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Effect of Additives on the Release of a Model Protein From PLGA Microspheres

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ABSTRACT The purpose of this study was to investigate the effect of 2 additives, poly(ethylene glycol) (PEG) 1000 and 1,2,3-tridecanoyl glycerol (tricaprin), on the physico-chemical characteristics and in vitro release of a model protein, bovine serum albumin (BSA), form poly(D,L-lactic-co-glycolic acid) (PLGA) microspheres. BSA-loaded microspheres were prepared by the double emulsion solvent evaporation method. Additives were incorporated into microspheres to modify the release of protein. The addition of PEG 1000 and tricaprin changed the surface characteristics of microspheres from smooth and nonporous to porous and dimpled, respectively. The in vitro release profiles showed that the additives significantly ($P < 0.05$) increased the early-stage release of BSA from microspheres.

Keywords: microspheres, poly(D,L-lactic-co-glycolic acid), bovine serum albumin, poly(ethylene glycol), tricaprin, morphology, and in vitro release.

INTRODUCTION

Owing to the development of recombinant DNA technology, a large variety of protein drugs, such as hormones, growth factors, and vaccines, became commercially available for therapeutic purposes [1] . However, the use of these protein drugs is limited clinically because proteins have unique requirements and limitations for delivery compared with low molecular weight molecules. Generally, they have short plasma half-lives, are incapable of diffusing through biological membranes, and are not stable in the gastrointestinal tract [2] , which makes oral bioavailability low. Many proteins currently being developed are aimed at chronic conditions where therapy may be required over months or years. Alternative administration by frequent injections to keep the protein drug at effective concentrations is tedious, expensive, and has poor patient compliance. Therefore, development of sustained release injectable dosage forms becomes necessary to overcome these problems.

Biodegradable polymeric matrix has been found promising for delivering proteins over a desired period of time. The use of biodegradable poly(D,L-lactic-co-glycolic acid) (PLGA) microspheres for the delivery of peptides and proteins has been widely reported [3-7] . However, some difficulties of using this delivery system cannot be ignored. One major problem is whether the release rate of protein follows a desired profile. From current studies, it can be seen that the protein release from biodegradable microspheres is governed by many factors [8,9] . These include the degradation rate of PLGA copolymer, which largely depends on the physical properties of polymer such as molecular weight, hydrophilicity, and the ratio of lactide to glycolide [8] . Processing conditions employed during preparation of microspheres determine the properties of the microspheres, such as the size, morphology, encapsulation efficiency, and drug distribution [9] . All these properties influence the release of drug from the delivery system. Among all of these variables, morphology of microspheres plays a key role in modulating drug release. Incorporation of additives into the delivery systems could be an attractive approach to modify the morphology as well as the release profiles of protein from microspheres [10,11] .

Poly(ethylene glycols) (PEGs) of higher molecular weights (4000 to 70 000) have been incorporated with poly(lactic acid)(PLA) or PLGA microspheres to study their effect on the in vitro release of proteins [12-14] . In this study, relatively low molecular weight PEG 1000 was incorporated into microspheres to see its effect on the in vitro release of a high molecular weight model protein, bovine serum albumin (BSA). Fatty

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acid esters with long alkyl chain length have been studied for their ability to accelerate hydrophobic drug release from microspheres. Isopropyl myristate (IPM) has been used to enhance the release rate of taxol [15] , aclarubicin [16] , and etoposide [17] from microspheres. We also used 1,2,3-tridecanoyl glycerol (tricaprin) to accelerate the in vitro release of etoposide from microspheres [18] . However, the effect of fatty acid esters on the release of hydrophilic macromolecules from PLGA microspheres has not yet been studied. In this study, we incorporated tricaprin into microspheres to see its effect on the release of highly hydrophilic BSA from microspheres.

The objective of this study is to investigate the effect of the incorporation of 2 types of additives, hydrophilic PEG 1000 and hydrophobic tricaprin, on the size, surface characteristics, encapsulation efficiency, and in vitro release of a model protein BSA from microspheres. All microspheres were prepared using a double emulsion solvent evaporation method. The morphological effects were analyzed to obtain a better understanding of the mechanism of BSA release from microspheres prepared with different amounts of additives. The investigation has been driven by the need for improving the delivery of effective doses of therapeutic large molecular weight proteins from PLGA microspheres.

