Investigation on Preparation and Protein Release of Biodegradable Polymer Microspheres as Drug-Delivery System

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ABSTRACT: Among the different approaches to achieve protein delivery, the use of polymers, especially biodegraded, holds great promise. This work aimed to study the preparation and protein release of a novel drug-delivery system based on human serum albumin (HSA) encapsulated into biodegradable polymer microspheres. The microspheres containing HSA were elaborated by the solvent-extraction method based on the formation of multiple *w*/*o*/*w* emulsion. The encapsulation efficiency (E.E.) of HSA was determined by the CBB method. Alginate/alginate and calcium chloride was added into an internal aqueous phase to investigate the protein loading efficiency, protein stability, and *in vitro* release profiles. Microspheres were characterized in terms of their morphology, size distribution, loading efficiency, and *in vitro* protein release. SDS–PAGE results showed that HSA kept its structural integrity during the encapsulation and release procedure. *In vitro* studies indicated that the microspheres with alginate added in the internal aqueous phase had a smaller extent of burst release. In conclusion, the work presents a new approach for macromolecular drugs (such as protein drugs, vaccines, and peptide drugs) delivery. © 2002 John Wiley & Sons, Inc. J Appl Polym Sci 84: 778–784, 2002; DOI 10.1002/app.10327

Key words: biodegradable; peptides; polymerization; drug delivery systems

INTRODUCTION

In recent years, polymers have found increasing applications in the pharmaceutical industry as matrices for drug-delivery systems. With the success of biotechnology and recombinant technology, proteins are being looked upon as future therapeutic agents. Controlled drug-delivery technology using biodegradable polymers as carriers represents one of the most rapidly advancing areas of science. Controlled-release microspheres prepared from biodegradable polymers such as polylactide (PLA) and polylactide-*co*-glycolide (PLGA) have been extensively evaluated with the object of extending the duration of drug or antigen release.[1,2] Compared with the commonly used PLA and PLGA, poly-DL-lactide-poly(ethylene glycol) (PELA) shows much potential in protein-delivery systems.[3] Such delivery systems offer numerous advantages compared to conventional dosage forms including improved efficacy, reduced toxicity, and improved patient compliance and convenience.[4]

But there are some problems for these polymeric drug-delivery systems. One of the problems

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is the structural or conformational change of proteins during the preparation of, storage of, and release from this type of drug-delivery system.[5,6] One reason for the protein structural or conformational change, or integrity loss, may be the harsh preparation or formulation conditions.[6,7] For example, two immiscible liquid phases (such as organic and aqueous phases) are usually involved during formulation and interfaces are created. Protein molecules were usually dispersed in an organic polymer solution using a high-speed homogenizer or a sonicater, yielding a water-in-oil microemulsion.[6] Such a microemulsion possesses an extremely high interfacial area. Proteins, which can be surface active, tend to migrate to the interface between the aqueous phase and the organic phase. The protein molecules at the interfaces may unfold and, consequently, the structure or conformation may change.[3] Another cause of the protein structural or conformational change may be due to the acidic microenvionment created inside the microspheres during polymer degradation and also to the reactivity of the protein with the polymer and/or its degradation products.[8] In these respects, a disadvantage of the classical PLA, PLGA, and even PELA microspheres, in particular, those prepared by the double-emulsion technique, is that the water-soluble drug is highly dispersed in the polymer matrix and, consequently, directly exposed to the above-indicated deleterious factors.

Therefore, the preservation of protein stability during encapsulation and release is essential for the development of a successful controlled release of protein drugs. Taking this into consideration, our strategy has been to develop a system composed of a stabilizing core, which contains the proteins, coated by a biodegradable polymer wall. The rational behind this system was that the protein would be stabilized in the core and would be released following the erosion of the polymer coat.[8]

In this work, we used alginate crosslinked with calcium chloride to prepare protein-loaded microcores. Alginates are anionic polysaccharides derived from brown algae and comprise D-manuronic and L-guluronic acid residues joined linearly by 1,4-glucosidic linkages. It has been reported that purified alginate is nontoxic and biodegradable when taken orally.[9,10] In addition, alginate has been found to have bioadhesive properties and can also be effective in protecting mucous membranes of the gastrointestinal tract.[10] They are widely used in biomedical applications and are capable of being processed under mild conditions. Reports indicate that the biological activity is very much retained in the calcium alginate encapsulating process.[9] Since the process to prepare particulates made from natural, hydrophilic materials involves the use of aqueous solvents, stability-related, toxicological, and environmental problems associated with organic solvents would be minimized.[11] Although alginate particulates can improve the protein loading efficiency and the preservation of protein stability, their degradation rate cannot be efficiently controlled and the protein release rate from alginate particulates cannot be maintained as sustained and gradual. So, we designed a new microsphere-delivery system consisting of alginate complex microcores surrounded by a PELA coat.

