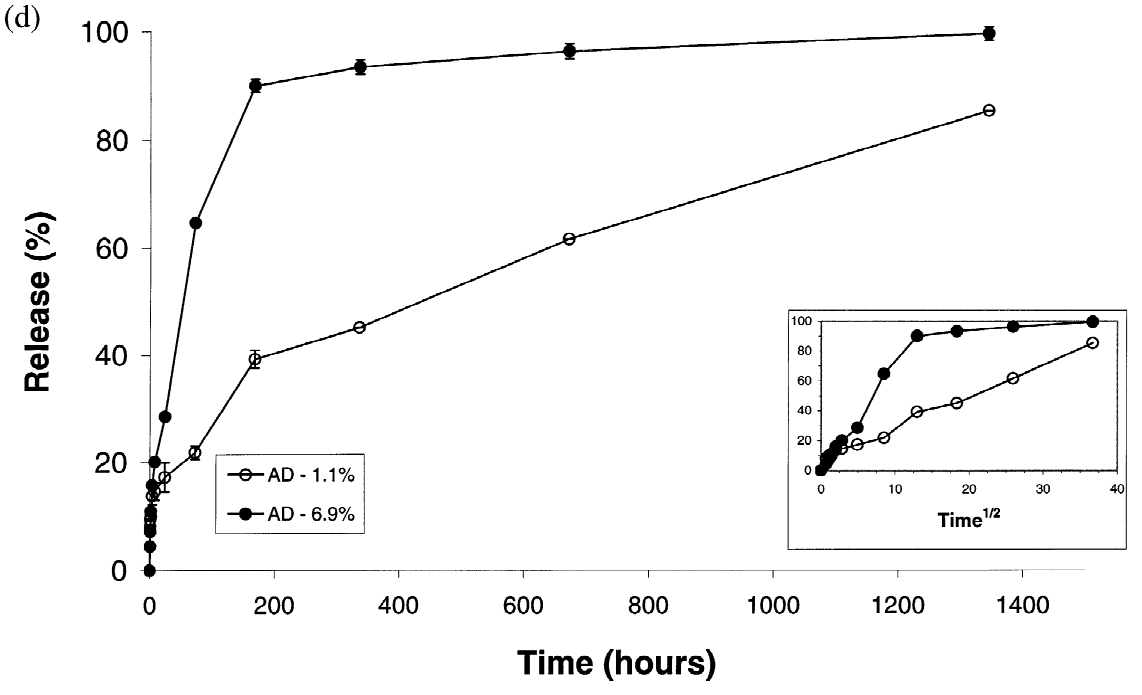


Fig. 1. (*continued*)

