DRUG DEVELOPMENT AND INDUSTRIAL PHARMACY, 19(11), 1243-1263 (1993)

EVALUATION OF A MICRORESERVOIR-TYPE BIODEGRADABLE MICROCAPSULE FOR CONTROLLED RELEASE OF PROTEINS

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ABSTRACT

For the controlled release of a model protein bovine serum albumin, a biodegradable microreservoir-type microcapsule was prepared. BSA was incorporated into the microcapsules with high efficiency of 96.1(±3.1) %. The encapsulation did not cause any changes in the molecular weight and conformation of BSA, which was proven by biochemical analyses such as gel electrophoresis, circular dichroism, and HPLC.

The compositions and fabrication technique of microcapsules were found to be closely related to the release of BSA from the microcapsules and their degradation. Depending on microcapsule formulations, the in vitro release profile of BSA was either monophasic or biphasic. The microcapsules provided various release rates. It was also possible to control the delay before the initiation of BSA release and total duration of its delivery.

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INTRODUCTION

Recent advances in genetic engineering have resulted in the commercialization of many protein-based pharmaceuticals. Even though much attention has been drawn to the delivery of proteins and peptides by noninvasive routes in the last decade[1], at the present time, the majority of proteins are still administered by parenteral routes. One of the approaches used to achieve a prolongation of their therapeutic levels is to deliver these proteins via biodegradable polymers fabricated to maintain the sustained release of proteins for a long period of time.

A number of biodegradable polymers have been developed for parenteral application for the sustained release of drugs[2], but the use of poly-d,l-lactide and/or poly-d,l-lactide-co-glycolide has prevailed because of their biodegradability and excellent tissue compatibility. As distinguished from their successful application as depot systems for the release of small peptides[3 - 7], the investigation on the application of these polymers for the delivery of proteins still remains an active area of recent research.

This article reports the preparation of a microreservoir-type microcapsule for the controlled release of a model protein, bovine serum albumin (BSA). The characterization of microcapsules and BSA before and after incorporation into the microcapsules was carried out. Furthermore, studies were also focused on the parameters affecting the release rate of BSA and the total duration of its delivery. This technology can be extended into immunization procedure, and the approach to controlled release of protein introduced in this study may be helpful in formulating a vaccine-containing polymeric device.

MATERIALS

Poly-d,l-lactide (PLA) and poly-d,l-lactide-co-glycolide (PLCG) were obtained from Birmingham Polymers Inc. (Birmingham, AL). PLCG

50:50 and 65:35 (with respective mole % of lactic acid:glycolic acid at 50:50 and 65:35) had intrinsic viscosities of 0.36 - 0.40 and 0.49, while PLCG 75:25 and PLA had those of 0.48 - 0.69 and 0.73 dL/g, respectively in chloroform at 25°C (Cannon-Fenske viscometer). A low-molecular-weight poly-d,l-lactic acid (Mw = 2,000, PLA 2000) was purchased from Boehringer Ingelheim (Ingelheim, F.R.G.). BSA (A7030), ^{14}C-methylated BSA (with a specific activity of 30.1 μCi/mg protein), sodium azide, and phosphate-buffered saline (PBS) were purchased from Sigma Chemical Co. (St. Louis, MO). Polysciences Inc. (Warrington, PA) was the supplier of polyvinylalcohol (88% mole hydrolyzed). All the other chemicals were reagent grade.

METHODS

Preparation of microcapsules: Microcapsules were prepared by a modified in-water drying method of Ogawa et al.[8] Briefly, BSA (20mg) was dissolved in water as the reservoir, and a polymer (0.6 - 0.7g) was dissolved methylene chloride (7 - 9ml): For the release study, the reservoir volume was fixed at 300 μl, unless otherwise indicated. The reservoir volume was changed from 200 μl to 600 μl to study the effect of variation in the reservoir volume on the inner morphology of the microcapsules. To make a water in oil (w/o) emulsion, the polymer and BSA solutions were homogenized with either Polytron (Kinematica GmbH, Luzern, Switzerland) or Microson XL2005 (Heat Systems, NY). The emulsion was poured into water (300 ml) containing 4 % polyvinyl alcohol with a constant stirring to make a w/o/w emulsion. The emulsion was stirred for 30 minutes to have the solvent diffuse out of the formed microcapsules. Then, using a peristaltic pump (Masterflex, Cole-Parmer, IL), water (700 ml) was added at a controlled rate into the emulsion to harden the microcapsules. The microcapsules were then collected by filtration, and dried in a vacuum. Within 48 hours, the microcapsules were hand-sieved and the size distribution was determined. The microcapsules with the size of 45 to 150 μm were retained for further studies.

The surface and inner structures of the microcapsules were examined using a scanning electron microscope (SEM, Amray 1400, Amray Inc., MA). For studying their surface structure, the microcapsules were coated for 60 seconds with gold palladium under an argon atmosphere. For studying the inner structure, the microcapsules were embedded in epoxy resin and cut before coating with gold palladium.

Gel electrophoresis: Three types of microcapsules were prepared using poly-d,l-lactide (PLA), PLCG 75:25 and PLCG 50:50. After storage at room temperature for 3 months, microcapsules were dispersed in PBS (pH 7.4). The BSA samples released from the microcapsules were compared with fresh BSA by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE). The BSA samples were mixed with a Tris-phosphate loading buffer and placed in boiling water for 3 min. After cooling, electrophoresis of BSA samples was carried out at 15 mA vertical slab gel in Tris-glycine buffer, pH 8.3 (25 mM Tris, 192 mM glycine, and 0.01% SDS). Later, the appearing protein bands were stained with 0.01% Coomassie blue solution containing 10 % acetate and 50% methanol, and the gel was destained.