MATERIALS AND METHODS

Materials

PLGA (50:50, inherent viscosity 0.63 dL/g in hexafluoroisopropanol at 30°C) was purchased from Birmingham Polymer, Inc (Birmingham, AL). BSA, PEG 1000, tricaprin, and poly(vinyl alcohol) (PVA) (MW 3000-7000) were obtained from Sigma Chemical Company (St Louis, MO). Methylene chloride was purchased from Fisher Chemical Company (Fair Lawn, NJ) and MicroBCA Kit from Pierce (Rockford, IL). All the other reagents were analytical grade.

Preparation of BSA-Loaded PLGA Microspheres

BSA-loaded PLGA microspheres were prepared by a double emulsion solvent evaporation technique [3,19] . Briefly, 100 µl of protein solution containing 30 mg of BSA in phosphate buffered saline (pH 7.4, 10 mM) was emulsified with 3 mL of methylene chloride solution containing 400 mg of PLGA to get the primary emulsion. The emulsification was carried out by ultrasonicator with a microtip (Sonifier® cell disruptor, Model W185, Heat System-Ultrasonics, Inc, Plainview, NY) for 30 seconds at 40 W. The primary emulsion was added into 100 mL of 2% PVA aqueous solution and stirred at 3000 rpm using a Silverson L4RT Homogenizer (Silverson Machines, East Longmeadow, MA) to form the second emulsion. The resultant double emulsion was then transferred into 800 mL of distilled water and magnetically stirred for 3 hours at room temperature to evaporate methylene chloride. The hardened microspheres were collected by suction filtration (Aspirator Pump, Cole-Parmer, Chicago, IL) and washed 3 times with deionized water and finally freeze dried using a lyophilizer (VIRTis, VIRTIS Co, Gardiner, NY) to obtain free-flowing powder. The dried microspheres were stored at 4°C under desiccation. The microspheres containing additive (PEG 1000 or tricaprin) were prepared in the same manner by dissolving the additive and PLGA into methylene chloride with different PLGA/additive ratios.

Microsphere Size Determination

The microspheres were dispersed in water containing 0.2% Tween 80. One drop of dispersed microspheres was mounted on a microscope slide with a cover slip. One hundred microspheres were counted for size using a Cole-Parmer Video Caliper (Model 49910-20, Cole-Parmer, Chicago, IL) under a 10x lens (Meiji Microscope, Osaka, Japan). The video caliper is a microprocessor-controlled video-based reticle generator that is projected onto a standard video monitor (CT-2086YD, Panasonic, Sacaucus, NJ). The calibrated caliper system was used to size microspheres projected onto the video monitor through the video microscope system.

Scanning Electron Microscopy (SEM)

A scanning electron microscope (JOEL SEM, Peabody, MA) was used to examine the shape and surface morphology of the microspheres. Freeze-dried microspheres were mounted on an adhesive stub and then coated with gold palladium under vacuum using an ion coater. The coated specimen was then examined under the microscope at 10 kV and photographed.

Protein Content Determination

Protein content was determined by dissolving quantitatively weighed microspheres (? 10 mg) into 1 mL of 1 N sodium hydroxide solution and then

neutralizing the solution by adding 1 mL of 1 N HCl. The resulting solution was then analyzed for total protein content by MicroBCA protein assay [20] . Samples were assayed in triplicate. Encapsulation efficiency of BSA in microspheres (ie, percentage of protein entrapped in the microspheres with respect to the total amount of protein added during the preparation of microspheres) was determined.

In Vitro Release

In vitro release studies were carried out by suspending 40 mg of microspheres into 1 mL phosphate buffered saline solution (10 mM, pH 7.4) in a centrifuge tube, incubating at 37°C in a water bath (Model 50 Reciprocal Shaking Water Bath, Precision Scientific, Winchester, VA), and shaking at 100 rpm. At predetermined time intervals, the samples were centrifuged at 4000g for 15 minutes (Sorvall® RC-5 Superspeed refrigerated centrifuge, DuPont Co, Newtown, CT). The supernatant was removed completely and replaced with the same amount of fresh buffer. The amount of the released protein was determined by MicroBCA protein assay [20] . In vitro release studies were carried out in triplicate for each batch of microspheres.