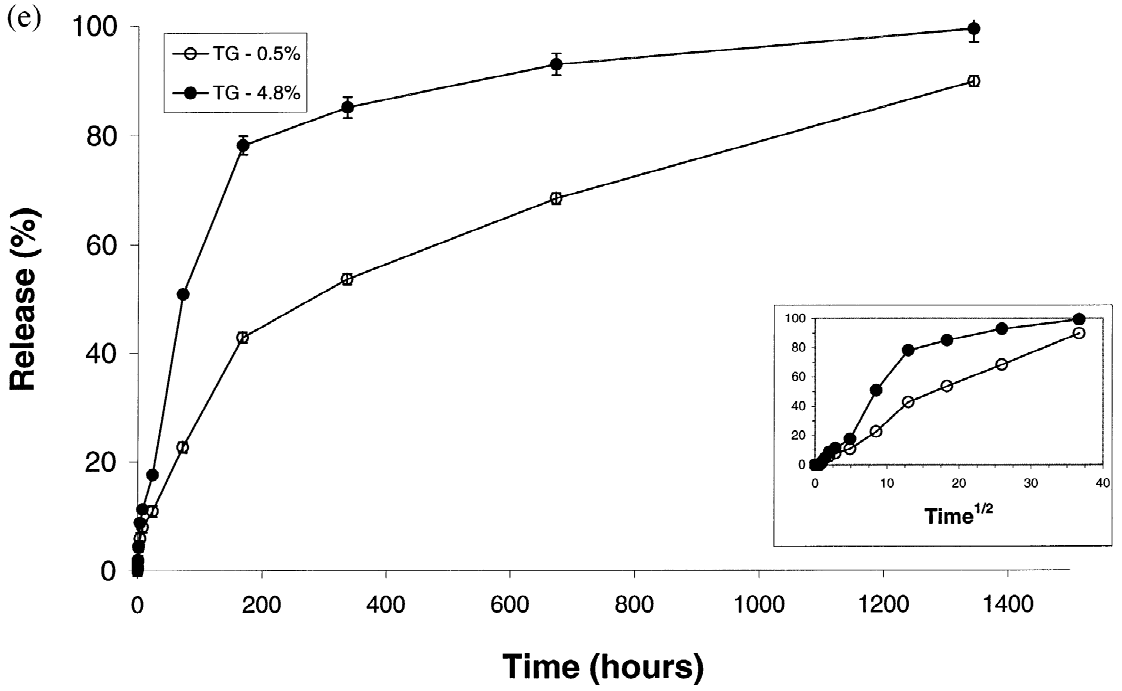


Fig. 1. (*continued*)

was too large to resolve in this system (Fig. 2). HPLC was also run on released proteins (data not shown) and confirmed the results of the SDS–PAGE. This additional data also told us that most of the CA being released up to 1 h was still in its native form. The nativity of proteins still unreleased from microspheres was not determined.

3.3. Polymer degradation analysis

PLGA MW loss was similar for all 2% loaded samples (Fig. 3). MW decreased to 70–80% of the original molecular weight (MW=12 068) by 1 day, to 40–50% by 1week, to 5–10% by 4 weeks, and to 5% by 6 weeks. As expected, mass loss results did not correspond to molecular weight loss (Fig. 4). Sample masses decreased to 95–99% of the original mass by 1 day, to 90–98% by 1 week, to 15–35% by 4 weeks, to 5% by 6 weeks, and to 2% by 8 weeks. Supplemental degradation studies of control microspheres revealed that unloaded PLGA microspheres degraded very similarly to those loaded with protein, indicating that encapsulated protein, regardless of molecular weight, does not affect degradation or erosion in this system. Control microsphere results were, therefore, taken to be representative of all types of microspheres examined in this study. The additional data points taken in this study give a better understanding of how all the polymer microspheres are actually degrading over time. Control microspheres decrease almost linearly to 33.4% of their original MW during the first week. They continue to degrade to 13.2% by the second week, and still retain 7.2% of their original molecular weight by the sixth week. Mass loss shows a decrease to 94.0% by the end of the first week, and a linear drop in mass to 14.7% by the fourth week. Table 2 shows MW and mass loss for unloaded control microspheres over discrete periods of degradation.

3.4. Microsphere sizing and imaging

SEM showed that all PLGA microspheres, whether protein-loaded or control, were spherical in shape and did not appear to be aggregated. The type of protein loaded did not appear to affect the