Circular dichroism (CD) analysis: Fresh BSA was dissolved in phosphate buffer (1 mM) containing NaCl (12 mM) and KCl (0.27 mM), pH 7.4. Each of 3 different microcapsules was suspended in the same buffer solution, and the sample containing BSA released from the microcapsules was passed through a filter membrane (0.22 μm). It was filled into a cell with path length of 0.1 cm, and spectra were recorded at the speed of 0.5 nm/sec from 194 to 240 nm by CD spectropolarimeter (Model 60DS, Aviv Associates Inc., NJ). All CD spectra were measured in triplicate, and corrected for nonideal solvent baseline. Mean residue ellipticity [θ] was calculated from the observed value (θobs):

$$[\theta] = \frac{\theta\text{obs} \times (\text{average residue weight})}{10 \times (\text{cell path length in cm}) \times (\text{mg/ml protein})}$$

Determination of BSA incorporated into microcapsules: The incorporation efficiency of BSA into microcapsules was calculated using ^{14}C-methylated BSA. After preparation, radiolabelled BSA-containing microcapsules were digested completely by 1N-NaOH. The pH of the solution was adjusted to 7, and its radioactivity was measured by liquid scintillation counter (LSC, Model 1214 RackBeta Excel, LKB Instruments Inc., MD). Incorporation efficiency (%) of BSA into microcapsules was determined as:

$$\text{Efficiency (\%)} = \frac{\text{Entrapped amount of dpm/mg of polymer}}{\text{Loaded amount of dpm/mg of polymer}} \times 100$$

Determination of BSA during in vitro release study: In addition to measuring ^{14}C-radiolabelled BSA by LSC, BSA was also analyzed by a gradient reverse-phase liquid chromatography (HPLC 1050, Hewlett Packard, NJ). Gradients were obtained by mixing solvent A (0.1% trifluoroacetic acid in water) and solvent B (0.1% trifluoroacetic acid in 5 % water and 95 % acetonitrile). The mobile phase was composed of 65 % solvent A and 35 % solvent B at time 0, and programmed multilinearly to 65 % solvent B over 6 minutes. The used column was Synchropak C4-300 (4.6 x 100 nm, HP Genenchem, CA). The flow rate was set at 0.8 ml/min, and the UV detector at 220 nm was used for detection.

In vitro BSA release study: Microcapsules (60 - 70 mg) were dispersed in PBS (4ml, pH 7.4) containing 0.02% polyvinylalcohol and 0.02% sodium azide, and kept in a shaking water bath (Model 129, Fisher Scientific, NJ) at 37°C. At each time interval, 0.5 ml of the medium was withdrawn for HPLC analysis, and the suspension was refilled with 0.5 ml of the medium. Sometimes, radiolabelled BSA-containing microcapsules were suspended in 0.1N-NaOH to accelerate the release of BSA. The suspensions were rotated at room temperature by Roto-Torque (Cole-

Parmer, IL). At each time interval, an aliquot of the medium was measured for radioactivity by LSC.

Tg measurement: The glass transition temperatures (Tg) of various microcapsules were measured with a differential scanning calorimeter (Perkin-Elmer DSC 7) using a heating rate of 10°C/min. Tg was taken as the midpoint in a thermogram as measured from extension of pre- and post-transition baselines.

pH monitoring of microcapsule suspension: Microcapsules (40 mg) were suspended in PBS (1.5ml) containing 0.02% sodium azide and 0.02% polyvinylalcohol at 37°C. The pH change in the microcapsule suspension was monitored continuously by MI-4154 pH probe (Microelectrodes Inc., NH).

RESULTS AND DISCUSSIONS

Figure 1 shows the size distribution of each of the microcapsules consisting of 3 different polymers PLA, PLCG 75:25 and PLCG 50:50, respectively. The size of most microcapsules in diameter ranges from 45 to 150 μm, which can be easily injected subcutaneously or intramuscularly.

The microcapsules possessed a smooth surface without dents (figure 2A). It is known that the surface morphology of microcapsules is affected by the rate of solvent diffusion and/or evaporation to a greater extent. The procedure reported here, which allows the microcapsules to undergo a slow hardening process in a w/o/w system, leads to the formation of microcapsules with a good surface morphology. The effect of a reservoir volume on the inner structure of PLCG 75:25 microcapsules is also illustrated in figures 2B - F. In the order of B to F, the reservoir volume increased from 0.2 ml to 0.6 ml with increment of an 0.1 ml, and the reservoirs inside the microcapsules became apparent, which can be positively correlated to the amount of the reservoir volume.

Table 1 shows the high encapsulation of BSA into the microcapsule. The actual BSA loading of the microcapsules was 2.75 %.

