ELSEVIER

International Journal of Pharmaceutics 219 (2001) 127–137

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

Preparation of gelatin microparticles by co-lyophilization with poly(ethylene glycol): characterization and application to entrapment into biodegradable microspheres

Takahiro Morita *, Yuji Horikiri, Takehiko Suzuki, Hiroyuki Yoshino

DDS Research Department, Discovery Research Laboratory, Tanabe Seiyaku Co., Ltd., 3-16-89 Kashima, Yodogawa-ku, Osaka 532-8505, Japan

Received 12 January 2001; received in revised form 15 February 2001; accepted 27 February 2001

Abstract

Gelatin microparticles were prepared by co-lyophilization with poly(ethylene glycol) (PEG) as a protein micronization adjuvant. Aqueous solutions containing gelatin and PEG at various mixing ratios were freeze-dried. The lyophilizates were dispersed in methylene chloride and subjected to particle size analysis. The particle size decreased as the PEG/gelatin ratio increased. The microparticles isolated from the suspension had spherical microdomains with sizes ranging from 1 to 10 μm, which indicated that phase separation between PEG and gelatin during freezing was involved in the formation mechanism of gelatin microparticles. By using this technology, gelatin microparticles with an average size of less than 10 μm, with high purity of more than 90% and with good dispersibility could be obtained with high yield. The gelatin microparticles with average sizes from 5 to 20 μm were applied to encapsulation into biodegradable PLGA/PLA microspheres via a solid-in-oil-in-water emulsion process. The entrapment efficiency was highly dependent on the particle size and the size distribution, signifying that solid microparticles with an average diameter of less than 5 μm and an maximal diameter of less than 10 μm would be required for effective encapsulation. These gelatin microparticles would be useful for studying and developing various drug delivery systems.

Keywords: Gelatin microparticles; Freeze-drying; Poly(ethylene glycol); Phase separation; Biodegradable microspheres; Solid-in-oil-in-water emulsion

1. Introduction

Over the past few decades, much research on various microparticulate systems as drug carriers has been done in the pharmaceutical field (Chen et al., 1987; Gupta and Hung, 1989). The number of studies using biodegradable and biocompatible materials such as gelatin (Ikada and Tabata, 1998), dextran (Stenekes et al., 1999), and collagen (Rossler et al., 1995), has been increasing. For fabricating microparticulate carriers from these hydrophilic polymer materials, spray drying

* Corresponding author. Tel.: +81-6-63002790; fax: +81-6-63002582.

E-mail address: t-morita@tanabe.co.jp (T. Morita).

0378-5173/01/$ - see front matter
PII: S0378-5173(01)00642-1

(Johnson, 1997; Maa et al., 1999) and a water-in-oil (W/O) emulsion method including the heat stabilization process or the cross-linking process (Gupta and Hung, 1989; Oner and Groves, 1993; Rossler et al., 1995) have been conventionally used. However, many such methods have disadvantages such as low recovery efficiencies, denaturation of materials by heat stress, and cytotoxic cross-linking agents.

Recently, we established a novel method for preparing protein microparticles, through the lyophilization of a poly(ethylene glycol)/protein aqueous mixture (Morita et al., 2000a). In short, during the freezing process, the formation of protein-rich droplets is induced by condensation of the system, resulting in protein microparticles dispersing in a PEG-rich continuous phase after drying. Because PEG is an amphiphilic polymer, the protein microparticles can be easily obtained as a suspension with the use of an appropriate organic solvent. In our previous study, model proteins like bovine serum albumin and superoxide dismutase were successfully micronized into spherical microparticles with diameters of less than 5 μm (Morita et al., 2000a,b). A mechanism based on aqueous phase separation would be applicable to other non-globular proteins, such as gelatin and collagen. For developing a drug carrier suitable for industrial production, this methodology would be useful.

The primary objective of this study was to examine the application of this protein micronization technology to the preparation of gelatin microparticles, using currently available grades of gelatin.

As promising biodegradable materials, poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA) have been used extensively for developing long-term releasing formulations of various therapeutic agents. In fact, leuprolide acetate-loaded microspheres (Luprin®; Ogawa et al., 1988) and human growth hormone-loaded microspheres (ProLease®; Tracy, 1998) are two examples which have been already put into use. Although several methods for preparing PLGA/PLA microspheres containing hydrophilic drugs have been proposed (Ogawa et al., 1988; Hayashi et al., 1994; Couvreur et al., 1997), there have been difficulties in entrapping protein drugs without loss of activity (Morlock et al., 1997; Sturesson and Carlfors, 2000). In this sense, a solid-in-oil-in-water (S/O/W) emulsion method would be better suited than a conventional water-in-oil-in-water (W/O/W) emulsion method for fabricating protein-loaded microspheres with regard to protein integrity (Putney and Burke, 1998). Our recent papers have also described a novel protein encapsulation method by applying the above-mentioned protein micronization technology to the S/O/W emulsion method (Morita et al., 2000b, 2001). However, little is known about the optimal properties of the solid protein particles to be encapsulated.

The second objective of this study was to examine the factors influencing the microencapsulation of solid microparticles into biodegradable PLGA/PLA microspheres by the S/O/W emulsion method. The effect of particle size on the entrapment efficiency and the initial burst release was examined, using various gelatin microparticles.