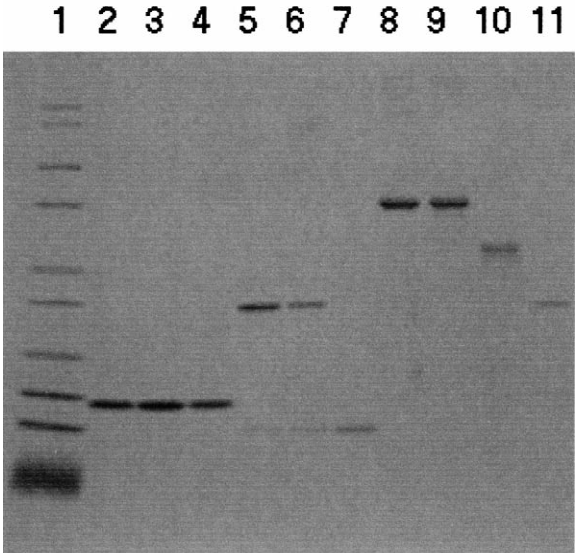


Fig. 2. SDS–PAGE of proteins released from high loaded PLGA microspheres. Lane 1: molecular weight marker; lane 2: lysozyme standard; lane 3: lysozyme at 30 min; lane 4: lysozyme at 8 weeks; lane 5: carbonic anhydrase standard; lane 6: carbonic anhydrase at 30 min; lane 7: carbonic anhydrase at 8 weeks; lane 8: BSA standard; lane 9: BSA at 30 min; lane 10: alcohol dehydrogenase standard; lane 11: alcohol dehydrogenase at 30 min.

morphology of the microspheres. When analyzed with Adobe Photoshop, the SEM micrographs showed no statistically significant differences between average particle size diameters for any of the microsphere groups (Table 3), except for the BSA group (0.374 ± 0.08 μm) which was significantly higher than the CA (0.211 ± 0.046 μm), TG (0.207 ± 0.044 μm), and control groups (0.196 ± 0.049 μm). Despite these differences, SEM microsphere size did not appear to be dependent on molecular weight of the protein loaded. Representative SEM micrographs of microspheres are presented in Fig. 5. Particle size analysis showed that microspheres had a volume size distribution median diameter of 2.23 μm and a range of 0.2–4 μm by dry powder analysis. Values obtained for both SEM and TEM fall within this range. The discrepancy in median is probably due to slight aggregation of spheres.

TEM micrographs were in agreement with SEM, confirming the spherical nature of specimens in each of the microsphere groups. TEM also provided additional information about the internal structure of microspheres. Unloaded control spheres were granular in appearance, but did not have true pores. Four

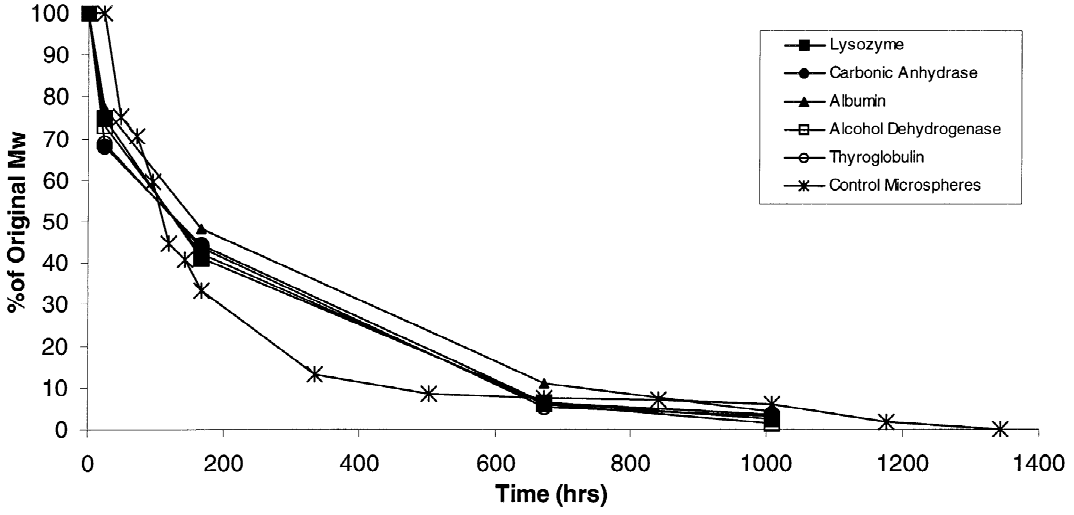


Fig. 3. Percent of original polymer molecular weight (MW) of various protein-loaded or unloaded control PLGA microspheres during degradation. Microspheres: (■) lysozyme; (●) carbonic anhydrase; (▲) bovine serum albumin; (□) alcohol dehydrogenase; (○) thyroglobulin; (*) control microspheres.