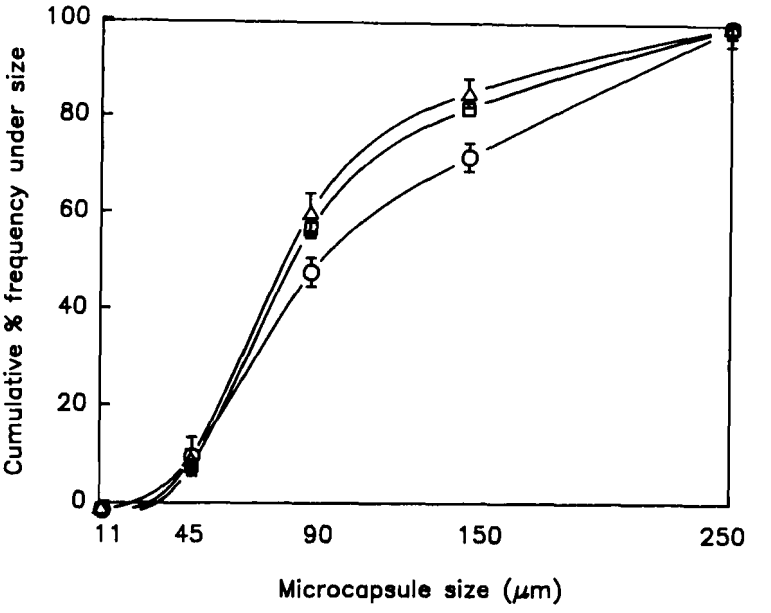

FIGURE 1
The size distribution of three types of microcapsules composed of PLA (Δ) , PLCG 75:25 (□), and PLCG 50:50 (○).

The fabrication of microcapsules may degrade proteins by harsh conditions such as their exposure to high shear forces and organic solvents, which can cause loss of their biological potency. Consequently, it is necessary to judge whether or not the processing of the microcapsule preparation imposes any harmful effects upon the native structure of BSA.

Figure 3 shows SDS-PAGE behaviors of BSA before and after incorporation into microcapsules. In every BSA sample, a small amounts of aggregates and some contaminants were observed above molecular weight markers of 66, and between 66 and 45 kilodaltons. However, all gel patterns of BSA samples released from the microcapsules after storage for 3 months at room temperature were exactly the same as observed with fresh BSA before encapsulation. It assures that the microcapsule fabrication does not cause any fragmentation and change of the molecular weight of BSA.

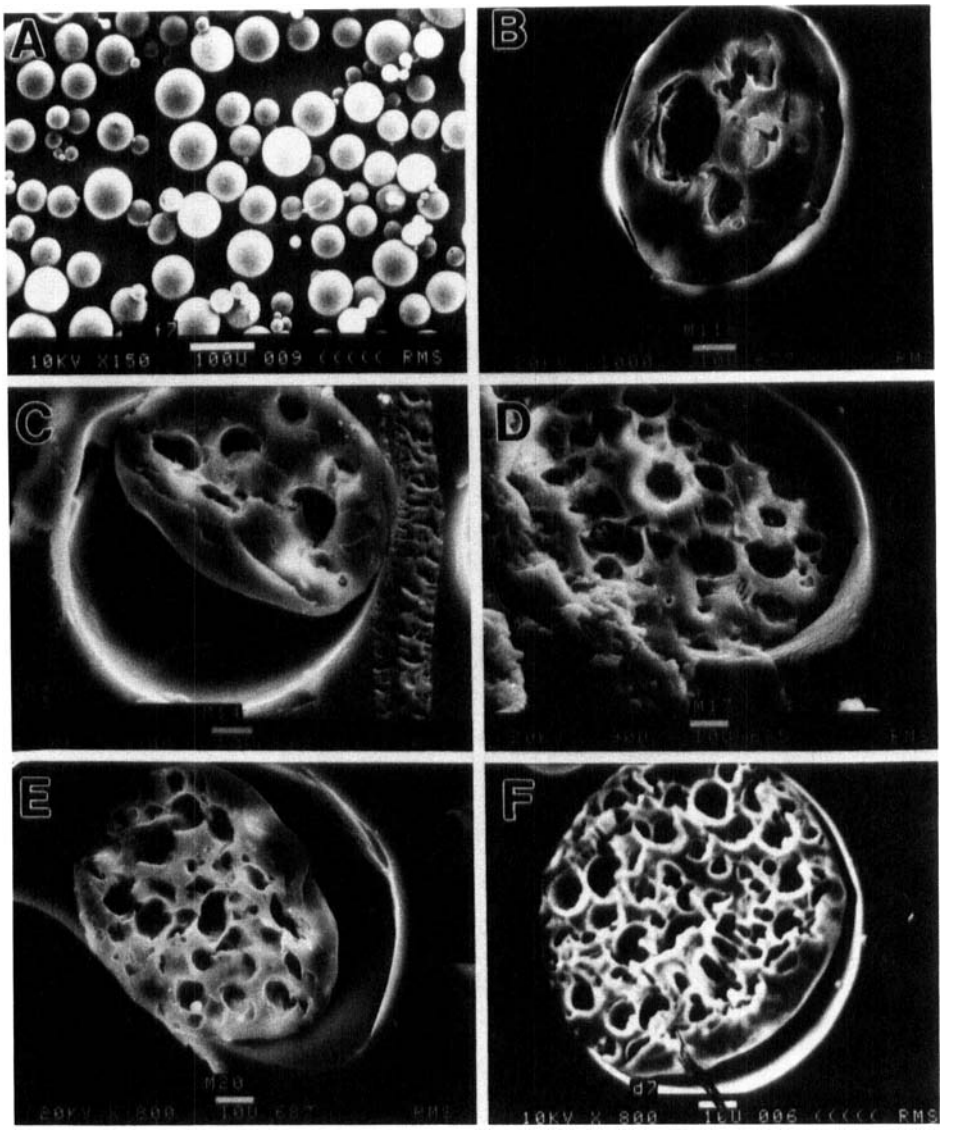

FIGURE 2

SEM pictures of PLCG 75:25 microcapsules: (A) shows their surface morphology Bar represents 100 μm; (B - F) pictures show the effect of variation in the reservoir volume on the inside structures of the microcapsules The size of bar is 10 μm. During the microcapsule fabrication, the reservoir volume was 0.2 ml (B), 0.3 ml (C), 0.4 ml (D), 0.5 ml (E) and 0.6 ml (F) with PLCG 75:25 (0.6 g) dissolved in methylene chloride.