Our previous study already confirmed that the microspheres prepared from PELA with a poly(ethylene glycol) (PEG) content of about 10% achieved the highest loading efficiency among PELA copolymers (PEG content: 5–50%) and PLA.[12] The biodegradable and biocompatible nature of PELA (PEG content: 1 0%) makes it a suitable candidate polymer for the development of control-delivery systems of water-soluble drugs, peptides, and vaccines. The hydrophilic domains of PELA copolymers could increase the affinity between the hydrophilic alginate complex microcores and the outer coat in the preparation process. Therefore, we selected the PELA copolymer as a microsphere matrix rather than PLA or PLGA polymers to investigate the preparation and protein release of biodegradable PELA microspheres. It was indicated that the core-coated microspheres have a higher protein efficiency than that of the conventional PELA microspheres. Protein drugs can be slowly released from the core-coated microspheres. The core-coated microspheres can stabilize the protein in the PELA matrix, which is the major advantage of the novel protein-delivery system over the conventional microspheres.

EXPERIMENTAL

Materials

Human serum albumin (HSA) was purchased from the Institute of Blood Transfusion, Chinese Academy of Medical Science. PEG (M_w = 6 kDa; Shanghai, China) and poly(vinyl alcohol) (PVA, 88% hydrolyzed, M_w = 130 kDa; Guangzhou,

China) were purchased from the Guangzhou Chemical Reagents Department. DL-Lactide (85%) was produced by the Chemical Factory of Hubei University (China). A PELA block copolymer containing 10% of PEG was synthesized by ring-opening polymerization as described previously.[13] Sodium alginate (3500 cps for a 2% solution at 25°C) was obtained from the Sigma Co. (St. Louis, MO). All other chemicals and solvents were of reagent grade or better.

Preparation of Microspheres

Preparation of Conventional HSA/PELA Microspheres (MS-0)

Conventional HSA/PELA microspheres were prepared by solvent extraction based on the formation of a modified double emulsion $w_1/o/w_2$ reported earlier.[14] After the complete removal of the organic solvent, the microspheres were collected by centrifugation (Tomy Seiko Co., Japan). The resultant microspheres were rinsed with distilled water and centrifuged three more times, then lyophilized overnight and stored at 4°C in a dessicator.

Preparation of HSA + alginate/PELA Microspheres (MS-1)

The procedure was essentially similar to the one described above. The different step was that the solution of sodium alginate of different concentrations was added in the internal aqueous phase (w_1) containing HSA under high-speed stirring.

Preparation of HSA-loaded Calcium Alginate Microcores and Coated with PELA Polymer (MS-2)

The method to prepare sodium alginate microcores was adapted from Bodmeier and Paeratakul.[15] First, a solution of sodium alginate (1.5%, w/v) was prepared in double-distilled water. HSA was dispersed in the alginate solution using a high-speed stirrer. Then, a solution of calcium chloride of different concentrations was added dropwise using a disposable syringe (21 gauge) under high-speed stirring. The gelation process began almost instantaneously and formed HSA-loaded calcium alginate microcores. The microcores were used as the w_1 phase, and the PELA-coated microspheres (MS-1) were prepared by the $w_1/o/w_2$ solvent-extraction method as described previously.[14]

Characterization of Microspheres

A scanning electron microscope (SEM, Amray, USA) was utilized to observe the surface characteristics and the morphology of the microspheres. The microsphere size and distribution were determined with a laser diffraction particle-size analyzer (Malven, Mastersizer 2000, United Kingdom).

The amount of HSA entrapment was measured by placing 100 mg of the microspheres in 1.5 mL of dichloromethane and extracting the HSA three times with 1.5 mL of double-distilled water. The HSA content of the extraction solution was determined using Bradford's method,[16] compared with a standard curve of data obtained by assaying known concentrations of HSA solutions. The amount of encapsulated (A.E.) HSA in the microspheres, given as a percentage, indicates the amount (mg) of HSA encapsulated per 100 mg of the microspheres. Also, the encapsulation efficiency (E.E.) of the process indicates the percentage of HSA encapsulated with respect to the total amount used for the preparation of the microspheres.

