

Biodegradable submicron carriers for peptide drugs: Preparation of DL-lactide/glycolide copolymer (PLGA) nanospheres with nafarelin acetate by a novel emulsion-phase separation method in an oil system

T. Niwa, H. Takeuchi, T. Hino, M. Nohara, Y. Kawashima *

Department of Pharmaceutical Engineering, Gifu Pharmaceutical University, Mitahora-higashi, Gifu 502, Japan

Received 13 June 1994; revised 23 August 1994; accepted 8 December 1994

Abstract

PLGA nanospheres, biodegradable polymeric carriers for peptide drugs, were prepared by a novel emulsion-phase separation method. The preparation was carried out in an oil phase system in order to improve the entrapment efficiency of water-soluble peptide. An LH-RH analogue (nafarelin acetate (NA)) was employed as a model peptide drug to investigate the encapsulation efficiency. An aqueous solution of the drug was emulsified by addition with stirring to a dichloromethane-acetone mixture containing dissolved PLGA. The gradual addition of Triester oil (caprylate and caprate triglyceride) into the resultant w/o emulsion induced phase separation of PLGA at the interface of aqueous droplets. It was found that the aqueous droplets effectively worked as a coacervation-inducing agent of the polymer. PLGA coacervates precipitated around the aqueous emulsion droplets containing the peptide which were hardened by evaporation of the solvent, producing spherical drug carriers. The presence of surfactant significantly reduced the size of the aqueous droplets, resulting in submicron-sized PLGA spheres (mean diameter, 500–800 nm). The recovery of drug entrapped in the nanospheres was markedly increased compared with our previous preparation technique in a water system. Further, optimum conditions in the present method for preparing nanospheres were established to enhance the recovery of nanospheres and the efficiency of drug entrapment.

Keywords: Emulsion; Phase separation; Nanosphere; DL-Lactide/glycolide copolymer (PLGA); Luteinizing hormone-releasing hormone analog; Coacervate; Biodegradation

1. Introduction

In recent years, major advances have been made in genetic engineering and culturing technology for producing many physiological potential

polypeptides as possible therapeutic agents. The use of these pharmacologically active polypeptides in diseases is fraught with problems. Since these molecules have a very short biological half-life and low permeability across through a biomucous membrane, frequent injections (i.e., subcutaneous, intramuscular or intravenous) of drug over a long therapeutic period are generally required

* Corresponding author.

to produce an effective therapy. Therefore, many prolonged-release devices composed of biodegradable and biocompatible polymers have been investigated in an attempt to overcome this practical disadvantage. Depot formulations such as injectable microcapsules with DL-lactide/glycolide copolymer (PLGA) (Sanders et al., 1984, 1985; Ogawa et al., 1988; Yamakawa et al., 1992) and implantable small cylinders with PLGA (Hutchinson and Furr, 1990) have already been developed to extend the therapeutic effect of peptide drugs. However, their sizes were too large to direct the drug to target tissues across the mucosal membrane or via systemic circulation.

Therefore, the present authors have investigated the preparation of submicron-sized particles (i.e., nanospheres), which would be valuable for application in the non-parenteral administration of polypeptide drugs, including the oral and transmucosal (nasal, pulmonary) routes (Niwa et al., 1993). We developed a novel spontaneous emulsification solvent diffusion method in the aqueous phase to prepare biodegradable PLGA nanospheres with peptide easily and reproducibly in a previous report (Niwa et al., 1994).

However, a major problem with this technique is the poor entrapment of water-soluble drugs, which tend to leak out of the internal organic phase into the continuous aqueous phase during preparation. Most bioactive polypeptide drugs are water-soluble. The aim of this study was to improve the entrapment efficiency of peptide drugs in PLGA nanospheres. To achieve this purpose, non-toxic oil was employed as an outer phase to prevent the leakage of drug out of the nanospheres. Polylactic acid (PLA) and PLGA microspheres containing hydrophilic drugs have already been prepared by the methods of phase separation (Ruiz et al., 1989), oil-in-oil (o/o) solvent evaporation (Wada et al., 1990; Stureson et al., 1993) and solvent extraction (partition) (Leelarasamee et al., 1988; Sato et al., 1988). However, no information is currently available on the preparation of submicron-sized drug carriers in an oil phase system due to the aggregation of the carriers. We investigated a new preparation method in an oil phase system applicable to hydrophilic peptide drugs.

Nafarelin acetate (NA), a luteinizing hormone-releasing hormone (LH-RH) analogue, was loaded in the devices as a model peptide drug. The mechanism of formation of nanospheres was clarified by observing the preparation procedure and by characterizing the physicochemical properties of the resultant nanospheres, such as particle diameter, surface topography and drug content. Furthermore, the optimum preparation conditions were established by selecting the appropriate types and suitable amounts of organic solvent and emulsifier.

