ELSEVIER

European Journal of Pharmaceutics and Biopharmaceutics 45 (1998) 41–48

European Journal of Pharmaceutics and Biopharmaceutics

Research article

Properties of a peptide containing DL-lactide/glycolide copolymer nanospheres prepared by novel emulsion solvent diffusion methods

Yoshiaki Kawashima *, Hiromitsu Yamamoto, Hirofumi Takeuchi, Tomoaki Hino, Toshiyuki Niwa [1]

Gifu Pharmaceutical University, Gifu, Japan

Received 10 February 1997; accepted 17 June 1997

Abstract

Novel emulsion solvent diffusion methods in water or oil were developed to prepare the peptide (TRH and elcatonin)-loaded PLGA nanospheres, via the coprecipitation of drug and polymer in the emulsion droplets induced by the diffusion of solvent. The PLGA nanospheres prepared by the emulsion solvent diffusion method in water were unimodally dispersed particles with an average diameter of about 250 nm, whereas those prepared by the emulsion solvent diffusion method in oil and the phase separation method exhibited bimodal distribution with average diameter of about 700 and 800 nm, respectively. The content and recovery of the peptide and nanospheres prepared by the emulsion solvent diffusion method in oil were superior to those of nanospheres prepared using other methods. The drug release properties from nanospheres depended strongly on their preparation processes. The nanospheres prepared by the emulsion solvent diffusion method in oil, released the drug continuously over 14 days, the rate of which was determined by the diffusion of drug in the rigid matrix structure of the nanosphere. The drug release behavior of nanospheres prepared by the phase separation method exhibited a triphasic release pattern with an initial burst and an induction period followed by the diffusion of drug through the walls of the reservoir type nanospheres, which were produced via the phase separation of polymer deposited around the emulsion droplets of the drug. © 1998 Elsevier Science B.V.

Keywords: DL-lactide/glycolide copolymer; Nanosphere; Emulsion solvent diffusion method; Elcatonin; Tyrotropin releasing hormone

1. Introduction

New carriers for peptide drugs which protect the peptide against enzymatic digestion and improve the absorption rate at the site have been proposed and studied as alternative drug delivery systems administered by intravenous injection. One of the most successful carriers is DL-lactide glycolide copolymer, PLGA, [1] or DL-lactic acid polymer, PLA [2] microspheres with leuprorelin acetate which provided sustained release of the peptide over a period of 1 or 3 months, respectively, after subcutaneous injection in male rats. Biodegradable nanospheres, including polyalkyl cyanoacrylate [3] and PLGA [4] nanospheres, are attractive carriers and have been studied with the peroral [5] and ophthalmic [6] routes of administration. Nanoparticles have also been investigated in order to target drugs that are passively and actively absorbed to specific tissues, by the surface modifications of the polymer [7].

Although the preparation methods of PLGA microspheres have been well established, techniques to prepare PLGA nanospheres are still under development.

---

* Corresponding author. Gifu Pharmaceutical University, Department of Pharmaceutical Engineering, 5-6-1 Mitahora-higashi, Gifu, 502 Japan.

[1] Present address: Pfizer Pharmaceuticals Inc., Japan.

PII S0939-6411(97)00121-5

The extremely large surface area of the particle could lead to aggregation or low drug entrapment during the preparation processes. In our previous reports [8,9], spontaneous emulsification solvent diffusion methods were developed to prepare PLGA nanospheres encapsulating either water—soluble or—insoluble drugs. In this process, the poured droplets of acetone-chlorinated hydrocarbons with PLGA and the drug were finely emulsified spontaneously into nanometer-sized spheres in the dispersing aqueous system caused by the rapid diffusion of acetone from the organic to the aqueous phase. Although this process improved dramatically the encapsulation efficiency of water insoluble drug, some modifications are required to prevent the loss of water soluble drugs into the dispersing medium during processing. Furthermore, the amount of organic solvent used should ideally be reduced and the chlorinated hydrocarbon replaced—due to the required time (> 3 h) for removal of the solvent from the nanoparticle.

In this paper, novel emulsion solvent diffusion methods were developed to prepare PLGA nanospheres in an organic dispersing medium, in order to improve the encapsulation efficiency of water soluble peptide drugs, e.g. elcatonin and thyrotropin releasing hormone (TRH). A modified emulsion solvent diffusion method in an aqueous dispersing medium was also developed to replace chlorinated hydrocarbon with methanol so as to improve the encapsulation of the water soluble peptides and to decrease the preparation time to less than 3 h. In addition, the drug release characteristics of the resultant PLGA nanospheres were compared with those prepared using the emulsion-phase separation method in the oil system that was described earlier [10].