Statistics

One-way analysis of variance (ANOVA) was employed to assess statistical significance. The significance level was 0.05.

RESULTS

Table 1 shows the effect of additives on particle size and encapsulation efficiency of microspheres. In case of PEG 1000, the encapsulation efficiency was reduced by adding PEG 1000 intooil phase during the emulsification. The encapsulation efficiencies of BSA within microspheres were 41.62% ± 0.06% and 38.82% ± 1.79% for PLGA/PEG ratios 2:1 (batch A1) and 1:1(batch A2), respectively, which were significantly lower ($P < 0.05$) than the control (45.14% ± 0.67%). Encapsulation efficiency of BSA in microspheres of batch B1 (PLGA/tricaprin 10:1) was 46.91% ± 1.14%, which is not significantly different ($P > 0.05$) than the control. Further increase in the tricaprin amount in microspheres (PLGA/tricaprin 2:1, batch B2) significantly ($P < 0.05$) lowered the encapsulation efficiency (30.98% ± 1.00%). The average size range among all batches of microspheres varied between 53 µm and 62 µm, and the size was independent of incorporation of additives into microspheres. **Figure 1** displays the surface morphology of microspheres containing additive PEG 1000. The control microspheres showed a smooth, nonporous surface while the microspheres with PEG exhibited a highly porous surface. The porosity of microspheres increased with an increasing amount of PEG. It was reported that a substantial fraction of the PEG dissolved in the methylene chloride phase of emulsion was extracted into the external aqueous phase. The leaching out of PEG during microsphere manufacturing from the polymer blends would form pores and channels through which drug could also pass from internal aqueous phase to external aqueous phase [12] .

Tricaprin has been successfully used to increase the release of etoposide from PLGA microspheres [18] . Unlike PEG 1000, tricaprin produced microspheres with a dimpled surface. The dimples increased in size with the increasing tricaprin concentration (**Figures 2** and **3**). The dimples are spherical in shape and arranged over the surface in a remarkably regular manner. We assume that the formation of dimples is the result of tricaprin leaching from the matrix during microparticle formation. When the PLGA/tricaprin ratio was 10:1 (batch B1), the diameters of most dimples on the surface were generally below 1 mm, and they increased up to 10 µm when the tricaprin amount was increased (PLGA/tricaprin 2:1, batch B2). These dimples generally opened up after a few days during in vitro release study. The smaller the dimples, the easier they will be broken (**Figure 3**).

Table 1. The effect of PEG 1000 and tricaprin on particle size and encapsulation efficiency of BSA-loaded PLGA microspheres

Batch No.	PLGA (mg)	BSA (mg)	PLGA/PEG ratio	PLGA/ tricaprin ratio	Encapsulation Efficiency (%) (mean ± SD)*	Size (µm) (mean ± SD)**
C (control)	400	30	—	—	45.14 ± 0.67	55.93 ± 15.55
A1	400	30	2:1	—	41.62 ± 0.06	52.73 ± 15.12
A2	400	30	1:1	—	38.82 ± 1.79	53.62 ± 11.34
B1	400	30	—	10:1	46.91 ± 1.14	58.22 ± 20.46
B2	400	30	—	2:1	30.98 ± 1.00	62.00 ± 20.88