2. Materials and methods

2.1. Materials

All gelatins used in this study were provided by Nippi Co. (Tokyo, Japan). Type A gelatins with an average molecular weight (MW) of 30 000 (A30) and 7000 (A7), produced by an acid processing, have a high isoelectric point (6.5–8.5). Type B gelatins with a MW of 30 000 (B30) and 7000 (B7), and type D gelatin with a MW of 3000 (D3), produced by an alkaline processing, have a low isoelectric point (4.5–5.5). Bovine serum albumin (BSA) was purchased from Sigma (St Louis, MO). Poly(ethylene glycol) 6000 (PEG6K), poly(DL-lactic acid) with a MW of 20 000 (PLA0020), and poly(DL-lactic-co-glycolic acid) with a lactic to glycolic acid copolymer ratio of 50:50 and a MW of 20 000 (PLGA5020) were obtained from Wako Pure Chemicals (Osaka, Japan). L-PLA with a MW of 110 000 (L-PLA00110) was purchased from Boehringer Ingelheim (Ingelheim, Germany). Micro-BCA protein assay reagent was obtained from Pierce Chemical

(Rockford, IL). All other reagents and solvents used were of reagent grade.

2.2. Preparation of gelatin microparticles

2.2.1. Lyophilization (Step 1)

A series of mixed solutions (1 ml) containing 4 mg of gelatin and an amount (0.2–28 mg) of PEG6K, prepared in glass vials, were frozen onto a pre-cooled shelf of a freeze dryer (RLE-52ES, Kyowa Vacuum, Japan) at −50°C. Freeze-drying was performed by maintaining the shelf temperature at −20°C for 3 h and 20°C for 12 h under a pressure of about 0.02 Torr.

For the preparation of the isolated microparticles of various kinds of gelatins, 25 ml of the aqueous mixture containing 100 mg of gelatin and a prescribed amount (100–500 mg) of PEG6K was lyophilized in the same manner.

2.2.2. Isolation of gelatin microparticles (Step 2)

The obtained lyophilizate was dispersed in an appropriate amount of methylene chloride under sonication. After removing large aggregates with a 150-μm mesh filter, the microparticles were collected with a 0.22-μm membrane filter and redispersed in fresh methylene chloride. This operation was repeated three times to remove the remaining PEG, and the final precipitates were dried under vacuum until they came to have a constant weight.

2.3. Characterization of gelatin microparticles

2.3.1. Particle size analysis

The freeze-dried samples (containing 4 mg of gelatin) or the isolated microparticles (about 5 mg) were dispersed with 1 ml of methylene chloride under sonication, and the resulting suspension was subjected to particle size analysis by a laser diffraction particle size analyzer (SALD-1100, Hitachi, Japan). Ethanol was used as a dispersing solvent.

2.3.2. Quantitative analysis of gelatin microparticles

The purity of the gelatin microparticles was determined by a Micro-BCA protein assay against each raw material.

2.3.3. Scanning electron microscopic study

The morphologies of the isolated gelatin microparticles were observed by scanning electron microscopy (SEM; Model S-2250, Hitachi, Japan).

2.4. Preparation of gelatin-loaded microspheres

Twenty-five milligrams of gelatin microparticles were completely dispersed in 1850 mg of methylene chloride under sonication. Then, prescribed amounts of PLGA, PLA, and L-PLA were added to the suspension to complete an organic phase. The total amount of solid materials was 500 mg. The mixing ratio of the three kinds of polymers (PLGA5020/PLA0020/L-PLA00110) was 40:54:6, which provided a polymer-alloy (phase-separated) structure comprised of an inner PLGA-rich phase and an outer PLA-rich phase in the organic phase (Matsumoto et al., 1997; Morita et al., 2001). The organic phase was added to 4 ml of a methylcellulose solution (0.25% w/v) maintained at 15°C, and emulsified with a polytron homogenizer (Kinematica Ag Littau, Switzerland) for 5 min at 8000 rev./min. The resulting emulsion was quickly poured into 400 ml of distilled water, and then the temperature of this emulsion was raised stepwise up to 30°C for 3 h under vigorous stirring using a propeller mixer at 400 rev./min. The hardened microspheres were collected with a 20-μm mesh filter, washed with an excess amount of distilled water, and finally freeze-dried.

2.5. Determination of gelatin content in microspheres

The total protein content in the gelatin-loaded microspheres was determined as described previously (Morita et al., 2000b). Briefly, microspheres (10 mg) were incubated for 1 h in 750 μl of dimethylsulfoxide, and then for a further 1 h after the addition of 2150 μl of a 0.5% sodiumlaurylsulfate/0.2 N NaOH solution at room temperature to be completely dissolved. After neutralization with hydrogen chloride, the protein concentration in this solution was measured by the micro-BCA protein assay. Results were presented as 'entrapment efficiency' (E%) values, which indicate the

percentage of gelatin entrapped in the microspheres with respect to the total amount of gelatin loaded in the process.

2.6. Determination of initial burst release in vitro

An in vitro burst release was determined as follows. Fifty milligrams of microspheres were incubated at 37°C in 10 ml of phosphate buffered saline (pH 7.4) containing 0.02% sodium azide, by rotating the test tube at 25 rev./min. After 1 h, each test tube was centrifuged at 2000 rev./min for 5 min, and 9 ml of supernatant was withdrawn. The supernatant was filtered through a 0.5-μm membrane filter, and assayed for protein release by the micro-BCA protein assay.