2. Material and method

2.1. Materials

PLGA with an average molecular weight of 20 000 and copolymer ratio of DL-lactide to glycolide being 50:50 (Wako Pure Chemical, Japan) was used as the biodegradable polymer. TRH (molecular weight: 363.4) and elcatonin (molecular weight: 3363.82) were supplied by Takeda Chemical (Japan) and Asahi Chemical (Japan), respectively. Polyvinyl alcohol (PVA-403, Kuraray, Japan) was used as a dispersing agent. Caprylate and caprate triglyceride (Triester F-810, Nikko Chemicals, Japan), non-toxic oils, with good biocompatibility and low viscosity, were chosen as the dispersing medium. Hexaglycerin condensed ricinoleate (HGCR) (Hexaglyn PR-15, Nikko Chemicals, Japan) and sorbitan monooleate (Span 80, Kishida Chemicals, Japan) are safe for oral administration and were employed as emulsifiers. All materials were used as received.

2.2. Preparation of PLGA nanospheres

2.2.1. Emulsion solvent diffusion method in water

In the previously reported emulsion solvent diffusion method, dichloromethane (0.5 ml) and relatively large amounts of acetone (15 ml per 100 mg PLGA) was used, which resulted in a relatively long evaporation time (3 h) [9]. Using a novel emulsion solvent diffusion technique, dichloromethane was eliminated from the process and the preparation time was reduced to less than 3 h. A schematic of the procedure is shown in Fig.

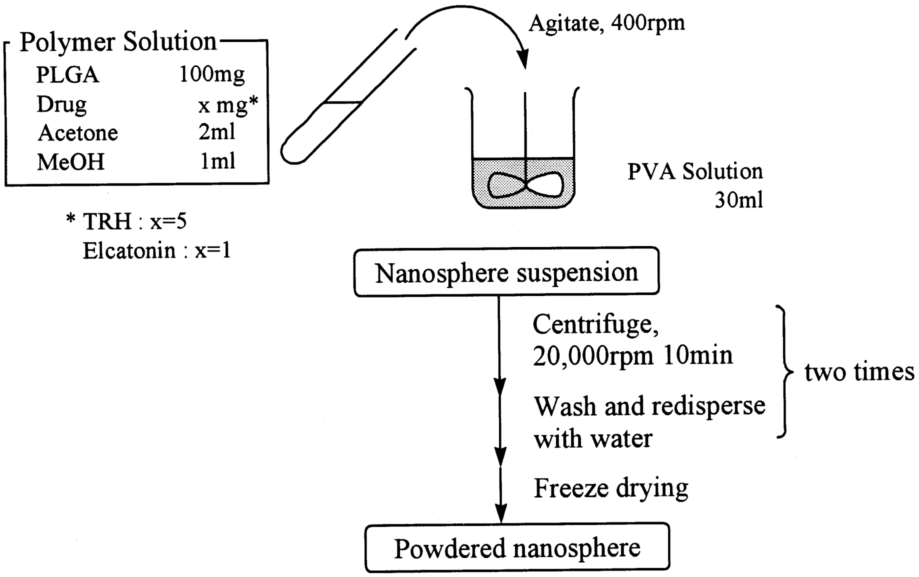

Fig. 1. Schematic procedure for the preparation of PLGA nanospheres with the emulsion solvent diffusion method in aqueous PVA solution (solvent diffusion method in water, WSD).

1. PLGA (100 mg) and the weighed drug (TRH, 5 mg or elcatonin, 1 mg) were dissolved completely in a mixture of acetone (2 ml) and methanol (1 ml). The resultant organic solution was poured into 25 ml of an aqueous PVA solution (1.0%, w/v) and stirred at 400 rpm using a propeller type agitator with three blades (Heidon 600G, Shinto, Japan) for 5 min. The entire dispersed system was then centrifuged (43 400 × g for 10 min; Kubota 7800, Kubota, Japan) and resuspended in distilled water. This process was duplicated. The resultant dispersion was dried using a freeze drying method.