TABLE 1

Incorporation Efficiency (%) of BSA into Microcapsules

Formulation[a]	Actual loading[b] / Theoretical loading	Efficiency (Mean±S.D.)[c]
0.7g PLCG 75:25	(282±9.0)/302	93.4±3.0
0.6g PLCG 75:25 0.1g PLCG 50:50	(511±15)/530	96.4±2.8
0.6g PLCG 75:25 0.1g PLA 2000	(518±16)/530	97.7±3.0

[a]The reservoir volume, containing total 20 mg of hot and cold BSA, was fixed at 0.3 ml [b]The unit is the radioactivity of BSA (dpm) per mg of microcapsule [c]The incorporation efficiency is 96.1±3.1%.

CD data comparing the secondary structure of BSA before and after incorporation into microcapsules are illustrated in figure 4. In the far-ultraviolet spectrum (200 to 240 nm), BSA exhibited a fork-shaped negative ellipticity band with maxima at 209 and 220 nm which was characteristic of an α-helix spectrum[9]. All spectra of BSA samples released from the microcapsules are consistent with that of Reed et al., indicating that there is little alteration in its secondary structure after the incorporation and storage within the microcapsules. All BSA samples also showed the same retention time and chromatogram in HPLC analysis. Liu et al. reported that moisture-induced aggregation of BSA which caused the loss of its solubility in water[10]. This resulted from an intermolecular S-S bond formation via the thiol-disulfide interchange reaction in BSA. They pointed out moisture-induced protein aggregation as a critical problem in the delivery and storage of protein. However, all these biochemical analyses reported here are indicative of the usefulness of this microreservoir-type microcapsule as a safe depot for delivering proteins.

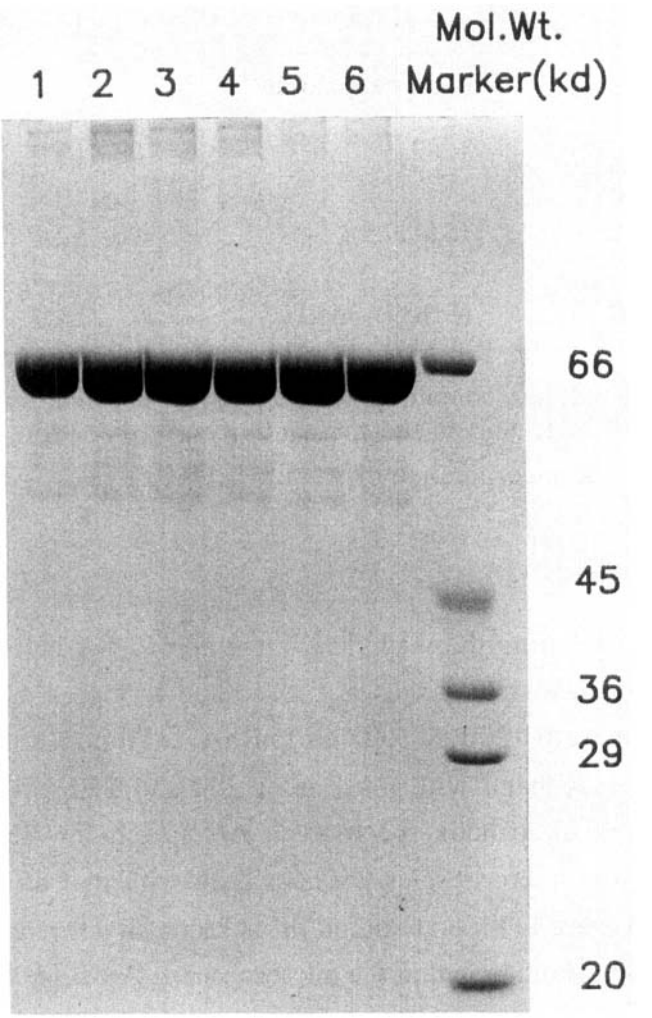

FIGURE 3
SDS-PAGE patterns of BSA before and after encapsulation into microcapsules. (1), fresh BSA before encapsulation; (2), BSA released from PLCG 50:50; (3), from PLCG 75:25; (4), from PLA microcapsules kept for 3 months at room temperature; (5) and (6), BSA kept for 2 and 4 weeks in PBS at 37°C.