*n = 3 **n = 100

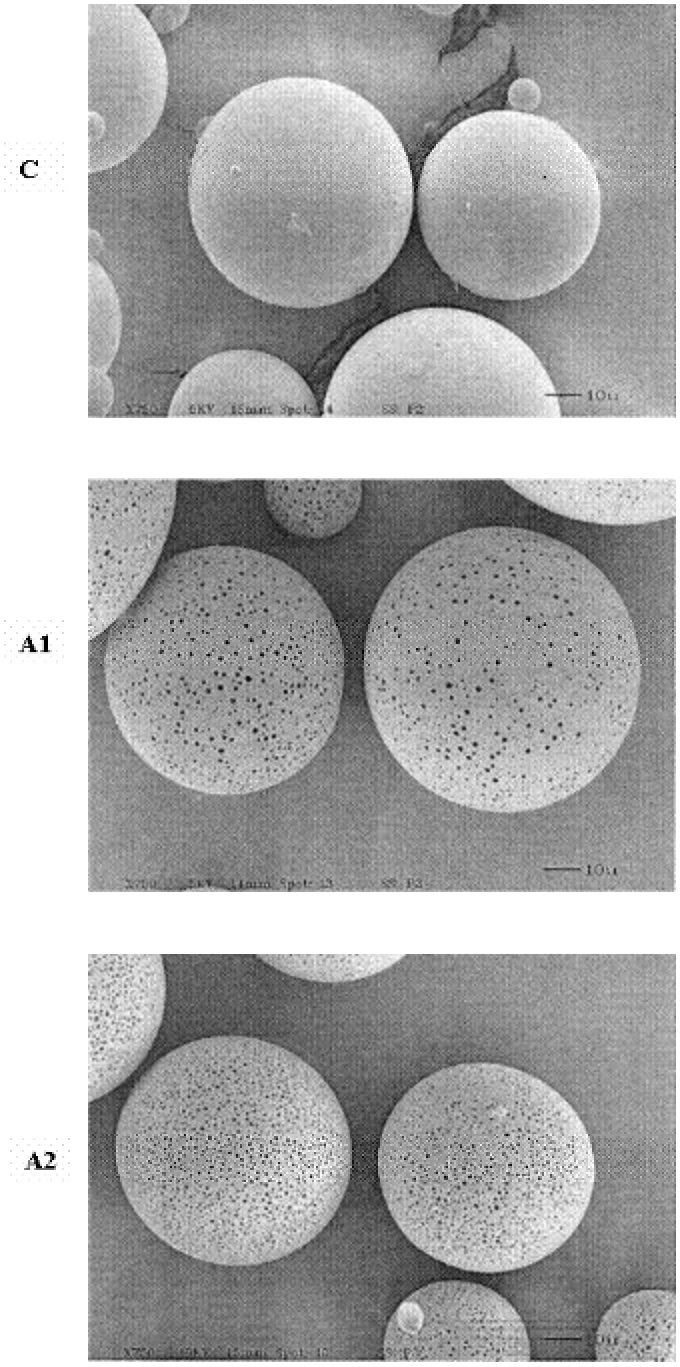


Figure 1. The surface characteristics of BSA-loaded microspheres with different PLGA/PEG ratios: C. Control, A1.2:1 (PLGA:PEG), and A2. 1:1 (PLGA:PEG).