2.2.2. Emulsion solvent diffusion method in oil

The PLGA (100 mg) and the weighed drug (TRH, 5 mg or elcatonin, 1 mg) were dissolved in a mixture of acetone (2 ml), methanol (1 ml) and Span 80 (100 mg). The resultant polymer–drug solution was emulsified into Triester F-810 containing 2% w/w HGCR (50 ml) using a high shearing homogenizer, (Pyscotoron®, Nition, Japan) for 5 min. Decomposition of the drug under high shear homogenization was not detected. The entire dispersed system was then centrifuged (43 400 × g for 10 min). The sediment was dispersed with hexane and centrifuged under the same running condition as above. The sediment was dispersed in an adequate volume of PVA solution (ca. 5 ml). An additional dispersing in distilled water (ca. 5 ml) was carried out. The resultant dispersion was then freeze dried. The schematic procedure for preparation is shown in Fig. 2. In the emulsion solvent diffusion methods in oil, the drug leakage from nanospheres during centrifugation and freeze drying were not found.

2.2.3. Emulsion phase separation method in oil

The drug (TRH, 5 mg or elcatonin, 1 mg) was dissolved in distilled water (0.5 ml) and then emulsified into the dichloromethane (15 ml)–acetone (0.5 ml) mixture containing the dissolved PLGA (100 mg) and Span 80 (100 mg), using a homogenizer (15 000 rpm) (Physcotoron). The addition of the triester oil dissolved 2% w/w of HGCR (30 ml) into the resultant water-in-oil (w/o) emulsion, induced the phase separation of PLGA when stirred with a magnetic stirrer. During evaporation of the dichloromethane under reduced pressure (for 3 h), the coacervated droplets enclosing the drug were transformed into nanospheres in the oily medium. The entire 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, Toyo Roshi, Japan) to remove 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 drug crystals, respectively, and then dried. The schematic procedure for the preparation is shown in Fig. 3.

2.3. Analysis of physicochemical properties of nanospheres

The average particle size of the PLGA nanospheres dispersed in the resultant aqueous system was deter-

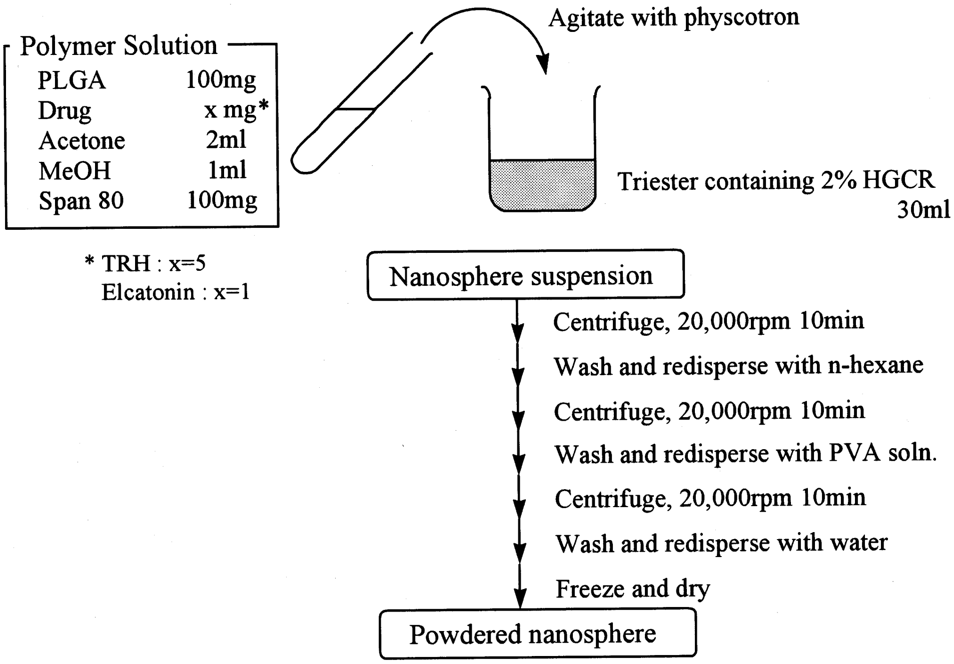

Fig. 2. Schematic procedure for the preparation of PLGA nanospheres with the emulsion solvent diffusion method in triester (solvent diffusion method in oil, OSD).