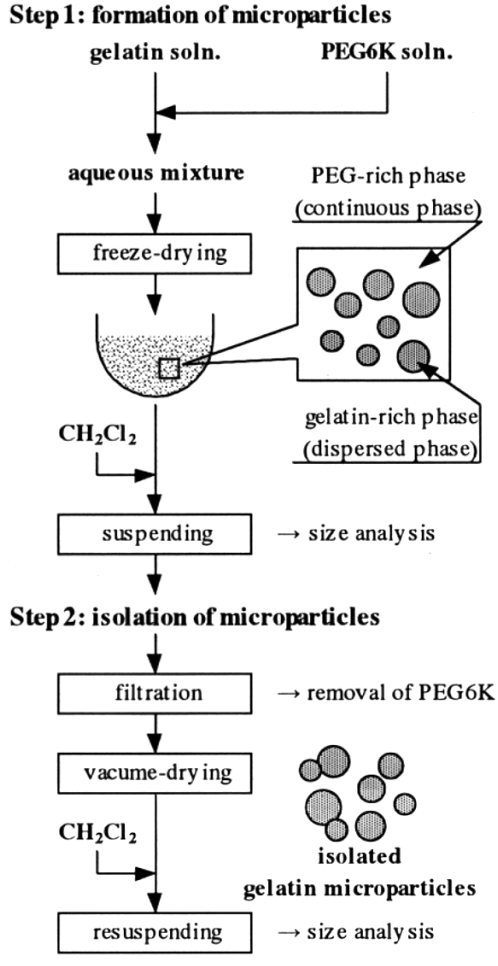
3. Results

3.1. Preparation of gelatin microparticles

Scheme 1 outlines the procedure for preparing gelatin microparticles, which consists of two major steps. The first step involving the formation of gelatin microparticles is the freeze-drying of a gelatin–PEG aqueous mixture. Details of the principle, based on the aqueous phase separation induced by freezing-condensation, were described in our previous paper using BSA (Morita et al., 2000a). Then, gelatin microparticles can be obtained as a suspension by dissolving PEG selectively with methylene chloride. For isolating gelatin microparticles from the suspension, repeated filtration using the same solvent would be sufficient in the second step, which can provide purified gelatin microparticles.

One of the keys to obtain gelatin microparticles is the mixing ratio with PEG. At first, the effect of PEG/gelatin mixing ratio on the size of gelatin microparticles was examined using gelatin A7 and PEG6K. A series of PEG6K/gelatin aqueous mixtures containing fixed amounts of gelatin (4 mg) and amounts of PEG6K from 0.2 to 28 mg were freeze-dried. After the addition of methylene chloride, the resulting suspensions were directly subjected to particle size analysis. Average diameters were plotted against the PEG6K/gelatin mixing ratio, and compared with the result of BSA in Fig. 1. The average sizes of gelatin microparticles decreased with an increase in the PEG6K/gelatin mixing ratio. In particular, microparticles with an average diameter of less than 10 μm could be obtained at a mixing ratio of more than three. However, the sizes of gelatin microparticles were apparently larger than those of BSA microparticles when compared at the same mixing ratio with PEG6K.

Analogous studies were performed using various gelatins with different physicochemical characteristics (A30, B30, B7, and D3). Similar results



Scheme 1. Schematic representation of the procedure for preparing gelatin microparticles.

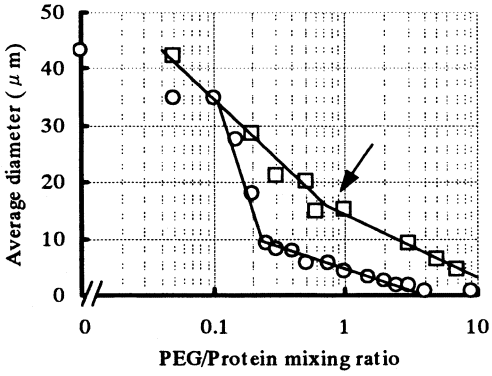


Fig. 1. The relationships between the PEG6K/protein mixing weight ratio and average diameters of protein microparticles. Open square represents the results of gelatin A7. Open circle represents the results of BSA which were quoted from Morita et al. (2000a).

as with gelatin A7 were obtained, in which the average sizes decreased with the increase in the PEG/gelatin mixing ratio (data not shown).

It should be noticeable that an apparent bending point exists around the mixing ratio of 0.25 in the case of BSA in Fig. 1. This point was discussed in our previous paper (Morita et al., 2000a), in relation to the critical point on the phase diagram of the PEG–BSA aqueous mixture system, at which phase transposition of the water-in-water emulsion should occur. Namely, PEG was more likely to form a dispersed phase than BSA below the bending point. In the case of gelatins, a bending point appeared around the mixing ratio of one (Fig. 1, shown by an arrow).

To examine the properties of gelatin microparticles in more detail, 100 mg of gelatin was loaded to prepare isolated microparticles using various grades of gelatins under typical conditions listed in Table 1. Although a recovery, shown in Table 1, varied (43–93%) with the kind of gelatin and the mixing ratio with PEG, several samples of isolated gelatin microparticles could be obtained.