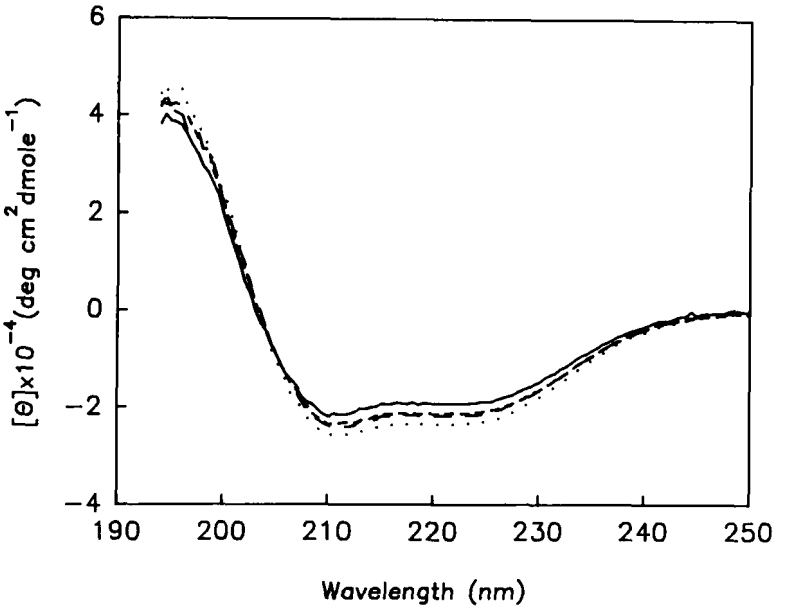

FIGURE 4
Circular dichroic spectra of BSA before and after incorporation into microcapsules. (——), fresh BSA before encapsulation; (- - - - -), BSA released from PLCG 50:50; (— —), from PLCG 75:25; (·····) from PLA microcapsules.

To accelerate the release of BSA, microcapsules were dispersed in 0.1N-NaOH acting as a strong base to attack ester linkages of the polymeric backbone. The release patterns of BSA are shown in figure 5. This data emphasize the crucial importance of a homogenization technique to make a w/o emulsion at the early stage of the microcapsule preparation. When an initial w/o emulsion was made by a Polytron homogenizer, 82.2% of BSA was released from the microcapsules after they were incubated overnight. On the contrary, the release of BSA continued for 17.5 days, when the homogenization was carried out by a Microson sonicator. Homogenization techniques seem to have a great impact on the determination of the release pattern of BSA, which can be attributed to their involvement in deciding the distribution of BSA within microcapsules and their morphology.

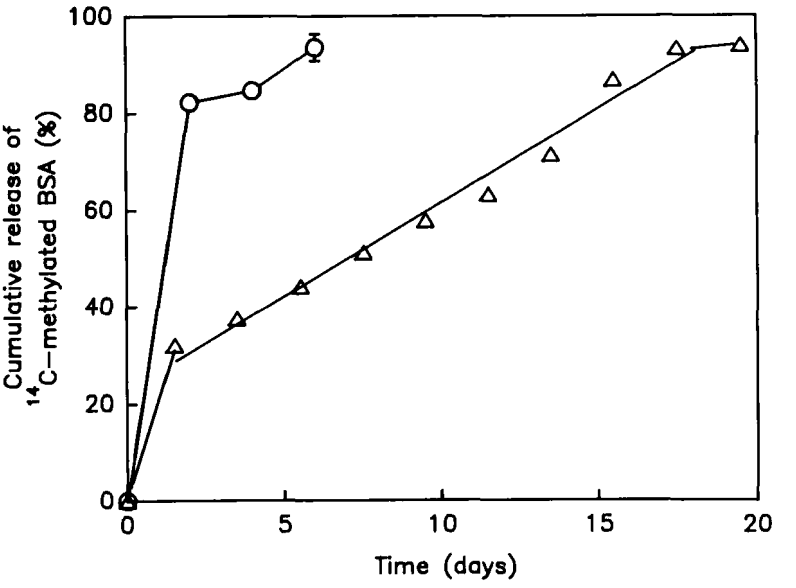

FIGURE 5
The effect of homogenization methods, which make a w/o emulsion at the early stage of the microcapsule preparation, on the release of ^{14}C-methylated BSA from microcapsules. The homogenization was carried out either using a Polytron (○Δ) or using a Microson sonicator (Δ).

Further release studies were investigated with the microcapsules prepared using a Polytron homogenizer, unless otherwise mentioned. In the following experiments, microcapsules were suspended in PBS for release study. Figure 6(a) represents the degradation of various microcapsules composed of different molar ratios of lactic to glycolic acids, which was defined as the pH change in the microcapsule suspension; The hydrolytic cleavage of the ester linkage of PLA and/or PLCG generates terminal carboxylic acid groups which are ionizable. As seen in the figure, copolymers ratios rich in lactic acid are much more stable to hydrolytic attack than those rich in glycolic acid. The release of ^{14}C-methylated BSA from the microcapsules in PBS is also illustrated in figure 6(b). To improve the slow release rate encountered with PLCG 75:25 microcapsule, the polymer composition was changed to PLCG

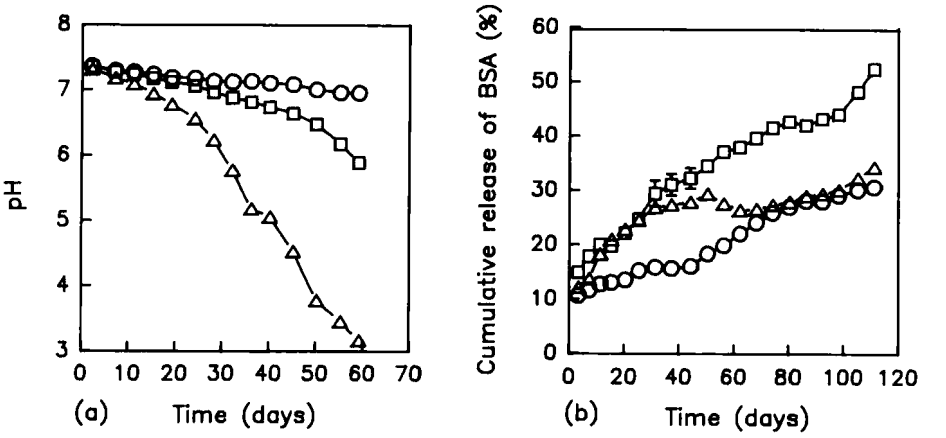

FIGURE 6

(a) The effect of molar ratios of lactic to glycolic acids on pH change in the microcapsule suspension. Microcapsules (40mg) were suspended in PBS (1.5ml), and pH was monitored continuously. The composition of the microcapsules are 0.7g of PLCG 75:25 (○), PLCG 65:35 (□), and PLCG 50:50 (Δ): (b) The release of the radiolabelled BSA from the same microcapsules in PBS.