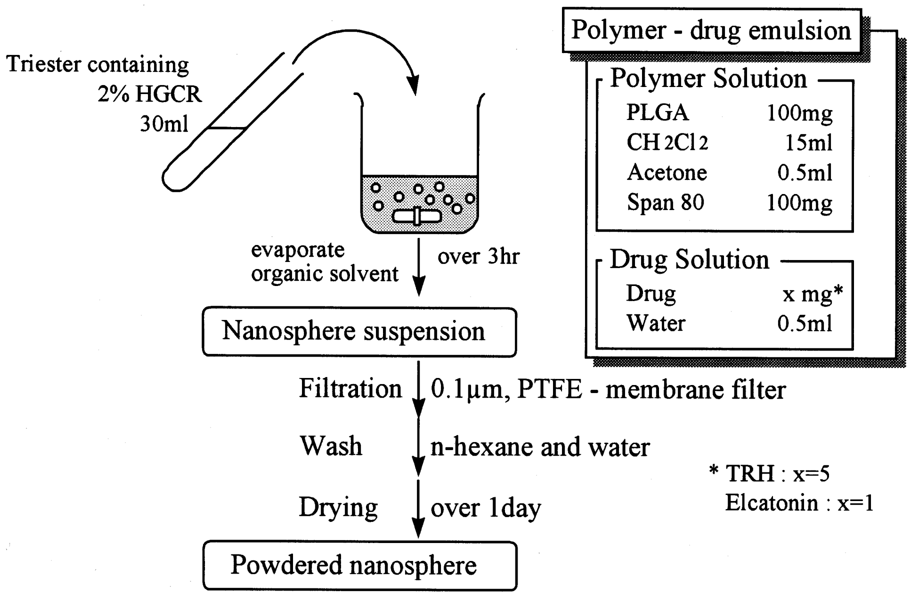

Fig. 3. Schematic procedure for the preparation of PLGA nanospheres in triester oil system (phase separation method, PS).

mined by a laser particle size analyzer (LPA 3100, Otsuka, Japan) and a photon correlator (LPA 300, Otsuka, Japan), based on a dynamic light scattering concept. The surface topography of the freeze dried nanospheres was observed by means of a scanning electron microscope (JSM-T330A, Nihon Denshi, Japan). The recovery of PLGA nanospheres was defined as the weight ratio of the freeze dried nanospheres to the initial loading of PLGA and drug. The freeze dried nanospheres were dissolved in acetonitrile, into which distilled water was added to preferentially precipitate the polymer. The drug content in the supernatant after centrifugation ($14\,600 \times g$ for 15 min, Kubota 7800, Kubota, Japan) was measured spectrophotometrically at 225 nm by means of a HPLC method (pump, PU-980; detector, 875-UV (Jasco, Japan); column, Inertsil ODS-2 (GL Sciences, Japan). The drug recovery and content in the nanospheres were calculated from Eqs. (1) and (2), respectively.

$$\text{Drug recovery}(\%) = \frac{\text{weight of drug in nanospheres}}{\text{weight of drug loaded in the system}} \times 100 \qquad (1)$$

$$\text{Drug content}(\%) = \frac{\text{weight of drug in nanospheres}}{\text{weight of nanospheres recovered}} \times 100 \qquad (2)$$

2.4. Analysis of drug release properties of nanospheres

The drug release properties from nanospheres were investigated in vitro. Fifty milligrams of nanospheres were dispersed in 15 ml of JP XIII No. 2 solution (0.2 M KH_2PO_4–0.2 M NaOH, pH 6.8) and (50 ml) shaken horizontally at 37°C and 60 strokes per min. One milliliter of the dispersion was withdrawn from the system at each time interval and centrifuged ($14\,600 \times g$ for 5 min). The resultant supernatant was removed and the precipitate was dissolved in 0.3 ml of acetonitrile to which distilled water was added to precipitate the polymer and to dissolve the drug in the resultant aqueous mixture. This suspension was centrifuged ($14\,600 \times g$ for 5 min) to remove the precipitated polymer. The drug remaining in the supernatant was determined using the HPLC method as described above.

3. Results and discussions

3.1. Micromeritic properties of PLGA nanospheres prepared with the emulsion solvent diffusion methods in water and oil

The scanning electron microphotograph (SEM) of PLGA nanospheres with elcatonin prepared by the solvent diffusion method in water is shown in Fig. 4. The particles were monodispersed spheres having a weight mean diameter of 250 nm with a polydispersity index between 0.02 and 0.06, irrespective of the type of drug loaded, as shown in Table 1. The freeze dried nanospheres were readily redispersed in aqueous medium by shaking manually, reproducing the original particle diameters before drying. It was assumed that PVA introduced into the system as a dispersing agent was adsorbed on the surface of the nanospheres during freeze drying as previously reported [11]. The PVA on the surface might improve the wettability of the nanospheres on redispersing.