2. Materials and methods

2.1. Materials

PLGA with an average molecular weight of 54116, whose copolymer ratio of DL-lactide to glycolide is 85:15 (henceforth referred to as PLGA(85:15)-54116) (Medisorb, Du Pont Co., USA) was used as biodegradable polymer. Nafarelin acetate (NA) ($[D-Nal(2)^6]$ LHRH) was supplied by Syntex Research (USA). **Caprylate and caprate triglyceride (Triester F-810**, Nikko Chemicals Co., Ltd, Japan) was selected as a non-toxic oil, which had higher biocompatibility and low viscosity. **Hexaglycerin condensed ricinoleate (HGCR)** (Hexaglyn PR-15, Nikko Chemicals Co., Ltd), sorbitan monooleate (Span 80, Kishida Chemicals Co., Ltd, Japan) and polyoxyethylene hydrogenated castor oil 10 (HCO 10, Nikko Chemicals Co., Ltd) were used as an emulsifier, which were acceptable for oral administration.

2.2. Preparation of PLGA nanospheres

The concept of the preparation method is based on the 'polymer phase separation technique' (Ruiz et al., 1989), in which the drug crystals, i.e., core material, are generally suspended in the polymer solution. However, it is practically impossible to comminute the crystals to nanometer-sized particles for dispersing in the system. In the present study, the drug was dissolved in the aqueous phase emulsified in an organic solution of the polymer. NA (3 mg) was

dissolved in 0.2 μm filtered water (0.5 ml). This aqueous solution was emulsified in a dichloromethane (15 ml)-acetone (0.5 ml) mixture containing dissolved PLGA(85:15)-54 116 (100 mg) and Span 80 (300 mg) under stirring at 15 000 rpm using a homogenizer (Physoctron, Nichion Irikakikai, Japan). The gradual addition (2 ml/min) of Triester oil dissolved 2% w/w of HGCR (30 ml) into the resultant water-in-oil (w/o) emulsion, inducing the phase separation of PLGA under stirring with a magnetic stirrer. The system was thermally maintained at 10°C for 10 min and then heated to 35°C for 50 min. During evaporation of dichloromethane under reduced pressure (for 2 h), the coacervated droplets were solidified into nanospheres in the oily solution. The whole dispersed system was filtered through a 400 mesh sieve (opening, 37 μm) and a poly(tetrafluoroethylene) membrane filter (pore size, 1.0 μm , PTFE; T100A047A, Advantec Toyo, Japan) to separate the aggregates and oil. The nanospheres remaining on the membrane filter were washed with *n*-hexane and water to remove the oil and unencapsulated free NA crystals, respectively, and dried. The schematic procedure for preparation is shown in Fig. 1.

2.3. Construction of phase diagram for preparation of PLGA nanospheres

0.5 ml of water dissolving dye (amaranth) was emulsified in the dichloromethane solution (15 ml) of PLGA(85:15)-54 116 at concentrations of 6.7, 20, 40, 50, 200, and 250 mg/ml. To test the preparation of PLGA coacervates, 1 ml of Triester oil containing HGCR 2% w/w was progressively dropped into the resultant w/o emulsion under stirring. Then, an aliquot withdrawn from the system was observed under an optical microscope (BH-2, Olympus, Japan). The phase diagram was constructed in order to determine the phase separation region yielding stable coacervates. A similar procedure was run in the absence of water to ascertain the role of water in phase separation.

2.4. Determination of optimum conditions for preparing nanospheres

The experimental parameters were varied as follows in order to establish the optimum conditions for the preparation of nanospheres to improve recovery and drug content:

- (1) Volume of water: 0, 0.03, 0.1, 0.5, 1, 2 and 5 ml.

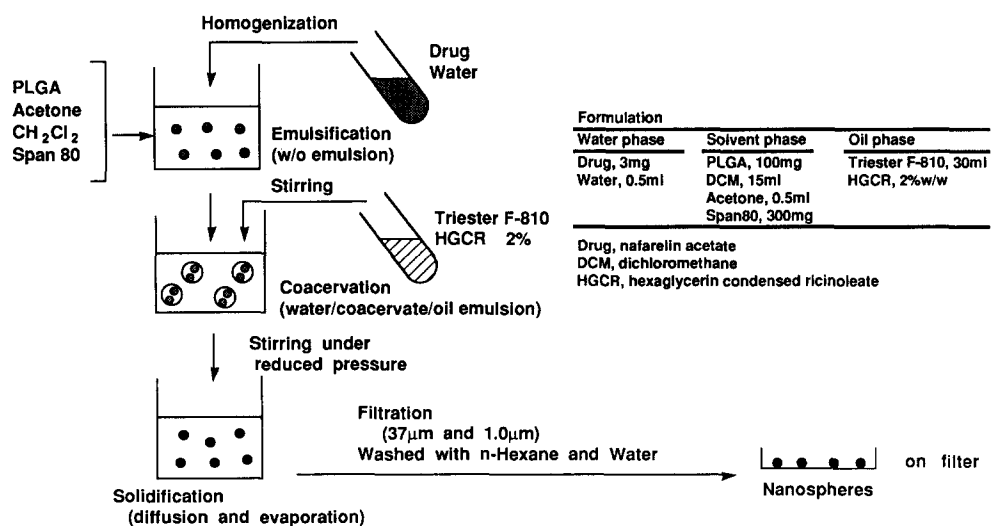


Fig. 1. Schematic procedure for the preparation of PLGA nanospheres in Triester oil system.