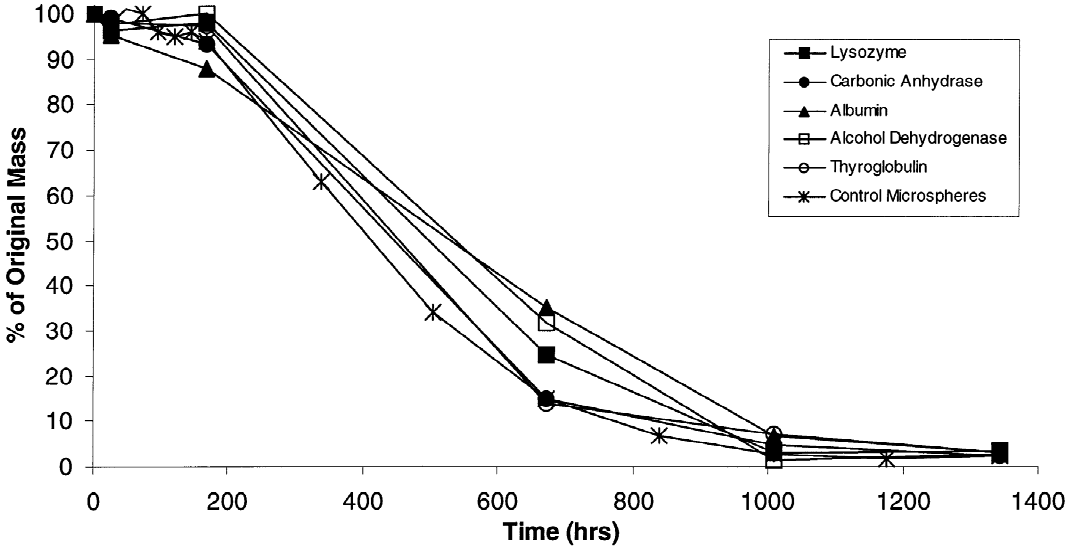


Fig. 4. Per cent of original total mass of various protein-loaded or unloaded control PLGA microspheres during degradation. Microspheres: (■) lysozyme; (●) carbonic anhydrase; (▲) bovine serum albumin; (□) alcohol dehydrogenase; (○) thyroglobulin; (*) control microspheres.

Table 2
Summary of polymer molecular weight (MW) loss and mass loss from control PLGA microspheres

Time period	MW lost (%)	Mass lost (%)
Up to 1 week	66.6	6.0
1–2 weeks	20.2	31.0
2–4 weeks	5.9	48.3
4–8 weeks	7.3	12.3

microsphere groups, however, those loaded with CA, BSA, AD, and TG, appeared to be porous. In addition, TEM was also used to obtain an estimate for particle size diameter (Fig. 6, Table 3). Although these results did not exactly coincide with those of SEM, they did further strengthen the assumption that the microspheres are in the sub-micron to 1-micron range. Results of TEM, and analysis by Adobe Photoshop software, yielded diameter values between 0.819 ± 0.156 μm (AD) and 1.077 ± 0.116 μm (CA) for the microsphere groups examined. TEM was also a useful tool in determining the average pore size diameter and pore size range (Fig. 6). Visible pores are ascribed to protein released from microspheres during processing for TEM and are therefore assumed to be synonymous with interior protein particle size. Overall, pores ranged from 3.22 to 151.61 nm (Table 3). Pores could not be visualized in LZ-loaded spheres by the methods employed. Protein particle images obtained by SEM (data not

Table 3
Summary of protein-loaded and control PLGA microspheres diameter and pore size as determined by SEM and TEM