In Vitro HSA Release Test

The *in vitro* HSA release profiles of the MS-0, MS-1, and MS-2 microspheres were determined as follows: Preweighed microspheres were placed in individual test tubes containing 15.0 mL of PBS (154 m*M*, pH 7.4). The tubes were kept in a thermostatted shaking air bath (Hualida Laboratory Equipment Co., China) that was maintained at 37°C and 100 cycles/min. At appropriate intervals, 1.0 mL of the release medium was collected by centrifugation and 1.0 mL of fresh PBS was added back to the test tube. The amount of HSA was measured by the Bradford protein assay as described above.

Polyacrylamide gel electrophoresis (PAGE)

The structural integrity of HSA extracted from microspheres and the release *in vitro* assay was detected by SDS–PAGE, compared with native HSA and reference markers. Protein samples were diluted with Tris buffer (pH 6.8) with 2% SDS. The electrophoresis of the samples was performed at a constant voltage of 200 V in a Tris/glycine/SDS buffer using a Bio-Rad MiNi-Protein II electrophoresis system. After migration, the gel was stained with Coomassie® Bright Blue in methanol–acetic-water (2.5:1:6.4) to reveal the protein and then destained and dried.

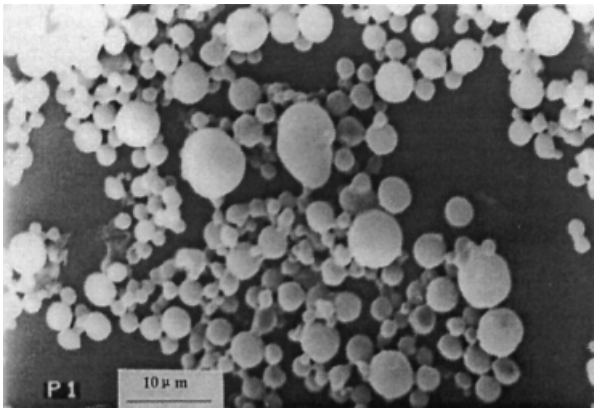

Figure 1 Dispersion pattern and morphology determined by SEM of core-coated PELA microspheres (MS-2) containing HSA.

RESULTS AND DISCUSSION

Characterization of Microspheres

The scanning electronic micrographs of the MS-0, MS-1, and MS-2 microspheres are almost identical as in our previous study.[12] Our early study already confirmed the formation of a new microsphere-delivery system consisting of calcium alginate complex microcores containing HSA surrounded by a PELA coat by DSC analysis and SEM observation.[17] A scanning electronic micrograph of the MS-2 microspheres is shown in Figure 1. They had a smooth spherical surface structure, devoid of pits and pores. The mean microsphere diameter was less than 5 μm, and a typical size distribution is shown in Figure 2. It was reported that small fluorescent particles under 10 μm are advantageous for the uptake by Peyer's patches (PPs), which are known to play a critical role in the oral immunization process and which finally transport into the draining lymph nodes.[18] So, the size and distribution are suitable for microspheres to reach the targeting sites efficiently.

Effect of Sodium Alginate of Internal Water Phase on Protein Loading Efficiency and Particle Size

The microspheres containing HSA were prepared by a double-emulsion *w*/*o*/*w* based on the solvent-evaporation method. An increase in the loading efficiency of the hydrophilic peptide[14] and a considerable increase in the particle size[19] were reported following the addition of a stabilizer to the peptide solution prior to emulsification. In the present work, the addition of sodium alginate into the internal water phase also produced a significant difference in the HSA loading efficiency, and no apparent effect on the particle size was observed (shown in Table I). There was an obvious increase in the loading efficiency initially with an increase in the concentration of sodium alginate in the internal water phase, and the largest loading efficiency was achieved when the concentration of sodium alginate was 3.0% (w/w), and over that concentration, a decrease in the loading efficiency was obtained by increasing the concentration of sodium alginate. It may be that the viscosity of the internal water phase increased by using an increasing concentration of sodium alginate, which supplied a high transfer resistance for protein expelling from the internal aqueous phase to the external aqueous phase during the second emulsification. Thus, an increased loading efficiency was obtained initially. The primary emulsion became unstable with more sodium alginate added into the internal water phase, resulting in a loading efficiency decrease.