3.2. Characterization of gelatin microparticles

Fig. 2 shows typical SEM images of the isolated gelatin microparticles (a–e) and BSA microparticles (f). Fig. 2(a) represents the product obtained from gelatin B30 at the mixing ratio with PEG6K of one (abbreviated to B30/1), in which large irregular-shaped (string-like) particles containing

Table 1
The characteristics of the gelatin microparticles

Micronization condition			Recovery[d] (%)	Properties of microparticles		
Protein[a]	Ratio[b] (PEG/protein)	Abbr.[c]		Diameter[e] (μm)		Purity[f] (%)
				Average	Range	
Gelatin A30	1	A30/1	43.1	16.5	0.7–43.9	95.3
	3	A30/3	67.2	10.4	0.6–39.6	99.3
Gelatin B30	1	B30/1	55.7	18.6	0.7–44.2	92.8
	3	B30/3	63.1	11.0	0.6–30.7	94.6
Gelatin A7	1	A7/1	68.6	17.2	0.7–44.2	94.4
	5	A7/5	74.5	5.0	0.6–21.7	96.7
Gelatin B7	1	B7/1	88.7	6.2	0.6–26.7	97.6
Gelatin D3	1	D3/1	93.7	5.2	0.6–22.7	84.4
BSA	1	BSA/1	98.9	6.6		97.6

[a] See Section 2.1. For example, 'A7' means a type A gelatin with a MW of 7000.
[b] Mixing ratio with PEG6K.
[c] Abbeviation of the micronized product.
[d] % recovery when 100 mg of gelatin was loaded.
[e] Determined with SALD-1100 after suspending in CH_2Cl_2. The volume-average diameters are shown.
[f] Calculated against each raw material.

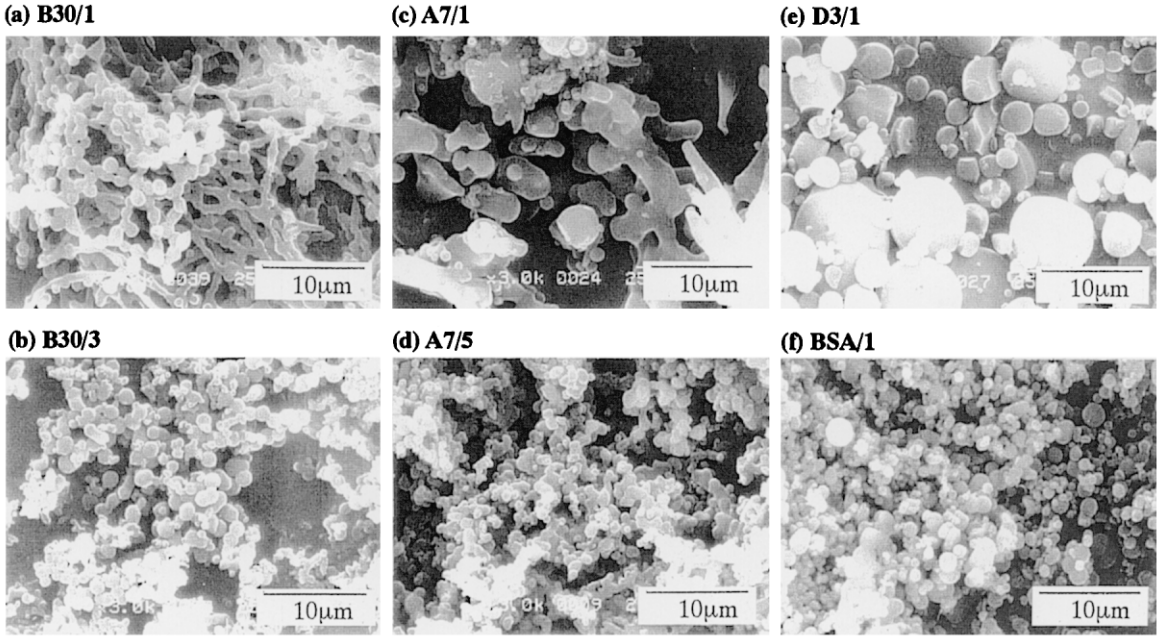


Fig. 2. Typical SEM images of the isolated gelatin microparticles (a–e) and BSA microparticles (f). See Table 1 for the abbreviation of the samples.

many spherical microdomains with an apparent size of about 1 μm were observed. In Fig. 2(b) showing the sample B30/3, well-separated spherical microparticles with a uniform size of about 1 μm were observed as was seen in BSA microparticles (Fig. 2(f)). Similarly, comparing A7/1 (Fig. 2(c)) with A7/5 (Fig. 2(d)), a higher PEG/gelatin mixing ratio provided more separated and smaller microdomains. For another example, in Fig. 2(e) showing the sample D3/1, spherical microparticles with various sizes from 1 to 10 μm were observed.

In Fig. 3(a), the size distribution of typical isolated gelatin microparticles (A7/5) after re-suspending in methylene chloride was compared with that before isolation. Although the size distribution of the isolated microparticles was somewhat broader, with a range from 0.5 to 20 μm, than that of BSA microparticles (Fig. 3(b)), it almost coincided with that before isolation, indicating good dispersibility of the isolated gelatin microparticles.

In Table 1, the characteristics, including an average diameter with size distribution range, purity and recovery, of all the isolated gelatin microparticles are summarized. From this table, we can observe the following. Firstly, microparticles with a smaller average size could be obtained with a higher PEG/gelatin mixing ratio, which was expected from the result in Fig. 1. Secondly, all samples had a wide size distribution, for example, ranging from 0.6 to 40 μm (A30/3). Thirdly, the purity of gelatin microparticles was, on the whole, higher than 90%, indicating that most part of PEG could be removed by the isolation treatment. Finally, high recovery ($\sim$93%) was attained, though it was lower than that of BSA microparticles (98.9%). Concerning all these properties of the gelatin microparticles, a higher PEG/gelatin mixing ratio provided more improved values (smaller size, narrower size distribution, higher recovery, and higher purity).