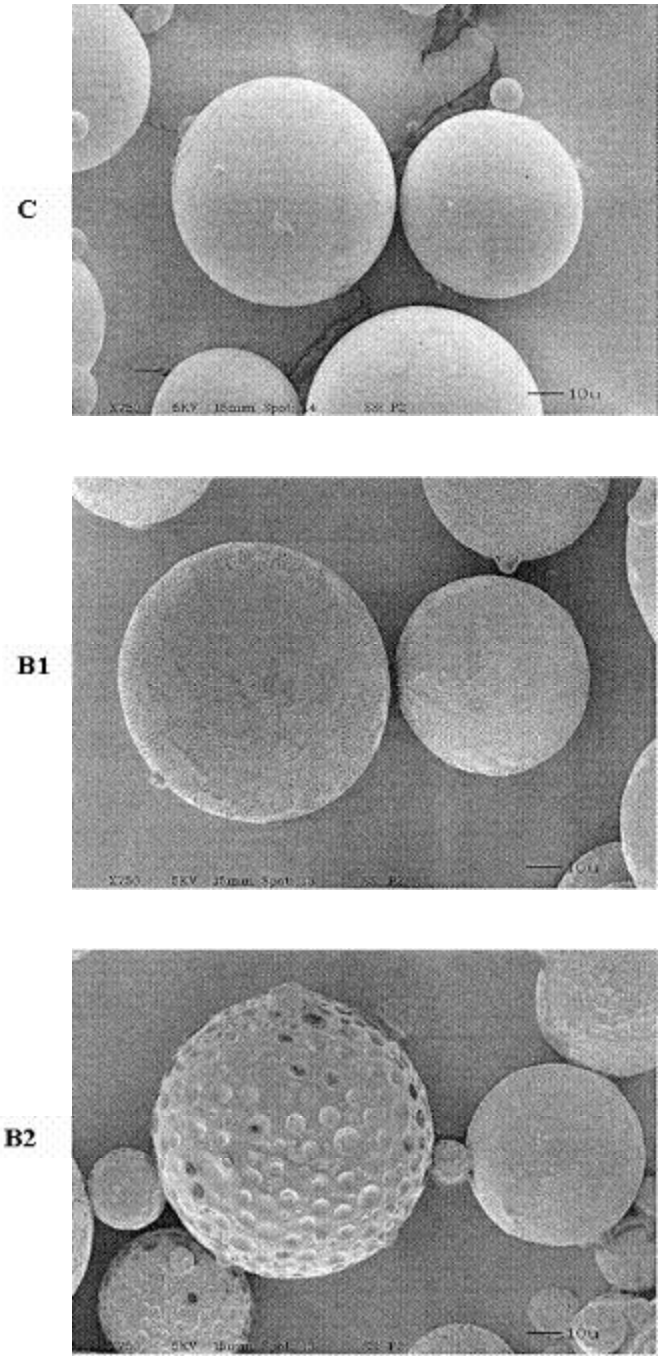
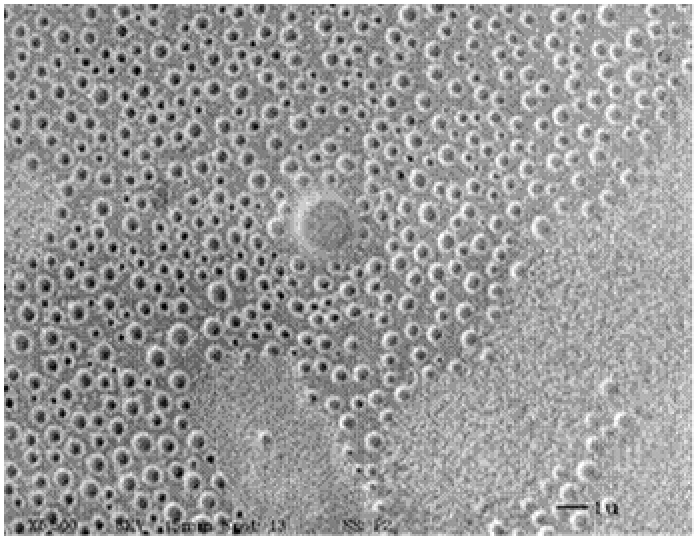
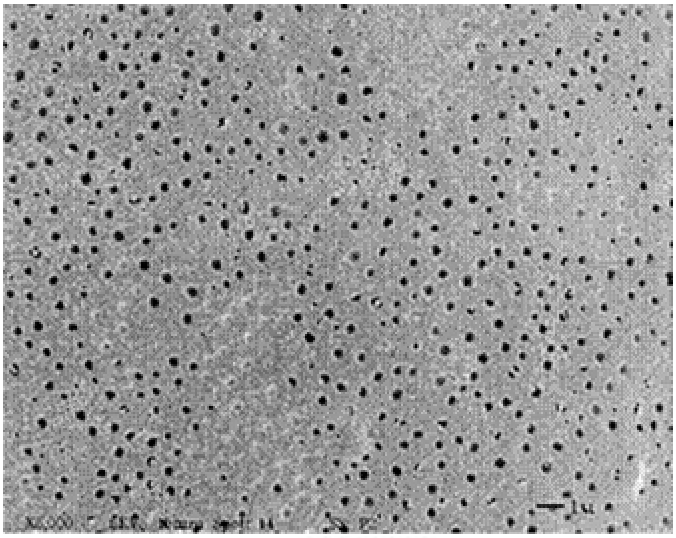