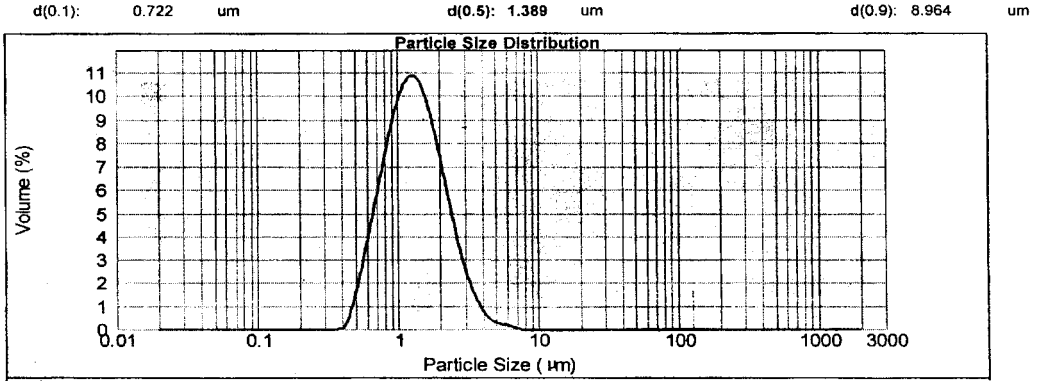

Figure 2 Microsphere size and distribution determined by a laser diffraction particle-size analyzer.

Table I Effect of Sodium Alginate of Internal Water Phase on the Characteristics of Microspheres

Concentration of Sodium Alginate (%, w/w)	Diameter (μm)	Standard Deviation	HSA Entrapment (%)	Loading Efficiency (%)
0	1.30	0.169	0.82	38.3
1.0	1.31	0.201	0.91	42.4
2.0	1.34	0.182	0.96	44.8
3.0	1.33	0.171	1.2	56.7
4.0	1.37	0.231	0.90	40.8

Effect of Sodium Alginate and Calcium Chloride of the Internal Water Phase on Protein Loading Efficiency and Particle Size

The effect of a new system composed of a stabilizing core, which contained the protein, coated by a biodegradable polymer wall on the protein loading efficiency and particle size is shown in Table II. The result almost corresponds to that in Table I. The largest loading efficiency (58.6%) was achieved when the concentration of sodium alginate was 3.0% (w/w) and the concentration of calcium chloride was 1.0% (w/w), and over those concentrations, the loading efficiency decreased with an increased concentration of sodium alginate and calcium chloride. There was an apparent effect on the particle size compared with Table I when the new core-coated system was formed. This may be the formation of calcium alginate microcores resulting in an increase in the particle size.

From Tables I and II, we can draw a conclusion that the effect of the concentration of sodium alginate or/and calcium chloride in the internal water phase on the protein loading efficiency was significant. It was critical to form a stable primary emulsion by selecting an appropriate concentration of sodium alginate or/and calcium chloride.

In Vitro HSA Release Profile from Microspheres

Figure 3 shows the percent release of protein from all samples of the microspheres against the incubation time. The HSA loading efficiency of the MS-0, MS-1, and MS-2 microspheres was 38.3, 56.7, and 58.6%, respectively. The HSA release profiles of all the samples consist of a burst release followed by a gradual release phase. The extent of the HSA burst release of the conventional PELA microspheres (MS-0) at the initial phase is about 27%, which is higher than the 18.9% burst release of HSA from the core-coated PELA microspheres (MS-2). The extent of the HSA burst release of the MS-1 microspheres is the largest (41%) on the first day. The conventional PELA microspheres (MS-0) shows 61.5% HSA release within 30 days, whereas the MS-1 and MS-2 microspheres produce about 59 and 49% HSA release within 30 days, respectively. The result indicated that the HSA burst release could be reduced and the sustained, gradual release profiles could be obtained by the core-coated microsphere system.

Table II Effect of Sodium Alginate and $CaCl_2$ of Internal Water Phase on the Characteristics of Microspheres

Concentration of Sodium Alginate (%, w/w)	Concentration of $CaCl_2$ (%, w/w)	Diameter (μm)	Standard Deviation	HSA Entrapment (%)	Loading Efficiency (%)
0	0	1.30	0.169	0.82	38.3
1.0	0.25	1.34	0.21	0.89	40.5
2.0	0.5	1.36	0.23	0.95	43.8
3.0	1.0	1.39	0.22	1.26	58.6
4.0	1.5	1.81	0.26	0.77	26.8

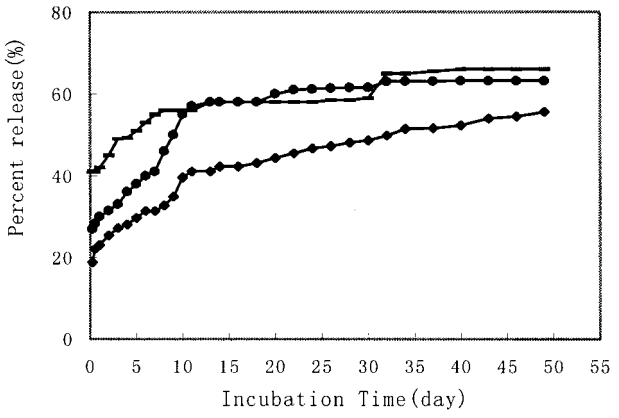

Figure 3 Percent release of HSA from (●) MS-0, (–) MS-1, and (■) MS-2 PELA microspheres containing HSA incubated in PBS at 37°C. Each point represents the mean of three individual samples of microspheres.