- (2) Volume of acetone: 0, 0.5, 1, 2, 5 ml.
- (3) Concentration of Span 80 in dichloromethane: 0, 0.33, 1 and 2% w/v.
- (4) Concentration of HGCR in Triester oil: 0, 1, 2, 4 and 5% w/w.
- (5) Concentration of PLGA in dichloromethane: 100 mg of PLGA(85:15)-54116 was dissolved in 5, 10, 15, 20 and 30 ml of dichloromethane.
- (6) Pouring speed of Triester oil into the w/o emulsion system: 30 ml of Triester oil was

added rapidly into the preparation system or over periods of 4.5, 10, 15 and 30 min.

2.5. Measurement of physicochemical properties of nanospheres

The mean diameter of nanospheres recovered on the membrane filter (1.0 μm) was measured by means of a dynamic light scattering method (LPA-300, Otsuka Electronics Co., Ltd, Japan)

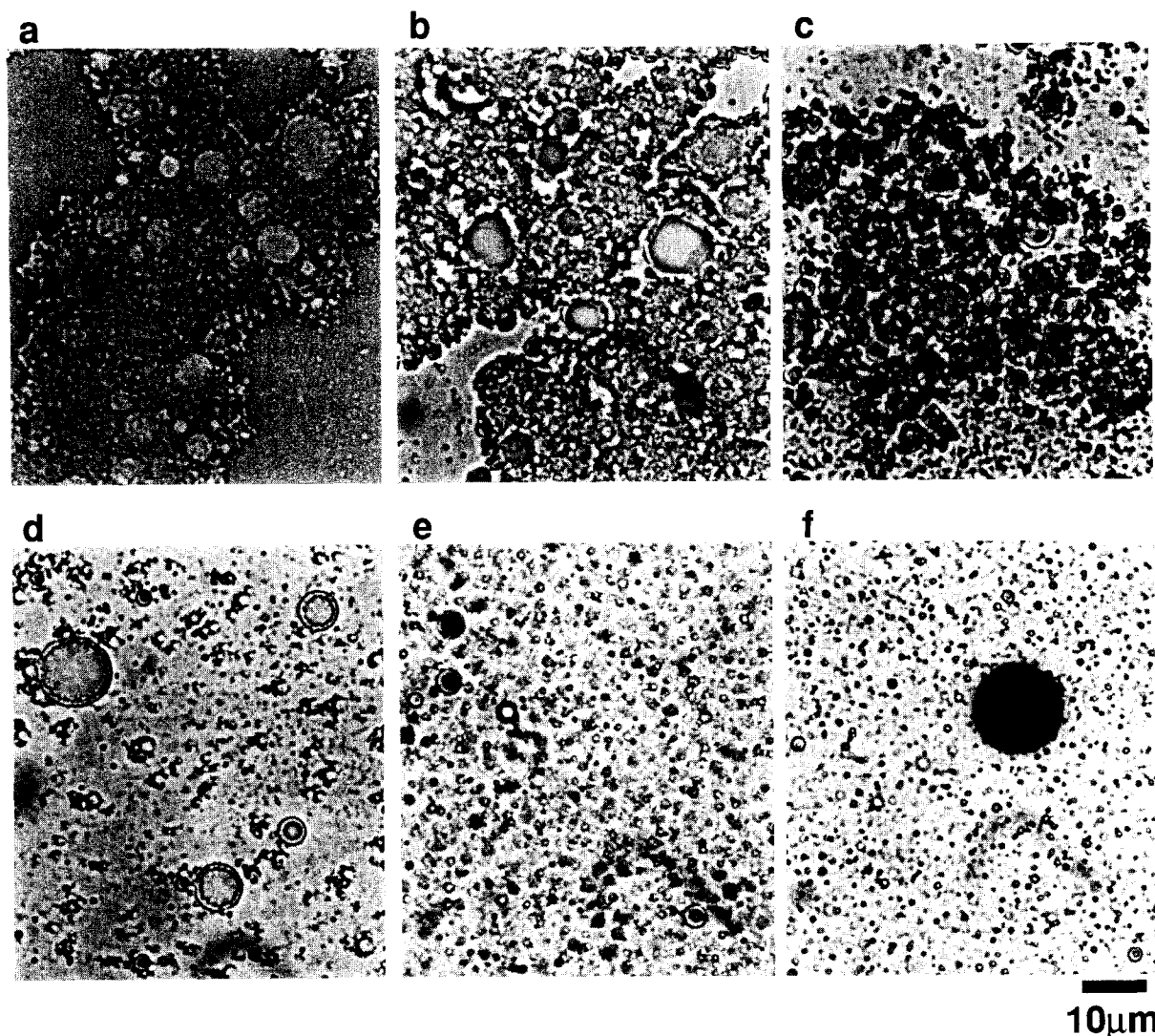


Fig. 2. Optical microphotographs following the process of preparation of PLGA nanospheres with addition of Triester oil. Volume of Triester oil: (a) 0 ml (w/o emulsion), (b) 2 ml, (c) 4 ml, (d) 6 ml, (e) 16 ml, and (f) 30 ml. PLGA: PLGA(85:15)-54116.