65:35. As expected, an enhancement in its release rate took place. On the contrary, a further shift to PLCG 50:50 did not increase the release of BSA. Moreover, in the later phase of the release, the amount of BSA released from PLCG 50:50 microcapsules became stagnant in despite of the most pronounced degradation substantiated by figure 6(a). It is generally assumed that the degradation of PLCG and PLA in forms of matrices and reservoirs is prerequisite to the release of proteins. However, based on the data shown in figure 6, the degree of polymer degradation should be precisely controlled, too. If a polymeric device is designed to undergo degradation in a massive and bulky manner, it will prohibit the release of multiple-charged proteins via possible complexations between carboxylic acid end groups of polymers and the proteins.

One technique to change drug permeability in the polymer and its degradability is to mix it with other polymers possessing different characteristics[11 - 15]. Therefore, blending PLCG75:25 with either a small

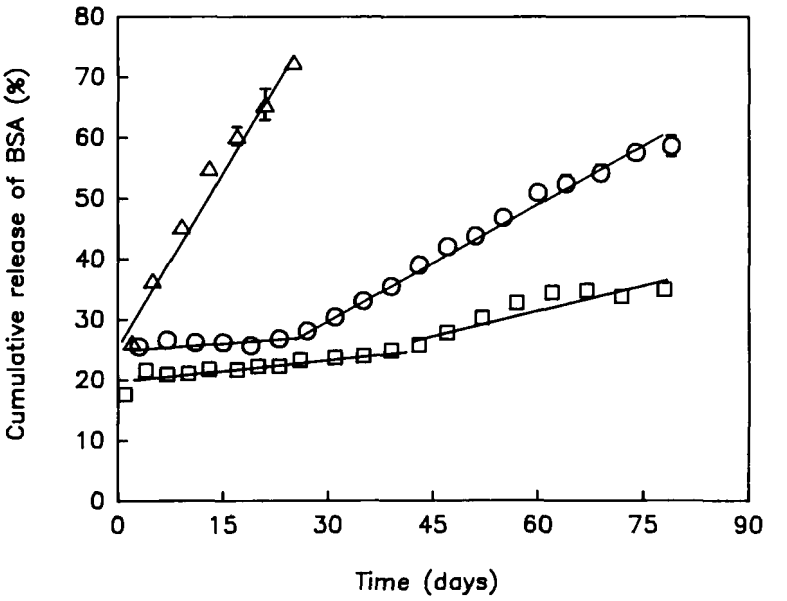

FIGURE 7
The release profiles of BSA from different microcapsules of which compositions are as follows: 0.7g PLCG 75:25 (□), 0.6g PLCG 75:25 plus 0.1g PLCG 50:50 (○) and 0.6g PLCG 75:25 plus 0.1g PLA 2000 (Δ). After 79 days, the release study was terminated because of BSA instability and low solubility below pH 5.0.

amount of PLCG 50:50 or a low-molecular-weight poly-d,l-lactic acid (PLA 2000, Mw = 2000) has been investigated to shorten the lag time required for significant initiation of microcapsule degradation and to increase the release rate of BSA. It is based on an idea that if microcapsules are made of a major fraction of PLCG 75:25 and minor fraction of PLCG 50:50 and/or PLA 2000, increased porosity will develop in the microcapsules. This will happen due to the rapid degradation of the minor components. However, the overall microcapsule structure will be maintained by the major component PLCG 75:25. Consequently, it can be expected that BSA release will improve as function of time, shortening the lag time observed in the microcapsules solely composed of PLCG 75:25.

TABLE 2

The in vitro release rates of BSA from microcapsules[a]

Formulation	Rate (μg of BSA/day/mg of microcapsule)	R[b]
0.7g PLCG 75:25[c]	0.054	0.983
0.7g PLCG 65:35[c]	0.085	0.990
0.6g PLCG 75:25 0.1g PLCG 50:50[d]	0.175	0.975
0.6g PLCG 75:25 0.1g PLA 2000[d]	0.522	0.988

[a]The loading amount of BSA is 27.5 μg/mg of microcapsule [b]R represents a correlation coefficiency [c]Calculated from the data in figure 6 [d]Calculated from the data in figure 7.

It was proven that the blending reduced a lag time and significantly increased the release rates of BSA, and these effects were dependent on the characteristics of the minor components (figure 7). When PLCG 75:25 was mixed with PLA 2000 undergoing a faster degradation than PLCG 50:50, the release of BSA was monophasic without an induction period. The release pattern could be expressed by zero-order kinetics with a rate constant of 0.522 μg / day / mg of microcapsule. This monophasic release is distinguished from multiphasic releases often observed with conventional microcapsules or microspheres[16 - 22]. Therefore it is possible to control the release rates of protein, lag times, and total duration of protein delivery by a proper microcapsule formulation focusing on precise control of its degradation. After the induction period, the release of BSA from the microcapsules composed of 0.6g PLCG 75:25 and 0.1g PLCG 50:50 could be described by zero-order kinetics, too. The relevant rate constants are summarized in table 2.