The release involved two different mechanisms, that is, diffusion of the protein molecules and degradation of the polymer matrix. The burst release of protein is associated with those protein molecules dispersing close to the microsphere surface, which diffuse out in the initial incubation time. Thus, the small burst effect of these core-coated microspheres is due to the preferential location of protein molecules within the deep sections of the microsphere matrix due to the existence of the alginate complex microcores within the PELA matrix. On the contrary, the slightly big burst effect of conventional PELA microspheres resulted from the preferential location of HSA within the shallow sections of the microsphere matrix. The largest burst effect of the MS-1 microspheres may be due to the existence of the hydrophilic sodium alginate mixed with HSA, which encouraged the HSA protein to diffuse out in the PBS medium. The proteins were gradually released from microspheres matrix, showing some similarities to the diffusion of macromolecules through a hydrogel-like structure after immersion in water.[20]

PAGE Behavior of HSA During Encapsulation and *In Vitro* Release

Figure 4 shows the SDS–PAGE results of HSA extracted and released from the core-coated microspheres (MS-2), which is typical. It can be seen that the samples, HSA before encapsulation and HSA extracted from these core-coated PELA microspheres (MS-2) and released from them during the initial 1, 3, 5, and 10 days, showed no additional peak of high and low molecular HSA. It is suggested that no chemical polymerization, noncovalent aggregation, or molecular hydrolysis occurred during these processes. Previous studies showed that protein drugs are usually denatured to some extent by the direct encapsulation in PLGA microspheres.[21,22] The microencapsulation of BSA within PLGA particles was recently shown to have severe noncovalent aggregation,[23] while the encapsulated protein was severely hydrolyzed within the fast degrading PLGA due to an acidic microenvironment generated from polymer degradation.[24] In our core-coated system, protein was entrapped within alginate complex microcores to avoid its contact with organic solvents directly and to help preserve the structural integrity of the protein. From the above discussion, it can be concluded that the core-coated microspheres can stabilize protein in the PELA matrix, which is the major advantage of the novel protein-delivery system over conventional microspheres.

CONCLUSIONS

The above results show that sodium alginate added into the internal water phase can improve protein loading efficiency but that it only increased the extent of the HSA burst release. A new microsphere-delivery system consisting of calcium alginate complex microcores surrounded by a PELA coat was prepared by a double-emul-

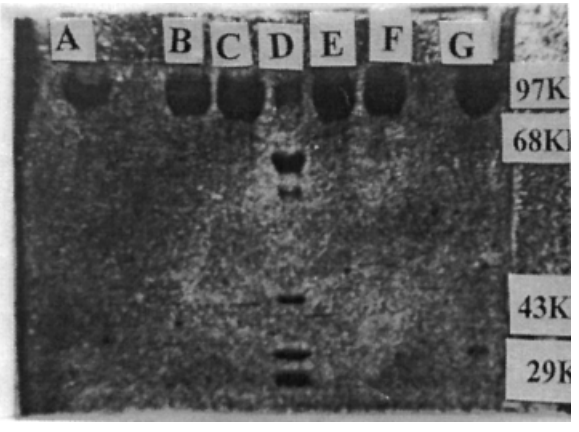

Figure 4 SDS–PAGE results of different HSA samples: (lane C) HSA before encapsulation; (lane B) HSA extracted from MS-2 microspheres; (lane D) molecular weight maker; (lanes A, E, F, G) HSA released from MS-2 microspheres after 1, 3, 5, and 10 days of *in vitro* release assay.

sion *w*/*o*/*w* based on solvent-extraction methods. The system made use of the advantages of a natural polymer and a synthetic polymer. The HSA burst release from these core-coated microspheres is the smallest within the first day. The novel delivery system for hydrophilic drugs, peptides, proteins, and antigens shows some advantages over the conventional system in improving the protein loading efficiency, achieving a more stable *in vitro* release profile, and preserving the structural integrity of protein encapsulated within and released from the microspheres. However, it is clear that a more detailed investigation is necessary to clarify the effect of the matrix polymer on the protein stability and antigen immunogenicity during the microsphere preparation and antigen-releasing procedure, the *in vitro* degradation mechanism, and influence factors and drug release profiles.

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