after redispersing in water. The aqueous droplet size of w/o emulsion and the size of resultant nanosphere dispersed in the oily suspension were analyzed according to a laser-based time of transition method (cis-1, Galai, Israel). The nanospheres dried under cooling at 5°C were observed under a scanning electron microscope (JSM-T330A, Nihon Denshi Co., Ltd, Japan). The recovery (%) of nanospheres was represented by the ratio of the weight of the resultant spheres passing through a 400 mesh sieve and recovered on a filter (1.0 μm pore size) to the total weights of PLGA and NA loaded. The amount of aggregated particles remaining on the sieve (37 μm opening) was excluded from the recovery values. The weighed nanospheres after drying in a desiccator were dissolved in acetonitrile, to which an aqueous solution of KH₂PO₄ (30 mM) was added to precipitate preferentially the polymer. The drug in the clear supernatant after centrifugation (10000 rpm, 15 min, Kubota 7800, Kubota Co., Ltd, Japan) was analyzed spectrophotometrically at 225 nm by means of an HPLC technique (pump, 880-PU; detector, 875-UV; Jasco, Japan; column, Wakosil 5C8; Wako Pure Chemical Ind., Japan) according to our report (Niwa et al., 1994). The drug recovery and content in the nanospheres are represented by Eq. 1 and 2, respectively.

drug recovery (%)

$$= \frac{\text{amount of drug in nanospheres}}{\text{amount of drug loaded in the system}} \quad (1)$$

drug content (%)

$$= \frac{\text{amount of drug in nanospheres}}{\text{amount of nanospheres recovered}} \quad (2)$$

The theoretical drug content is 2.9%, as calculated from the amounts of drug and polymer loaded.

3. Results and discussion

3.1. Emulsion-phase separation method in an oil system

Fig. 2 illustrates the sequence of events occurring in the PLGA solution following the progressive addition of Triester oil. Firstly, a w/o emulsion was formed when the aqueous solution of dye was poured into the organic phase (Fig. 2a). When the Triester oil, which does not act as a solvent for PLGA, was gradually introduced into this emulsion medium, the phase separation of polymer started around the fine aqueous droplets after pouring 2–4 ml of oil (Fig. 2b,c). Discrete aqueous droplets covered with coacervates were clearly observed in the system when 6 ml of oil added (Fig. 2d). Further addition of Triester oil caused a further increase in volume of coacervates and hardened their droplets without any unfavorable aggregation (Fig. 2e,f). After the evaporation of dichloromethane under heating and reduced pressure, the PLGA coacervates

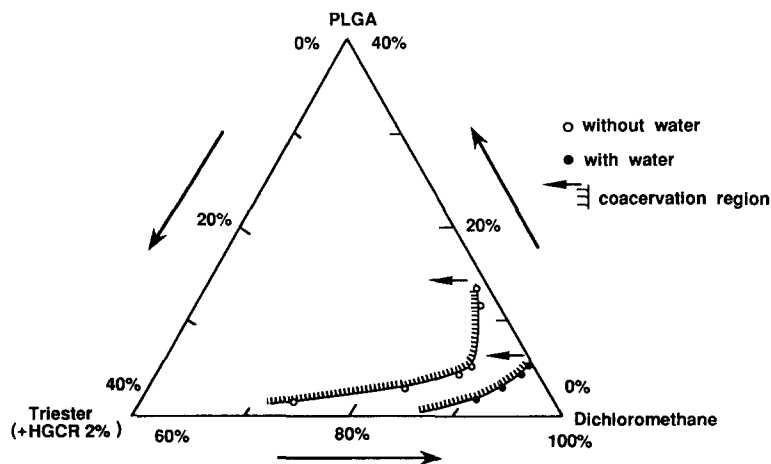


Fig. 3. Triangular phase separation diagram for the formation of PLGA coacervate droplets. PLGA: PLGA(85:15)-54 116.

preferentially precipitated around the emulsion droplets containing the dye and were transformed to red-colored spherical particles.

The phase separation region of the polymer was found as shown in the triangular diagram in Fig. 3 expressing the respective weight proportions of PLGA(85:15)-54116, Triester oil and dichloromethane. Phase separation occurs in the left zone of the boundary curves in this diagram. It was found that the region generating the coacervate droplets obviously expanded with the presence of water in the system, indicating that the aqueous droplets worked as a coacervation-inducing agent of PLGA. Furthermore, no aggregation of coacervate droplets was observed on pouring of excess Triester oil and a stable dispersion of PLGA coacervates could be obtained in the entire region on the left side of the separation curve. Span 80 and HGCR were assumed to work as a protective colloid to prevent effectively the coacervates from aggregation, as discussed later.