3.3. Application of gelatin microparticles to encapsulation into PLGA/PLA microspheres

In the previous section, gelatin microparticles with various average sizes (from 5 to 20 μm) were prepared. Using these microparticles, the effect of

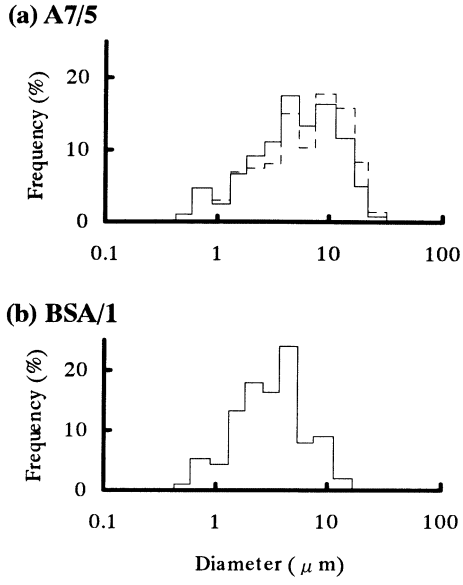


Fig. 3. The size distributions of gelatin microparticles (A7/5) before (a: dotted line) and after (a: solid line) isolation treatment, and BSA microparticles (b).

particle size of gelatin microparticles on the entrapment efficiency into the PLGA/PLA microspheres by the S/O/W emulsion method was examined. Microspheres were prepared by the 'polymer-alloys method' using a mixture of PLGA and PLA (a mixing ratio of 4:6). Fig. 4 shows typical optical microscopic images of the organic phase, in which both microparticles prepared from type A gelatin (Fig. 4(a)) and type B gelatin (Fig. 4(b)) were dominantly localized in the inner PLGA-rich phase. By emulsifying such an organic phase in water, via a S/O/O/W emulsion, reservoir-type microspheres could be obtained. Final sizes of the microspheres were approximately 40 μm, which were independent of the formulation.

In Fig. 5(a), the E% values for gelatin microparticles into the microspheres were plotted against their average diameters. Apparently, the E% values were well correlated to the average particle size of the microparticles, in that smaller particles were entrapped with greater efficiency. Fig. 5(a) also suggested that particles with an average diameter of less than 5 μm would be required for attaining E% of more than 90%.

In Fig. 5(b), the relationship between the initial burst release and average diameters of the gelatin microparticles is shown. The initial burst release for all formulations was small, ranging from 8 to 14%, an advantage of the reservoir-type microspheres. A significant correlation was not observed, but the initial release was slightly reduced by loading smaller particles.

4. Discussion

4.1. Mechanistic consideration of gelatin microparticle formation

Our previous paper introduced a new micronization technology for protein drugs includ-

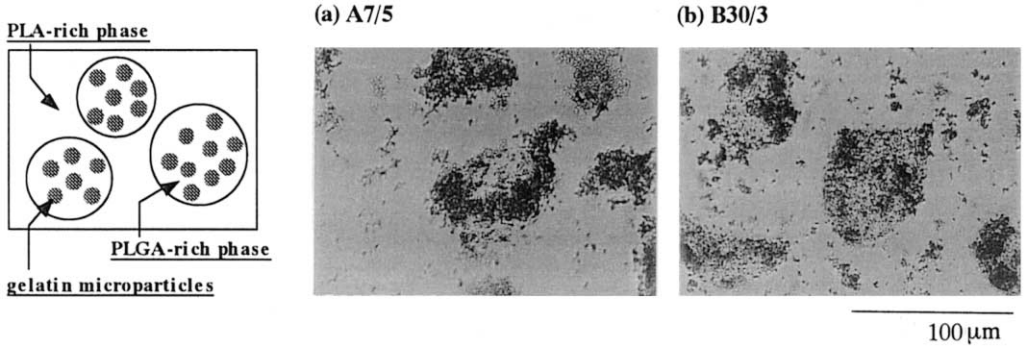


Fig. 4. The localization of gelatin microparticles in the organic phase observed with an optical microscope (DIAPHOT, Nihon Kohgaku Ltd., Japan). The polymer composition of PLGA/PLA was 2:3, which provided an inner PLGA-rich phase and an outer PLA-rich phase, as shown in the left scheme.