Microsphere	SEM diameter (μm)	TEM diameter (μm)	TEM pore size range (nm)	TEM pore size average (nm)
Lysozyme	0.260 ± 0.056	0.959 ± 0.400	N/A	N/A
Carbonic anhydrase	0.211 ± 0.046	1.077 ± 0.116	3.22–151.61	41.23 ± 30.65
Bovine serum albumin	0.374 ± 0.080	0.908 ± 0.207	7.90–100.00	32.61 ± 10.25
Alcohol dehydrogenase	0.255 ± 0.070	0.819 ± 0.156	5.26–102.63	34.48 ± 18.04
Thyroglobulin	0.207 ± 0.044	0.898 ± 0.225	15.79–60.56	32.84 ± 9.99
Control (blank)	0.196 ± 0.049	0.913 ± 0.273	N/A	N/A

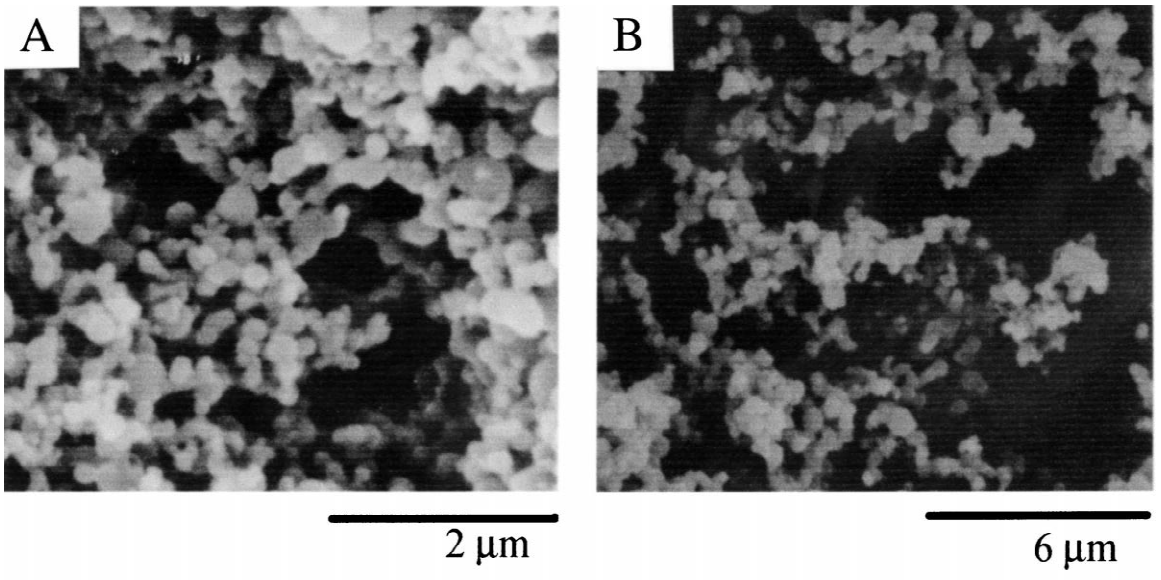


Fig. 5. Scanning electron micrographs of PLGA microspheres preceding degradation. (A) Carbonic anhydrase-loaded microspheres (×15K), (B) control microspheres (×5K).

shown) were likewise analyzed and found to have size averages similar to those of the microsphere pores obtained with TEM.

By SEM, microspheres loaded with AD were found to have protein particles with an average size of 63.5±12.82 nm and those loaded with LZ had a very similar average size of 57.48±10.34 nm. The similarity between protein particle sizes and microsphere pore sizes supports the theory that pores seen by TEM are due to protein encapsulation. This information further supports the assumption that diffusion of protein out of the microspheres is occurring through the pores. No porous structures were observed for control microspheres.

Microspheres encapsulating the smallest proteins were shown by TEM to have either no detectable pores or a large percentage of pores smaller than 20 nm (Fig. 7). The fraction of pores in this size range decreased with increasing protein molecular weight. Conversely, the percentage of pores 40 nm or larger was higher for microspheres encapsulating larger proteins.