3.2. Submicronization of PLGA spherical particles

In order to explain clearly the coacervation-inducing effect of water, the influence of the volume of water loaded in the system on the particle size of PLGA nanospheres was investigated (Fig. 4A). The mean diameter of nanospheres was almost the same independent of the volume of water in the range of 0.1–5 ml. In contrast, the

absence of water or the presence of a very small volume of water (0.03 ml, which was completely miscible with 15 ml of dichloromethane) in the system resulted in a significant increase in the size of PLGA spheres. In these systems, a w/o emulsion was not formed. Therefore, phase separation occurred randomly in the system and the coacervates aggregated to form large droplets of coacervate, leading to the formation of microspheres. This result suggested that preferential coacervation occurred around the aqueous droplets, i.e., the interface between water and oil rather than the water molecule dissolved.

On the other hand, the content of NA in the nanospheres diminished with increase in the volume of water (Fig. 4B). The amount of coacervate droplets of PLGA became insufficient to entrap the larger volume of aqueous droplets, which resulted in thinning of the coacervate film and leakage of the aqueous solution of NA from nanospheres. However, when NA was loaded as a suspension in the organic phase (i.e., volume of water = 0 ml), the encapsulation efficiency was inferior (drug recovery = 8.7%) to that in the presence of water, indicating that water was also effective for improving the entrapment of NA in nanospheres. Furthermore, water could potentially prevent the labile peptide from denaturation in direct contact with the organic solvents.

The addition of acetone in the organic solution of PLGA led to an increase in the drug content

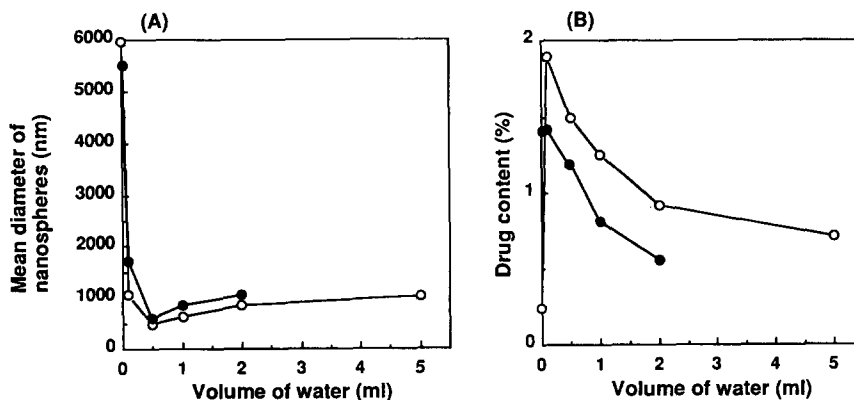


Fig. 4. Effects of volume of water on the mean diameter (A) and drug content (B) of PLGA nanospheres. (○) With acetone (0.5 ml); (●) without acetone in the polymer solution. PLGA: PLGA(85:15)-54116.

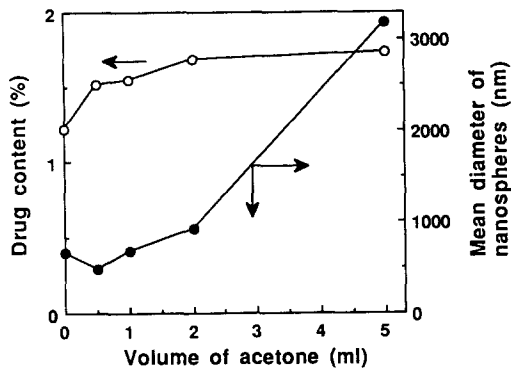


Fig. 5. Effects of volume of acetone on the drug content (○) and mean diameter (●) of PLGA nanospheres. PLGA: PLGA(85:15)-54116.

in nanospheres, since acetone enhanced the affinity between aqueous and coacervate phases because of its amphiphilic nature (Fig. 4B and 5).

The excess volume of acetone caused the aggregation of nanospheres due to insufficient emulsification of the aqueous droplets in the organic phase. A 0.5 ml volume of acetone was selected as optimal for preparing nanospheres. Consequently, the recovery of drug entrapped in the nanospheres was dramatically increased compared with our previous preparation technique in the aqueous system (drug content $\leq 0.5\%$) (Niwa et al., 1994).

Since the aqueous droplets played an important role in the preparation of submicron-sized spheres, it was assumed that the size of the nanospheres would be dependent on the size of aqueous droplets in the w/o emulsion, which could be effectively controlled by changing the concentration of Span 80 in dichloromethane. When Span 80 was not used as an emulsifier in the organic phase the w/o emulsion droplets

Table 1
Relationship between aqueous droplet size of w/o emulsion and resultant particle size of nanospheres

Surfactant	Concentration (%)	Droplet size (μm)	Particle size (μm)
Span 80	0.00	1.38	5.36
	0.33	0.84	0.91
	1.00	0.66	0.80
	2.00	0.62	0.76
HCO 10	1.00	2.12	7.65

Aqueous droplet size and particle size were measured by laser based time of transition analysis method. The concentration of surfactant was represented by the weight of surfactant to the volume of dichloromethane (15 ml).