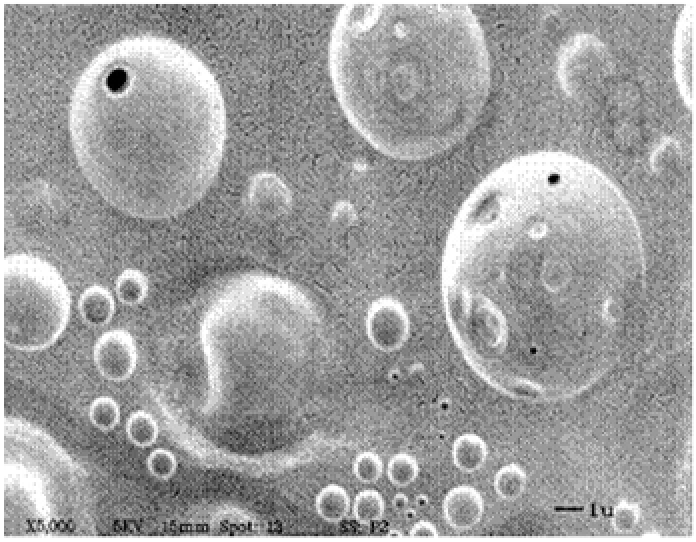
Figure 2. The surface characteristics of BSA-loaded microspheres with different PLGA/tricaprin ratios: C. Control, B1.10:1(PLGA:tricaprin), and B2.2:1(PLGA:tricaprin).



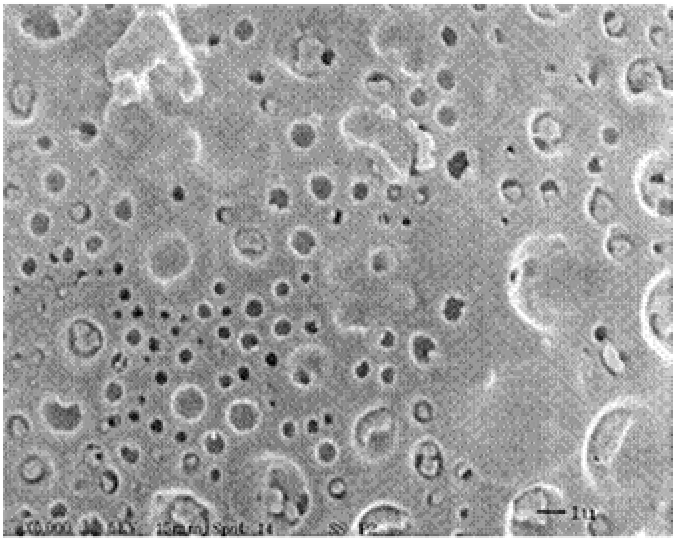
B1 (a)



B1 (b)



B2 (a)



B2 (b)

Figure 3. Surface characteristics of microspheres incorporated with tricaprin before and after 1 day release. B1.10:1(PLGA:tricaprin) A); B2.2:1(PLGA:tricaprin) a: before release, b: after 1 day release.

Typical triphasic release pattern was observed for most formulations: an initial burst phase (days 0-2), an intermediate phase (days 2-45), and a third phase (days 45-65). The protein released during the initial release phase was mainly due to protein desorption and diffusion from the surface and small pores on the surface of microspheres. The intermediate phase is typical for high molecular weight polymer because it requires a relatively long degradation time before sufficient erosion of polymer matrix is achieved for protein release [21] . In this phase, interconnected pores are emptied of protein. Further release of protein

trapped in the polymer matrix is largely restricted because of the very low diffusivity [22] .The third phase of the in vitro release happens because of erosion of matrix. In this investigation, it could be seen that the total amount of incorporated BSA could not be released during 2 months of the in vitro release study. It was suggested that the protein could denature and form insoluble aggregates during in vitro release. This insoluble fraction will account for the incomplete protein release from microspheres, which could not match the degradation rate of PLGA [23] . Studies on denaturation and aggregation of BSA during microsphere preparation are under way in our laboratory.