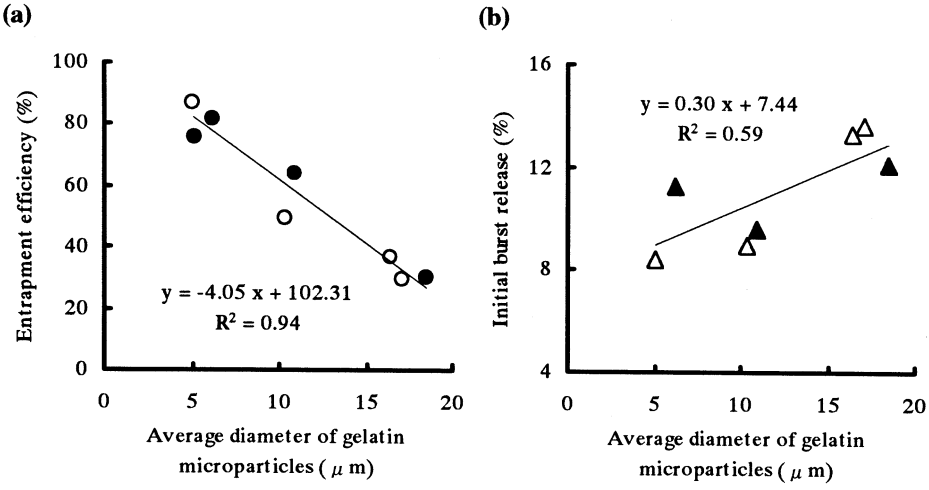


Fig. 5. (a) The relationship between the $E\%$ value and the average size of gelatin microparticles. (b) The relationship between the initial burst release of gelatin from microspheres and the average size of gelatin microparticles. In both figures, open symbols represent the results for type A gelatins, and closed symbols represent the results for type B gelatins.

ing a freeze-drying process for a PEG–protein aqueous mixture (Morita et al., 2000a). The principle of this method is based on the phase separation induced by freezing-condensation, and after the subsequent drying process, spherical microdomains of protein scattered in a continuous solid phase of PEG can be produced, in a particular condition range (Scheme 1, step 1). Because PEG can be dissolved into methylene chloride, a suspension of micronized protein particles can be obtained (Scheme 1, step 2).

In this study, various commercial grades of gelatins were applied to this technology for obtaining gelatin microparticles, using PEG6K as a micronization adjuvant. As shown in Fig. 2, spherical microdomains were formed after co-lyophilization with PEG. These morphologies indicate that the phase separation between gelatin and PEG during freezing is included in the particle formation process. The sizes of obtained gelatin microparticles were considerably influenced by the PEG6K/gelatin mixing ratio (Fig. 1). The significant change in diameter (a bending point) shown in Fig. 1 would indicate 'phase transposition' of a water-in-water emulsion formed during the freezing process. Namely, gelatin dominantly forms a dispersed phase above this bending point, while PEG forms a dispersed phase below this point. Therefore, when the PEG6K/gelatin mixing ratio is near the bending point (around 1), partial phase transposition from PEG-in-gelatin to gelatin-in-PEG would result in the formation of the aggregated particles, as was seen in Fig. 2(a,c).

The effect of characteristics of gelatins on the micronization property must be discussed. Generally, phase separation is caused by the incompatibility between different kinds of polymer (Tjerneld, 1992; Heller et al., 1997; Izutsu and Kojima, 2000). Therefore, it should be appropriate to consider that non-globular (linear) proteins like gelatins are more miscible with PEG which is also a linear polymer, than globular proteins like BSA. Namely, in the phase separated condition, a certain amount of gelatin would exist in the PEG-rich phase, and vice versa. Because a fraction of gelatin existing in the PEG-rich continuous phase might not be micronized, it would be removed as large aggregates during recovery, which may account for the lower recovery efficiencies of gelatin microparticles than BSA microparticles (Table 1). Another important factor influencing the particle formation should be the molecular weight of gelatins. In Table 1, the higher recovery and

smaller size were attained by using the lower molecular weight of gelatins, when compared at the same PEG/gelatin mixing ratio. Although, in general, phase separation will occur at lower concentration by increasing the molecular weight of polymers, the results cannot be simply explained by the molecular weight of gelatins. That is because, in the case of this study involving the lyophilization process, the miscibility of gelatins with PEG, the viscosity of the system, and the degree of condensation during the freezing might complicatedly affect the final sizes (Table 1) and morphologies of the isolated particles (Fig. 2). To clarify this point, further investigation including the phase diagram study would be necessary.

4.2. Possibility as a method for preparing gelatin microparticles

The characteristics of the gelatin microparticles prepared in this study were already explained in Section 3.2 using Table 1. Even though some factors involved in the particle formation, such as freezing rate, remain to be studied, the method is useful for several reasons.

Firstly, the entire process is simple and practical (Fig. 1), using only currently available technology, and can provide products with a higher yield than the conventional spray-drying process (less than 50% according to our recent small-scale experiment). In addition, the procedure itself could be easily changed from a laboratory scale to an industrial scale.

Secondly, the particle size can be easily tailored from the mixing ratio with PEG, signifying the possibility to obtain microparticles with a single-micron size. Although only PEG6K was used in this study, PEGs with different molecular weights would be applicable as a micronization adjuvant.

Thirdly, the purity of the gelatin microparticles is extremely high (Table 1), which would be due to the nature of aqueous phase separation. Although a trace amount of PEG might be left in the products, the method will always provide high quality products without any cytotoxic agent.

4.3. Encapsulation of gelatin microparticles into PLGA/PLA microspheres by S/O/W emulsion method

Putney and Burke (1998) have recently reported the protein encapsulation into PLGA microspheres by the S/O/W emulsion method, which would be better suited in terms of protein integrity than the conventional W/O/W emulsion method. Further, Matsumoto et al. (1997) reported a procedure for entrapping solid microparticles of hydrophilic drugs into reservoir-type microspheres by the polymer-alloys method. This technology is based on the phase separation phenomenon between PLA and PLGA, and the selective localization of solid drug microparticles in the PLGA-rich phase. However, in the application of protein drugs to these methods, much remains to be elucidated regarding the optimal properties of the solid protein particles for attaining high entrapment efficiency.