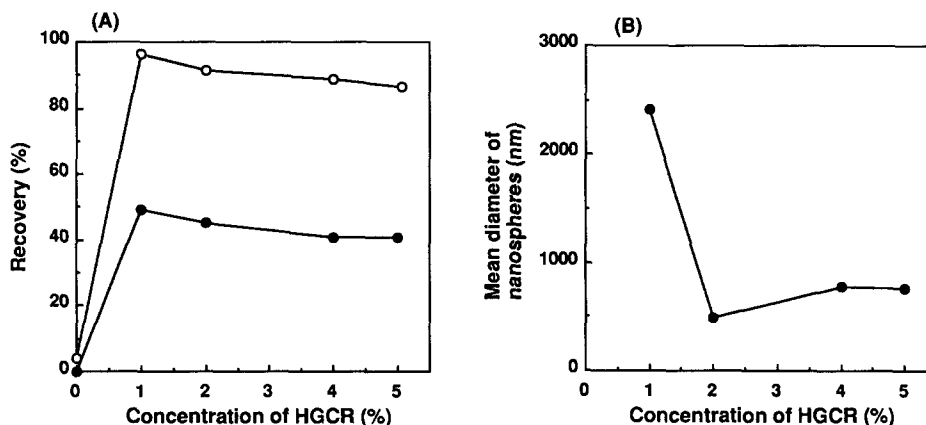


Fig. 6. Effects of HGCR concentration in Triester oil on the recoveries of nanospheres (A, ○) and nafarelin acetate (A, ●), and the mean diameter of nanospheres (B). PLGA: PLGA(85:15)-54116.

were unstable and became larger than $1\ \mu\text{m}$ in size, leading to the formation of microspheres (Table 1). HCO 10 was not as useful as Span 80 at reducing the particle size of the w/o emulsion droplet. In the presence of Span 80, the sizes of both aqueous droplets and resultant particles were almost equal and submicron-sized particles were successfully prepared. This finding indicated that phase separation of PLGA was induced at the interface between water and oil, and each aqueous droplet was individually covered with PLGA coacervate without aggregation. The optimum concentration of Span 80 was determined to be 2%, since at 2% or more of Span 80 the size and recovery of nanospheres were minimized ($\approx 520\ \text{nm}$) and maximized ($\approx 92\%$), respectively.

Fig. 6 shows the effect of the concentration of HGCR in Triester oil on the preparation of nanospheres. In the absence of HGCR, the recovery of nanospheres was very low because particles larger than $37\ \mu\text{m}$, which were removed with a 400 mesh sieve, were preferentially prepared. The addition of HGCR drastically improved the recovery of nanospheres and decreased the mean diameter of nanospheres. HGCR was assumed to work as a protective colloid for the coacervation droplets during preparation. The HGCR molecules adsorbed on the surface of droplets might prevent the coalescence of droplets. The recovery of nanospheres

was over 90% when the appropriate concentration of HGCR was chosen, whereas that of NA in nanospheres was less than 50%. The decrease in drug content in nanospheres was attributed to the leakage of drug localized on the surface and in the water channels in nanospheres during washing by water.

Fig. 7 shows scanning electron microphotographs of typical nanospheres with NA prepared by the present method. The photographs revealed that nanospheres had a discrete spherical structure without aggregation and their size distribution was very sharp, ranging from 400 to 800 nm, which is consistent with the values measured by the light scattering method. The surface of nanospheres were found to be a rigid film structure without any drug crystals.

3.3. Optimization of preparation conditions for PLGA nanospheres

With increasing volumes of dichloromethane dissolving a fixed weight of PLGA(85:15)-54116 (100 mg) the mean diameter of nanospheres decreased considerably as shown in Fig. 8. A higher concentration of PLGA in dichloromethane might increase the frequency of fusion of colliding particles due to the generation of more viscous coacervates, resulting in the lower recovery of nanospheres due to the production of larger

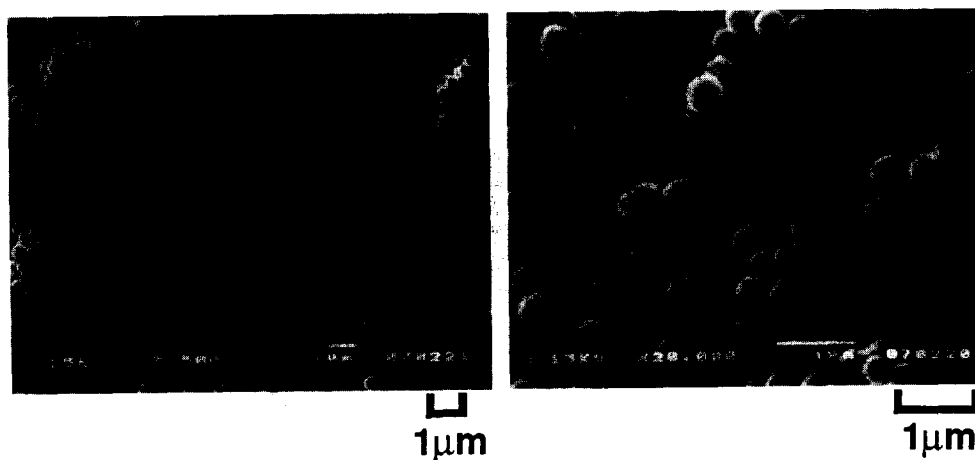


Fig. 7. Scanning electron microphotographs of PLGA(85:15)-54116 nanospheres with nafarelin acetate.