It was also discovered that microspheres encapsulating smaller proteins appeared to have an open branched network throughout the microsphere (Fig. 6) while those encapsulating larger proteins had pores towards the outer layers and appeared open nearer the surface while having a more dense structure nearer the inner layers of the microsphere.

4. Discussion

Polymeric delivery systems based on PLGA degrade by bulk erosion [22], a process marked by diffusion of water into the polymer matrix, swelling, and subsequent degradation brought about by hydrolysis of covalent bonds. PLGA drug delivery demonstrates a protein release profile based on the inherent degradation properties of the polymer and displays distinct, predictable phases of release that are the research focus of many scientific groups [23–25].

The first stage observed is one that is characterized by the rapid release of active agent in a diffusion-controlled manner. The existence of this phase relies on the initial penetration of water into the polymer matrix which initiates release of protein molecules entrapped very close to the surface of the matrix. Water solvates the protein molecules within the pores close to the surface, and additional protein molecules migrate through the interconnecting pores within the

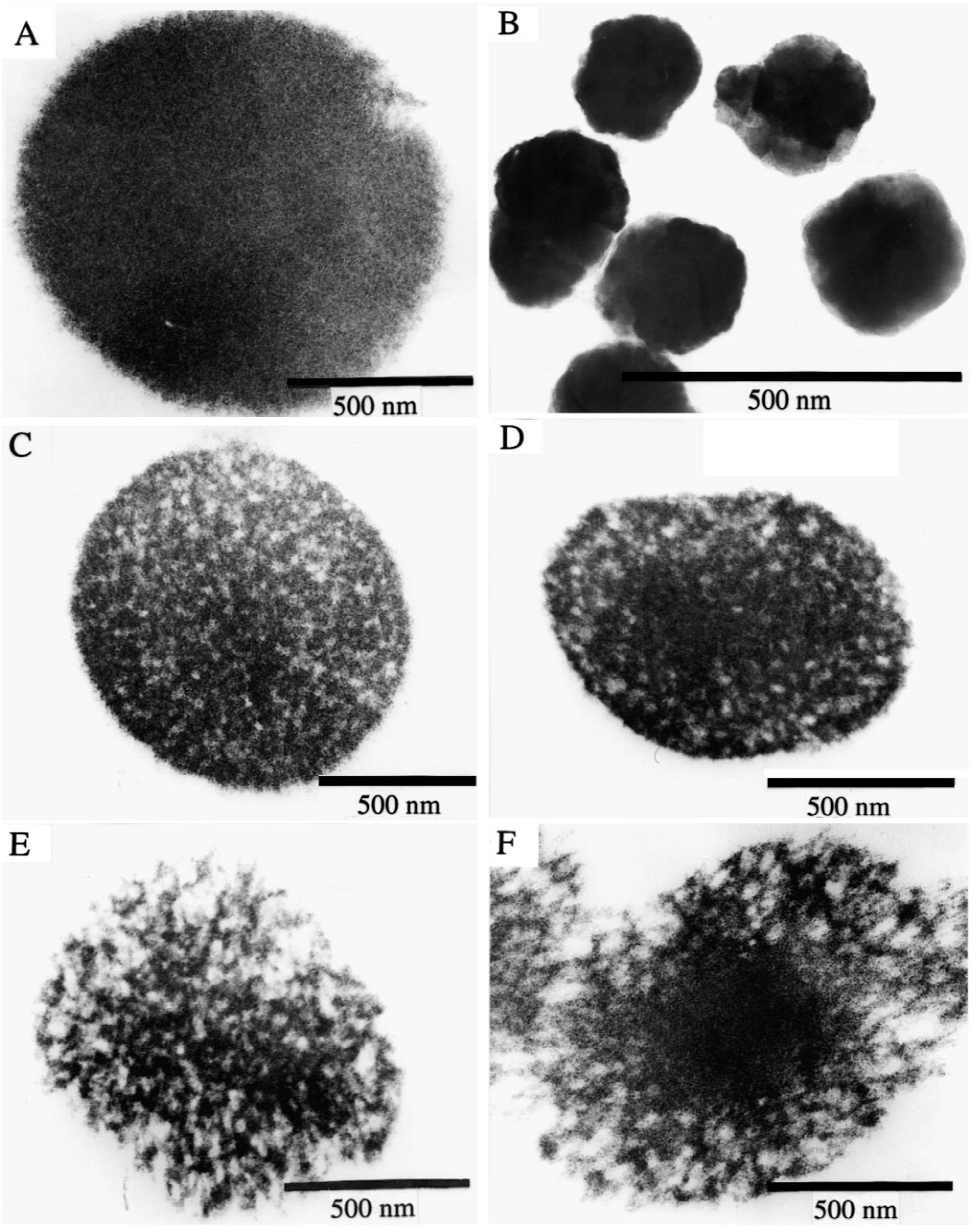


Fig. 6. Transmission electron micrographs of PLGA microspheres preceding degradation. (A) Control microspheres (×38K); microspheres with high loadings of (B) lysozyme (×69K); (C) carbonic anhydrase (×31K); (D) BSA (×38K); (E) alcohol dehydrogenase (×38K), (F) thyroglobulin (×38K).

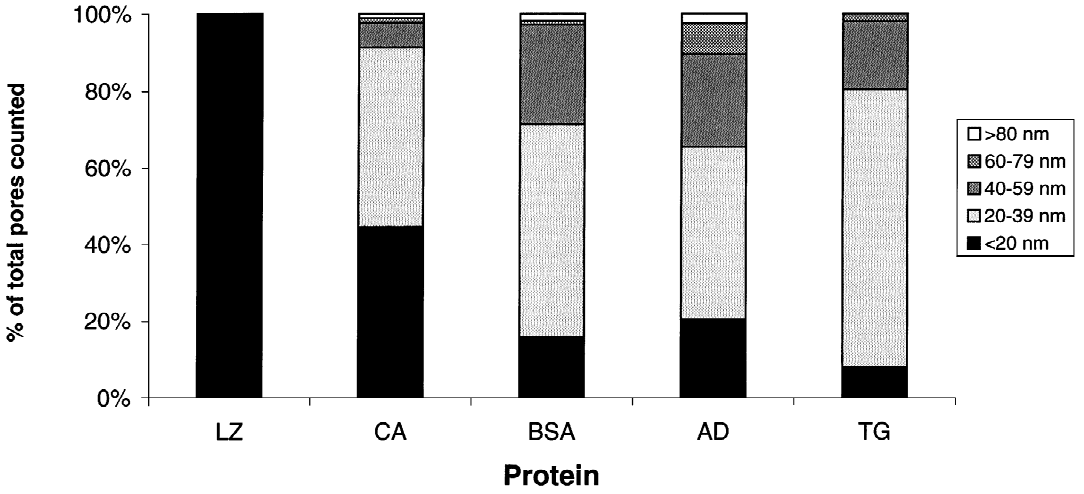


Fig. 7. Pore size distribution for protein-loaded PLGA microspheres.

polymer matrix [26–28]. These pores are assumed to contribute to the efficient release of protein from the higher loaded microspheres which have a larger network of interconnecting channels and, therefore, more access to the surrounding medium than the lower loaded microspheres.