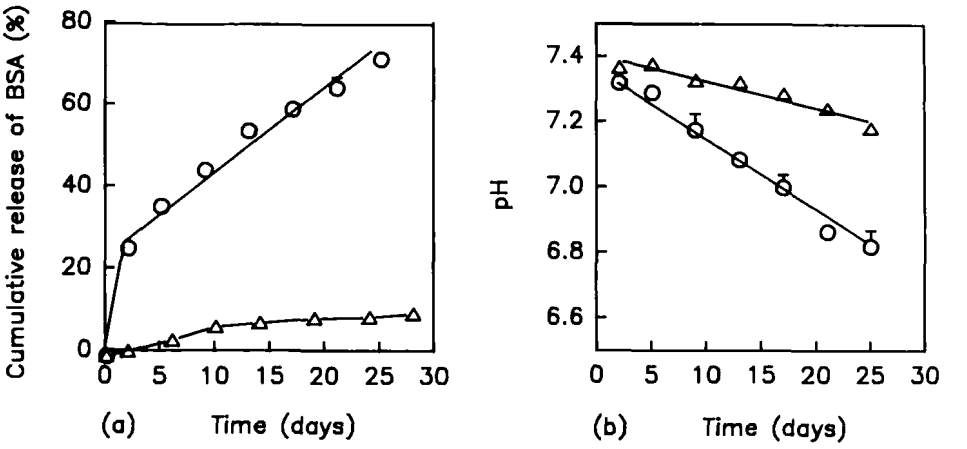

FIGURE 8

(a) The release of BSA from the microcapsules produced by different homogenization methods. (○) shows the release of BSA from the microcapsules consisting of 0.6g PLCG 75:25 and 0.1g PLA 2000. A Polytron is used for making an initial w/o emulsion. The microcapsules composed of 0.4g PLCG 75:25 and 0.2g PLA 2000 are prepared by a Microson sonicator (Δ). (b) The pH change in the microcapsules suspensions during the release study. A Microson sonicator produces the microcapsules which are much more stable at the hydrolytic chain cleavage of the polymers.

During the release study, the pH in the microcapsule suspension composed of 0.6g of PLCG 75:25 and 0.1g of PLCG 50:50 dropped below 5 after 79 days. It is reported that albumin undergoes a pH-dependent N-F conformational transition at acidic pH range. At neutral pH, it exists in N-state and is infinitely soluble. But when pH drops to acidic condition, albumin becomes F-state, losing its water-solubility. In addition, it can form dimers and even higher oligomeric forms via direct formation of disulfide linkage between molecules, and its aggregation is highly favored at low pH[23]. Because of these reasons, the in vitro release study in PBS was terminated when the pH in the microcapsule suspension became less than 5.

An additional result emphasizing the importance of the homogenization method is illustrated in figure 8. The microcapsule prepared using a

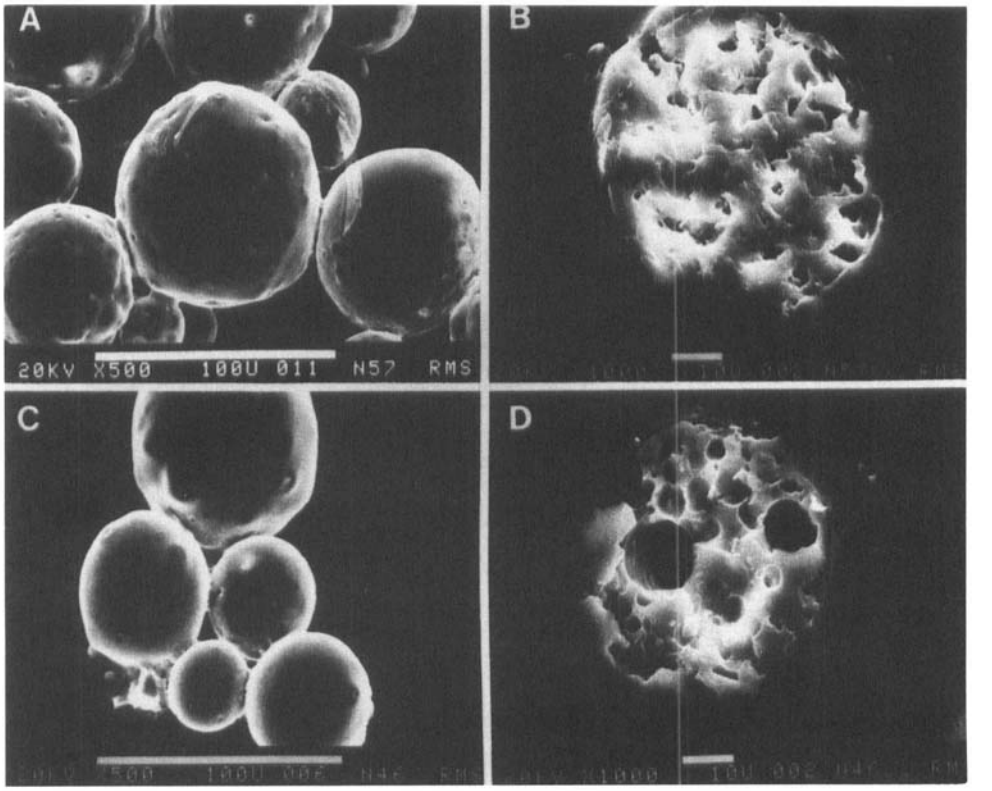

FIGURE 9
SEM analysis of the microcapsules incubated in PBS at 37°C for 59 days. A and B are the surface and inside structures of microcapsules composed of 0.7g PLCG 75:25. C and D represent those of microcapsules consisting of 0.6g PLCG 75:25 and 0.1g PLCG 50:50. Bar represent 100 μm in A and C, and it stands for 10 μm in B and D.