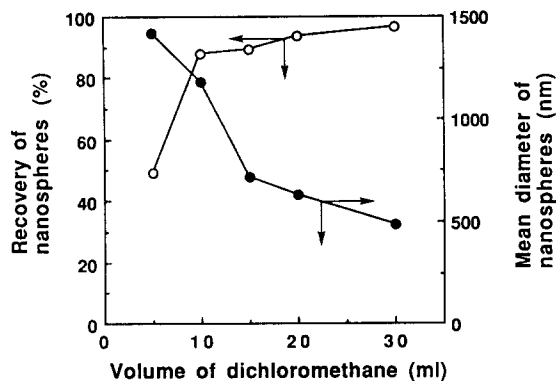


Fig. 8. Effects of the concentration of the polymer solution on the recovery (○) and mean diameter (●) of PLGA nanospheres. 100 mg of PLGA(85:15)-54116 was dissolved in a given volume of dichloromethane.

nanospheres. The drug content in nanospheres remained almost constant ($\approx 1.5\%$) independent of the concentration of polymer solution.

The recovery of nanospheres was not influenced by the pouring speed of non-solvent (Triester oil) into the w/o emulsion system, however, rapid addition caused the aggregation of coacervate droplets, resulting in an increase in the diameter of nanospheres (Fig. 9). This result indicated that the pouring speed might not influence

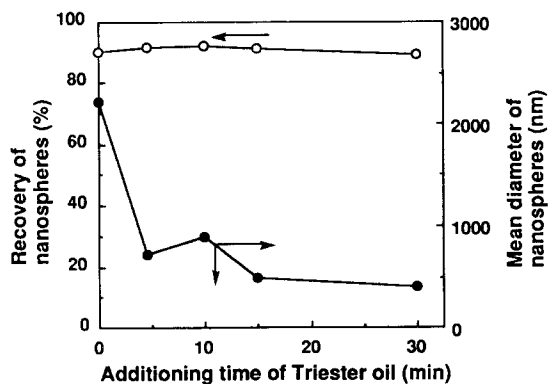


Fig. 9. Effects of pouring speed of Triester oil into w/o emulsion system on the recovery (○) and mean diameter (●) of PLGA nanospheres. PLGA: PLGA(85:15)-54116. 30 ml of Triester oil was added to w/o emulsion system for a given time.

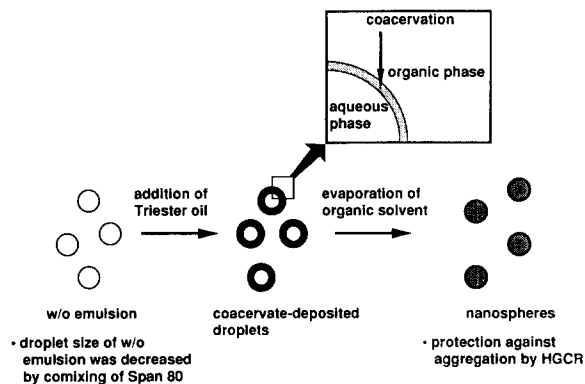


Fig. 10. Preparation mechanism of PLGA nanospheres by the emulsion-phase separation method in an oil system.

significantly the recovery and the size of the nanospheres over the range of 1–6 ml/min.

4. Conclusion

To summarize, the procedure and mechanism of preparation of PLGA nanospheres are illustrated in Fig. 10. The aqueous solution of the drug was emulsified in the organic phase dissolved the polymer. The size of the aqueous droplets was significantly decreased to the order of submicron by comixing lipophilic surfactant, i.e., Span 80. When Triester oil was poured into this w/o emulsion system, phase separation was induced at the interface between the aqueous and oily phase, having a higher potential energy. This phenomenon is consistent with the literature, in which the deposition of coating material was aided by a reduction in the total interfacial energy of the system (Deasy, 1984). As a result, the aqueous droplets were completely covered with the resultant coacervate droplets of PLGA. Therefore, this technique was designated the 'emulsion-phase separation method' in an oil system. The evaporation of the organic solvent from the dispersing medium reduced the solubility of PLGA and deposited it around the aqueous droplets containing NA, forming the nanospheres with the drug. HGCR effectively worked as a protective colloid against aggregation of coacervates, leading to the formation of discrete submicron-sized particles.