Figures 4 and **5** show that the release of BSA from the control microspheres is very low. Less than 8% of the protein was released after 7 days. The incorporation of PEG 1000 or tricaprin significantly increased BSA release in the first 2 days in comparison to the control. The early-stage release was increased with an increasing PEG 1000 content in the microspheres (**Figure 4**). However, the release rate during the intermediate and later stages did not increase to a large extent. The greater content of PEG in microspheres released the surface or near-surface BSA to a greater extent because of PEG's solubility in the release medium. However, the later release of BSA from inside the microsphere matrix mostly depended on the degradation of polymer. A modification of the release characteristics by varying the molecular weight of PEG may be possible. Morita et al [24] found that microspheres with 3% loading of PEG 6000 had slightly faster release of protein than microspheres with 3% loading of PEG 70 000. The difference in release rate of the protein was explained on the basis of different dissolution rates of PEGs after hydration in the microenvironment in matrices. It is suggested that the combination of different molecular weight PEGs would be a better way to modify the release of protein from microspheres. In the case of tricaprin, the early-stage release of BSA also increased from microspheres produced with PLGA/tricaprin ratio of 10 (batch B1) in comparison to the control. Further increase in the tricaprin concentration into microspheres (batch B2) led to a decrease in BSA release (**Figure 5**). Tricaprin, a hydrophobic compound, significantly increased the release of a hydrophobic drug, etoposide, from PLGA microspheres [18] . However, highly hydrophilic BSA may encounter difficulties in partitioning and diffusing through the tricaprin-filled channels of microspheres. The above difficulties are increased by increasing the tricaprin content in the microspheres. Therefore, the initial BSA release from microspheres of batch B2 was lower than the release from batch B1.

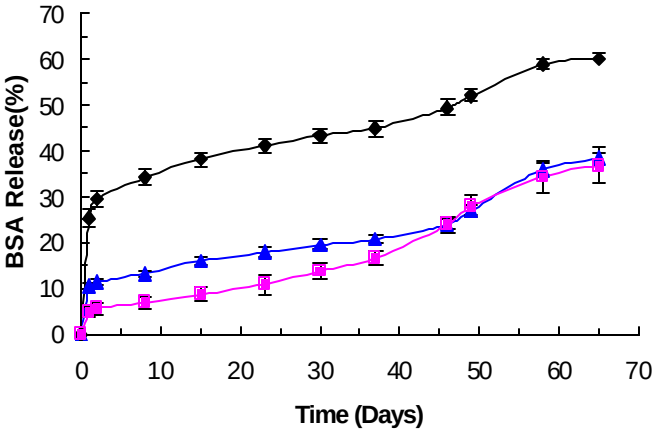


Figure 4. In vitro release profiles of BSA-loaded microspheres prepared with different PLGA/PEG ratios. (Keys: ■ control; ▲ 2:1(PLGA:PEG); and ◆ 1:1(PLGA:PEG)).

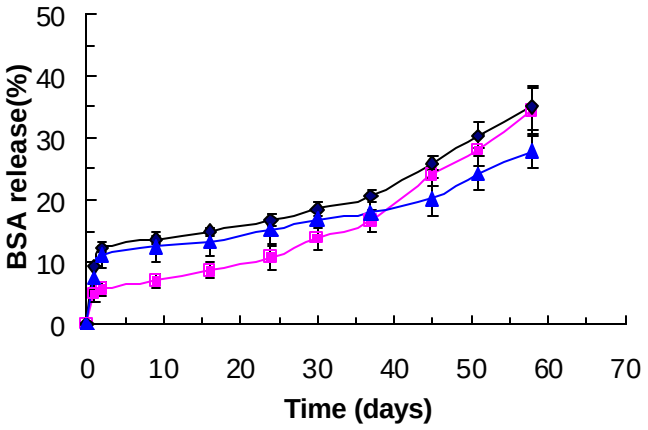


Figure 5. In vitro release profiles of BSA-loaded microspheres prepared with different PLGA/tricaprin ratios. (Keys: ■ control; ▲ 10:1 (PLGA:tricaprin); and ◆ 2:1 (PLGA:tricaprin).).

CONCLUSION

This study shows that the incorporation of additives PEG 1000 or tricaprin significantly ($P < 0.05$) increased the early-stage release of BSA from PLGA microspheres in comparison to the control. The difference in the release profiles between control and additive containing microspheres is closely related to their surface morphology.

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