In this paper, gelatin microparticles with various sizes were used as tools to examine the influencing factor on the entrapment efficiency of solid protein particles into microspheres by the polymer-alloys method. The localization of solid drug microparticles depends on the interrelationship of the solubility parameters of PLGA, PLA and drug (Matsumoto et al., 1997). Most hydrophilic compounds have a reportedly higher affinity to PLGA than PLA. Because both type A gelatin and type B gelatin, in this study, were dominantly localized in the inner PLGA-rich phase (Fig, 4), reservoir-type microspheres could be obtained via a S/O/O/W emulsion process. However, E% values were in the range from 30 to 90%, which apparently depended on the average sizes of the gelatin microparticles (Fig. 5(a)). Additionally, the initial burst releases were slightly reduced by using smaller sizes of microparticles (Fig. 5(b)).

Gelatin microparticles prepared in this study have a broad size distribution as shown in Table 1 and Fig. 3. To elicit the optimal size for effective encapsulation, the size distribution should be taken into consideration. Fig. 6 represents cumulative size distribution profiles of four typical batches of gelatin microparticles ((a) A30/3; (b) B30/1; (c) A7/5, and (d) D3/1). In each profile,

supposing that the smaller fraction of the gelatin microparticles can be entrapped in the microspheres, a critical diameter (CD) was estimated by applying $E\%$ to cumulative percentage on the size distribution curve. The calculated CD values, which might mean the maximal diameter to be entrapped, were around 10 μm (9.8–13.1 μm) in all cases. Therefore, solid microparticles with an average diameter of less than 5 μm (Fig. 5(a)) and an maximal diameter of less than 10 μm (Fig. 6) would be required for effective encapsulation into the reservoir-type microspheres. The CD value of 10 μm would be mainly related to the dispersibility of the particles, signifying that particles larger than 10 μm would be unstable or easily precipitated in the organic phase. Further, the CD value might closely depend on the final size of the reservoir-type microspheres (about 40 μm in this study), which can be tailored by the preparation variables, such as the viscosity of the organic phase, the PLGA/PLA mixing ratio, and the strength of emulsification. Although some factors, such as shape and surface electric charge of gelatin microparticles, might be attributed to the variation of $E\%$ and the degree of initial burst release, these considerations would be useful for designing protein microparticles for microencapsulation by the S/O/W emulsion method.

As another application of gelatin microparticles, they have some potential to work as a stabilizer for the bioactive proteins encapsulated in microspheres. Li et al. (1997) reported that BSA was stabilized in PLGA microspheres by being co-encapsulated with gelatin. Because various protein drugs would be possibly micronized with gelatin in the micronization process, such an application would be plausible.

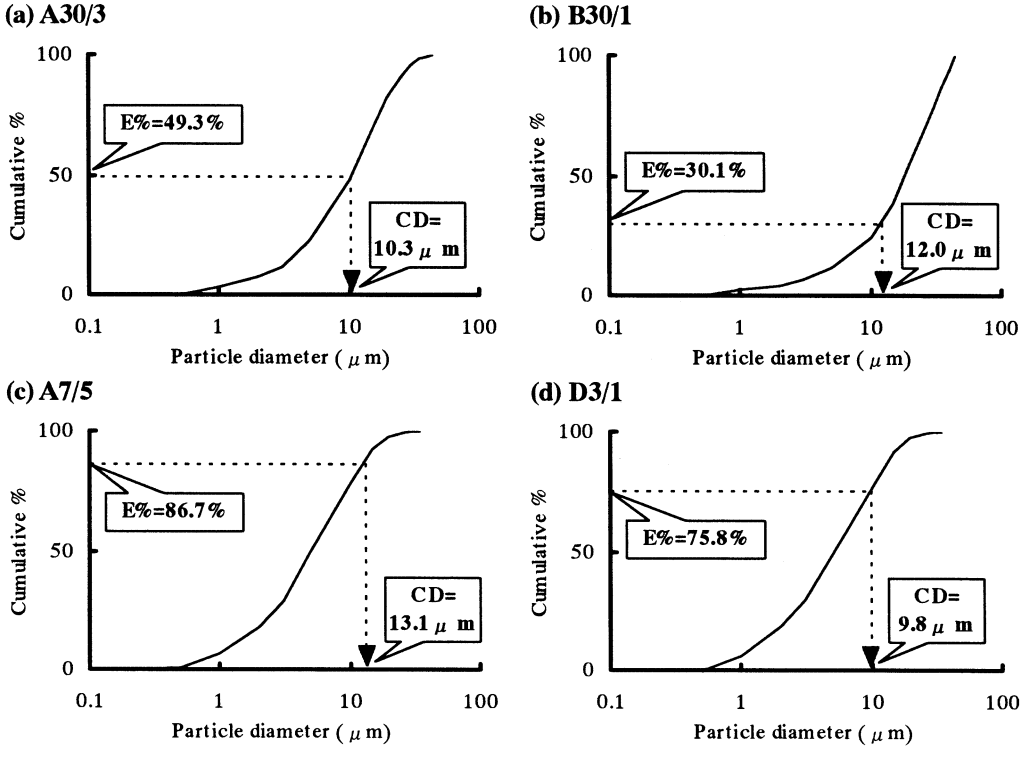


Fig. 6. The relationships between the size distribution of the gelatin microparticles and the entrapment efficiency into reservoir-type microspheres. A critical diameter (CD) was calculated by applying the $E\%$ value to the cumulative% on each size distribution curve, supposing that the smaller fraction of the gelatin microparticles can be entrapped.