Because all aqueous droplets of the drug were successfully entrapped in the coacervates, the drug recovery in the particles was dramatically improved compared with our previous preparation method of nanospheres in an aqueous system (Niwa et al., 1994). The present technique is extremely useful to encapsulate various water-soluble polypeptide drugs in biodegradable PLGA nanospheres. Furthermore, this method can avoid the use of coacervation-inducing agents, e.g., polyisobutylene, usually employed in the phase separation process for the preparation of microcapsules (Samejima et al., 1982; Lin, 1985).

The present nanospheres are possibly applicable for pulmonary applications of peptide drugs via an aqueous aerosolized form by nebulization, since they can readily be separated from the oil phase without coalescing during filtration and the drying process and the resultant powdered nanospheres can be easily redispersed in aqueous solution. The diameter of mist nebulized was not varied by suspending the nanospheres in the solution. The preliminary drug release test from the PLGA nanospheres in vitro proved that the present nanospheres had the property of the sustained release of peptide. We are preparing another paper to focus on the drug release behavior of nanospheres.

Acknowledgements

The supplies of NA and PLGA from Syntex Research and Du Pont are sincerely acknowledged. This project was partly supported by a grant in aid of the Japan Health Sciences Foundation (code no. 1-3-7).

References

- Deasy, P.B., Microencapsulation and related drug processes. *Drugs and Pharmaceutical Sciences*, Vol. 20, Dekker, New York, 1984, pp. 64–69.
- Hutchinson, F.G. and Furr, B.J.A., Biodegradable polymer systems for the sustained release of polypeptides. *J. Controlled Release*, 13 (1990) 279–294.
- Leelarasamee, N., Howard, S.A., Malanga, C.J. and Ma, J.K.H., A method for the preparation of polylactic acid microcapsules of controlled particle size and drug loading. *J. Microencapsul.*, 5 (1988) 147–157.
- Lin, S.Y., Influence of coacervation-inducing agents and cooling rates on the preparation and in vitro release of bleomycin hydrochloride microcapsules. *J. Microencapsul.*, 2 (1985) 91–101.
- Niwa, T., Takeuchi, H., Hino, T., Kunou, N. and Kawashima, Y., In vitro drug release behavior of DL-lactide/glycolide copolymer (PLGA) nanospheres with nafarelin acetate prepared by a novel spontaneous emulsification solvent diffusion method. *J. Pharm. Sci.*, 83 (1994) 727–732.
- Niwa, T., Takeuchi, H., Hino, T., Kunou, N. and Kawashima, Y., Preparations of biodegradable nanospheres of water-soluble and insoluble drugs with DL-lactide/glycolide copolymer by a novel spontaneous emulsification solvent diffusion method, and the drug release behavior. *J. Controlled Release*, 25 (1993) 89–98.
- Okawa, Y., Okada, H., Yamamoto, M. and Shimamoto, T., In vivo release profiles of leuprolide acetate from microcapsules prepared with polylactic acids or copoly(lactic/glycolic) acids and in vivo degradation of these polymers. *Chem. Pharm. Bull.*, 36 (1988) 2576–2581.
- Ruiz, J.M., Tissier, B. and Benoit, J.P., Microencapsulation of peptide: a study of the phase separation of poly(DL-lactic acid-co-glycolic acid) copolymers 50/50 by silicon oil. *Int. J. Pharm.*, 49 (1989) 69–77.
- Samejima, M., Hirata, G. and Koida, Y., Studies on microcapsules: I. Role and effect of coacervation-inducing agents in the microencapsulation of ascorbic acid by a phase separation method. *Chem. Pharm. Bull.*, 30 (1982) 2894–2899.
- Sanders, L.M., Kent, J.S., McRae, G.I., Vickery, B.H., Tice, T.R. and Lewis, D.H., Controlled release of a luteinizing hormone-releasing hormone analogue from poly(DL-lactide-co-glycolide) microspheres. *J. Pharm. Sci.*, 73 (1984) 1294–1297.
- Sanders, L.M., McRae, G.I., Vitale, K.M. and Kell, B.A., Controlled delivery of an LHRH analogue from biodegradable injectable microspheres. *J. Controlled Release*, 2 (1985) 187–195.
- Sato, T., Kanke, M., Schroeder, H.G. and DeLuca, P.P., Porous biodegradable microspheres for controlled drug delivery: I. Assessment of processing conditions and solvent removal techniques. *Pharm. Res.*, 5 (1988) 21–30.
- Stureson, C., Carlfors, J., Edsman, K. and Anderson, M., Preparation of biodegradable poly(lactic-co-glycolic) acid microspheres and their in vitro release of timolol maleate. *Int. J. Pharm.*, 89 (1993) 235–244.
- Wada, R., Hyon, S.H. and Ikada, Y., Lactic acid oligomer microspheres containing hydrophilic drugs. *J. Pharm. Sci.*, 79 (1990) 919–924.
- Yamakawa, I., Tsushima, Y., Machida, R. and Watanabe, S., In vitro and in vivo release of poly(DL-lactic acid) microspheres containing neurotensin analogue prepared by novel oil-in-water solvent evaporation method. *J. Pharm. Sci.*, 81 (1992) 808–811.