Although pore size averages were not significantly different from one microsphere type to the next, the pore size distribution might indicate the mechanism of protein release from microspheres. Microspheres encapsulating larger proteins had a preponderance of larger pores close to the surface which might allow initial solvation of large protein clusters on the surface. This solvation would lead to a quicker penetration of the microsphere by water, causing the formation of interconnecting channels or the solvation of protein in pre-existing channels within the microsphere [27,28]. Both of these effects could lead to the continuous release and diffusion through pores seen for microspheres encapsulating larger proteins. Microspheres encapsulating smaller proteins, however, seemed to have a higher percentage of small pores distributed more evenly throughout the microsphere. This type of structure might entrap proteins within the interior of the microsphere due to the collapsing of pores brought on by a lowered T_g of the amorphous polymer. Since these pores are smaller already, collapse would most likely yield a lag effect. Additional entrapped protein could only be released in the advanced stages of degradation which occurred, in this study, at 4 weeks.

A lag effect is evident as the rate of release decreases for smaller proteins following the diffusion phase. This period is marked by a release rate that has decreased from that of the diffusion phase and begins when the polymer MW has dropped to 74.7% of the original (MW=9020) for LZ at both low and high loadings and to 70.4% (MW=8424) for CA at low loading period. At low loadings, the larger proteins, AD and TG, do not experience this transition period, and diffusion-like behavior, which is most likely through the pores, appears to continue for a much longer period of time. The intermediate molecular weight protein, BSA, exhibits a lag phase after which there is no additional phase of protein release. Most of the protein has already been released in the first phase and whatever small portion remains is released slowly during the lag phase.

In the final phase of protein release, microsphere degradation continues to occur homogeneously. At the beginning of this stage, the MW of the polymer has decreased to 6.3% of the original (MW=831) for LZ and to 6.3% of the original (MW=829) for CA. By this time, mass of the polymer has dropped to 24.6% of the original mass for LZ and to 14.7% for CA by the creation and widening of pores by erosion. Degradation eventually proceeds to the point of eradication of any existing microsphere structure

and consequential release of remaining encapsulated protein [28]. In this system, MW loss is considerable from time zero to 4 weeks and far exceeds mass loss in the initial stages of degradation which only becomes significant at 1 week since it must be preceded by hydrolysis. This phenomenon is due to the fact that monomers which are hydrolyzed from the polymer must escape the confines of the microsphere to contribute to mass loss [29]. Only microspheres loaded with the smallest proteins, LZ and CA, exhibit third phases. Microspheres with low loadings of CA or LZ most likely had a more pronounced third phase than the higher loaded microspheres because a smaller per cent of the loaded protein was already released in the diffusion phase, due to fewer interconnecting channels and less accessibility to the surrounding media at early stages in the lower loaded microspheres.

In this study, it is assumed that by 4–8 weeks, the erosion of the PLGA matrix had proceeded to form a large enough mesh size and provide a sufficient aqueous environment to allow true solution/diffusion of the smallest proteins, which had been entrapped very efficiently within small pores in the microspheres. Once the polymer has eroded enough to release protein nearer the center of the microsphere and overcome the impedance of the swelling effect, the protein will again be quickly released, as in the first phase. It is believed, even at this late stage of microsphere degradation, that the low molecular weight polymer is still capable of retaining the encapsulated protein molecules. This effect is not seen for the larger proteins, however, and it could very well be that the release seen here is predominantly due to diffusion in pores, or true solution/diffusion in a now rubbery and water swollen polymer matrix, in addition to degradation.

5. Conclusion

The proteins examined in this study span a wide spectrum of molecular weight, with the smallest being 14.3 kDa and the largest 669 kDa. Each of these proteins, when encapsulated by this novel phase inversion technique and released from PLGA microspheres, exhibits a unique release profile. It is assumed that diffusion is the main contributor to protein release in the first phase. A combination of diffusion, degradation, and swelling yield the lag phase. The third phase is predominantly controlled by degradation for small proteins, while diffusion in pores seems to be the predominant mechanism for large proteins throughout the duration of the experiment. Release from microspheres did not appear to be dependent on average protein particle size within the microsphere, but mechanism of release did appear to be somewhat dependent on protein molecular weight as well as pore size distribution and location of pores. Perhaps the most exciting observation is that release from very small particles lasted for the entirety of the experiment, spanning 56 days.

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