5. Conclusion

In the present study, the preparation of gelatin microparticles was investigated by applying co-lyophilization with PEG. Although optimization would be necessary, gelatin microparticles with a single-micron size and high purity were obtained with high recovery efficiency. As demonstrated in the application for microencapsulation, this technology would be useful for studying and developing various drug delivery systems.

References

Chen, Y., Willmott, N., Anderson, J., Florence, A.T., 1987. Comparison of albumin and casein microspheres as a carrier for doxorubicin. J. Pharm. Pharmacol. 39, 978–985.

Couvreur, P., Blanco-Proeto, M.J., Puisieux, F., Roques, B., Fattal, E., 1997. Multiple emulsion technology for the design of microspheres containing peptides and oligopeptides. Adv. Drug Deliv. Rev. 28, 85–96.

Gupta, P.K., Hung, C.T., 1989. Albumin microspheres. II: Applications in drug delivery. J. Microencapsul. 6, 463–472.

Hayashi, Y., Yoshioka, S., Aso, Y., Po, A.L.W., Terao, T., 1994. Entrapment of proteins in poly(L-lactide) microspheres using reversed micells solvent evaporation. Pharm. Res. 11, 337–340.

Heller, M.C., Carpenter, J.F., Randolph, T.W., 1997. Manipulation of lyophilization-induced phase separation: Implications for pharmaceutical proteins. Biotechnol. Prog. 13, 590–596.

Ikada, Y., Tabata, Y., 1998. Protein release from gelatin matrices. Adv. Drug Deliv. Rev. 31, 287–301.

Izutsu, K., Kojima, S., 2000. Freeze-concentration separates proteins and polymer excipients into different amorphous phases. Pharm. Res. 17, 1316–1322.

Johnson, K.A., 1997. Preparation of peptide and protein powders for inhalation. Adv. Drug Deliv. Rev. 26, 3–15.

Li, J.K., Wang, N., Wu, X.S., 1997. A novel biodegradable system based on gelatin nanoparticles and poly(lactic-*co*-glycolic acid) microspheres for protein and peptide drug delivery. J. Pharm. Sci. 86, 891–895.

Maa, Y.-F., Nguyen, P.-A., Sweeney, T., Shire, S.J., Hsu, C.C., 1999. Protein inhalation powders: spray drying vs spray freeze drying. Pharm. Res. 16, 249–254.

Matsumoto, A., Matsukawa, Y., Suzuki, T., Yoshino, H., Kobayashi, M., 1997. The polymer-alloys method as a new preparation method of biodegradable microspheres: Principle and application to cisplatin-loaded microspheres. J. Controlled Release 48, 19–27.

Morita, T., Horikiri, Y., Yamahara, H., Suzuki, T., Yoshino, H., 2000a. Formation and isolation of spherical fine protein microparticles through lyophilization of protein-poly(ethylene glycol) aqueous mixture. Pharm. Res. 17, 1367–1373.

Morita, T., Sakamura, Y., Horikiri, Y., Suzuki, T., Yoshino, H., 2000b. Protein encapsulation into biodegradable microspheres by a novel S/O/W emulsion method using poly(ethylene glycol) as a protein micronization adjuvant. J. Controlled Release 69, 435–444.

Morita, T., Horikiri, Y., Suzuki, T., Yoshino, H., 2001. Applicability of various amphiphilic polymers to the modification of protein release kinetics from biodegradable reservoir-type microspheres. Eur. J. Pharm. Biopharm. 51, 45–53.

Morlock, M., Koll, H., Winter, G., Kissel, T., 1997. Microencapsulation of rh-erythropoietin, using biodegradable poly(D,L-lactide-co-glycolide): protein stability and the effects of stabilizing excipients. Eur. J. Pharm. Biopharm. 43, 29–36.

Ogawa, Y., Yamamoto, M., Okada, H., Yashiki, T., Shimamoto, T., 1988. A new technique to efficiently entrap leuprolide acetate into microcapsules of polylactic acid or copoly(lactic/glycolic) acid. Chem. Pharm. Bull. 5, 1095–1103.

Oner, L., Groves, M.J., 1993. Preparation of small gelatin and albumin microparticles by a carbon dioxide atomization process. Pharm. Res. 10, 1385–1388.

Putney, S.D., Burke, P.A., 1998. Improving protein therapeutics with sustained-release formulations. Nat. Biotechnol. 16, 153–157.

Rossler, B., Kreuter, J., Scherer, D., 1995. Collagen microparticles: preparation and properties. J. Microencapsul. 12, 49–57.

Stenekes, R.J.H., Franssen, O., van Bommel, E.M.G., Crommelin, D.J.A., Hennink, W.E., 1999. The use of aqueous PEG/Dextran phase separation for the preparation of dextran microspheres. Int. J. Pharm. 183, 29–32.

Sturesson, C., Carlfors, J., 2000. Incorporation of protein in PLG-microspheres with retention of bioactivity. J. Controlled Release 67, 171–178.

Tjerneld, F., 1992. Aqueous two-phase partitioning on an industrial scale. In: Harris, M. (Ed.), Poly(Ethylene Glycol) Chemistry: Biotechnical and Biomedical Applications. Plenum, New York, pp. 85–102.

Tracy, M.A., 1998. Development and scale-up of a microsphere protein delivery system. Biotechnol. Prog. 14, 108–115.