Polytron homogenizer provides a controlled release of BSA in PBS, while that using a Microson sonicator dose not (figure 8a). It is also very interesting to note that the microcapsules prepared using a Microson sonicator is much more stable to the hydrolytic chain cleavage than that prepared using a Polytron homogenizer, even though the former was formulated to undergo much faster degradation than the latter (figure 8b). Based on the data in figures 5 and 8, it can be concluded that the homogenization method seems not only to decide the distribution of BSA

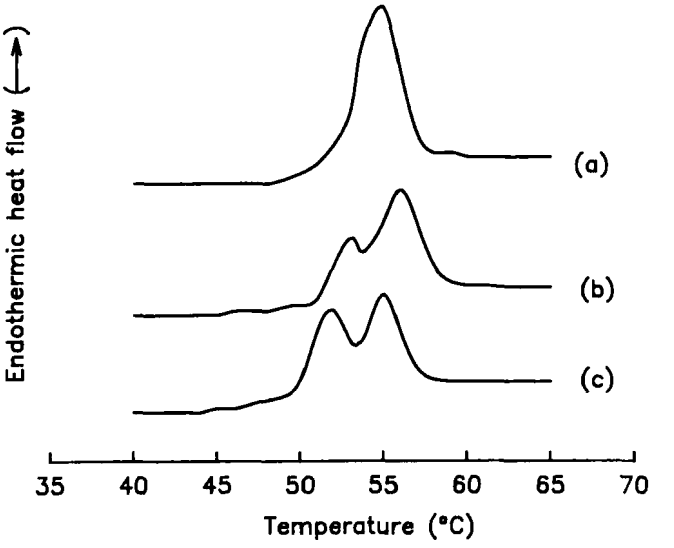

FIGURE 10
DSC thermograms of the microcapsules with different blending ratios of PLCG 75:25 to 50:50. The composition(%) of PLCG 50:50 is 18.8 (a), 37.5 (b), and 50 (c).

inside microcapsules and their morphology, but also to determine their susceptibility to hydrolytic degradation.

Microcapsule degradation was also observed by SEM. Microcapsules with 2 different compositions were used: the one exclusively composed of PLCG 75:25 (0.7g), and the other, of PLCG 75:25 (0.6g) and PLCG 50:50 (0.1g). After being kept in PBS at 37°C for 59 days, the microcapsules were investigated with SEM analysis (figure 9). Even though the surface structure of both microcapsules looked similar, there was a significant difference in the inside of the microcapsules. The presence of a minor fraction of PLCG 50:50 made a big contribution to the facilitation of their degradation, consequently causing an increase in the release rate of BSA as shown in figure 7. The observation that the inside of the microcapsule was degraded much faster than the surface can be explained by the autocatalytic effect of microcapsule degradation suggested by Spenlehauer et al.[24]. When the acidic fragments of polymers are produced by cleavage

of the polyester backbone, they remain entrapped inside microcapsules because of their inability to diffuse out. This causes an acceleration of the autocatalytic hydrolytic process inside microcapsules, while the surface of microcapsules is diluted by the external fluid.

Additionally it was observed that an increasing molar ratio of glycolic acid to lactic acid shifted the Tg of the microcapsules to a lower temperatures: Tg of PLCG 75:25 was 55.1°C, and of PLCG 50:50, 49.3°C. The thermogram of the microcapsule composed of PLCG 75:25 and 50:50 was also investigated as means of judging their miscibility. Figure 10 shows the thermograms of 3 microcapsule formulations. From the order of (a) to (c), the percentage of PLCG 50:50 of the microcapsule composition increased from 18.8 to 37.5, and 50%. When it exceeded 37.5%, 2 distinct peaks appeared in the thermograms. This is indicative of 2 separate polymeric domains within the microcapsule. The incomplete mixing of 2 polymers in the microcapsule may provide a high burst release of BSA. When the microcapsules composed of PLCG 75:25 (50%) and 50:50 (50%) were incubated in PBS at 37°C for 3 days, 43.7(±3.4) % of BSA released.

CONCLUSIONS

The microreservoir-type microcapsules can act as a safe depot system for the controlled release of protein for a long period of time as substantiated by biochemical analyses. The incorporation efficiency of BSA into the microcapsules is almost complete, 96.1(±3.1)%. The fabrication techniques, especially homogenization methods to make an initial w/o emulsion at the early stage, have an important effect on the release of BSA from the microcapsules. The PLA and/or PLCG have been known to undergo a homogeneous bulk erosion, and therefore, the release of macromolecules from the polymeric devices is often multiphasic. However, as shown in this report, a proper microcapsule formulation provides a constant release rate of protein. The release rate, together with

the total duration of its delivery, can be controllable. This approach depends on precise control of the microcapsule degradation, which gradually increase drug permeability and the porosity of microcapsule membrane.

ACKNOWLEDGMENTS

The authors wish to express their appreciation for the Johnson and Johnson fellowship awarded to HongKee Sah, and wish to thank the kind assistance of Mr. Rajesh Patel in using scanning electron microscope.

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