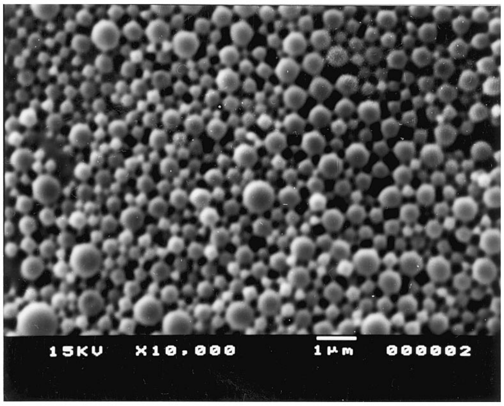

Fig. 4. The scanning electron microphotograph of PLGA nanospheres containing elcatonin prepared by the emulsion solvent diffusion method in water.

The SEM of the nanospheres produced without and with Span® 80 dispersed in the oil system (triester) are shown in Fig. 5a and b, respectively. The particles having a diameter larger than 10 μm were mixed in the system as found in Fig. 5a, compared with the nanospheres obtained with Span 80 (Fig. 5b). In the emulsion solvent diffusion method in water, the diffusion rate of the water miscible organic solvent was very rapid, and the turbulence of the interface of the emulsion droplets occurred due to the Marangoni effect [12], resulting in spontaneous droplet formation in the submicron range. The polymer immediately precipitated, due to the reduced solubility of PLGA at the interface of the resultant emulsion droplets. The emulsion droplets in the oil system coalesced into larger particles during agitation. The slower diffusion rate of the organic solvent into the dispersing oil medium than that in water caused a delay in the hardening of the emulsion droplets by precipitation of the polymer. Therefore, in the oil system, a proper surfactant (dispersing agent) was required to prevent the coalescense of the emulsion droplets and prepare the submicronized particles. Nanospheres containing elcatonin prepared with Span 80 were found to have an average diameter of 700 nm with a polydispersity index, 0.2–0.3, as shown in Fig. 5b. The size distribution of nanospheres prepared in this system became bimodal due to the different dispersing mechanisms from that in the aqueous system.

The nanospheres prepared by the phase separation method also exhibited bimodal particle size distribution, with a weight mean diameter and polydispersity index of 800 nm and 0.3–0.5, respectively. These findings were attributed to the coalescences of some of the coacervates droplets. Further investigations should be conducted to explain these phenomena.

Table 1
Effects of preparation method on nanosphere recovery, drug content and drug recovery

	WSD		OSD		PS	
	TRH	Elcatonin	TRH	Elcatonin	TRH	Elcatonin
Nanosphere recovery (%)	85.7	92.6	74.4	77.7	54.5	66.9
Drug content (%)	0.0594	0.208	1.989	0.567	0.663	0.0303
Drug recovery (%)	1.07	19.5	31.2	44.5	7.59	2.05

WSD, emulsion solvent diffusion method in water; OSD, emulsion solvent diffusion method in oil; PS, phase separation method.

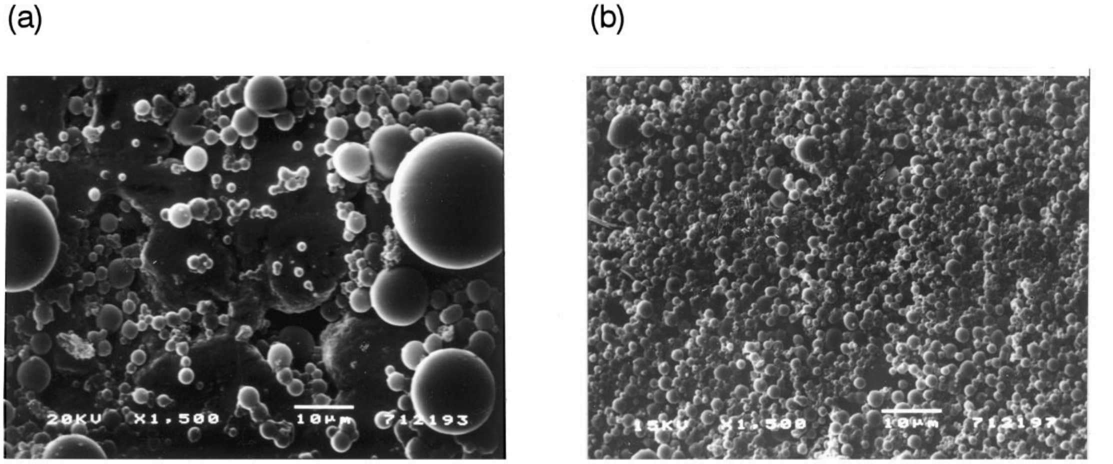

Fig. 5. Effect of surfactant on particle diameter of PLGA nanospheres containing elcatonin. Amount of Span® 80: (a) 0 mg; (b) 100 mg.

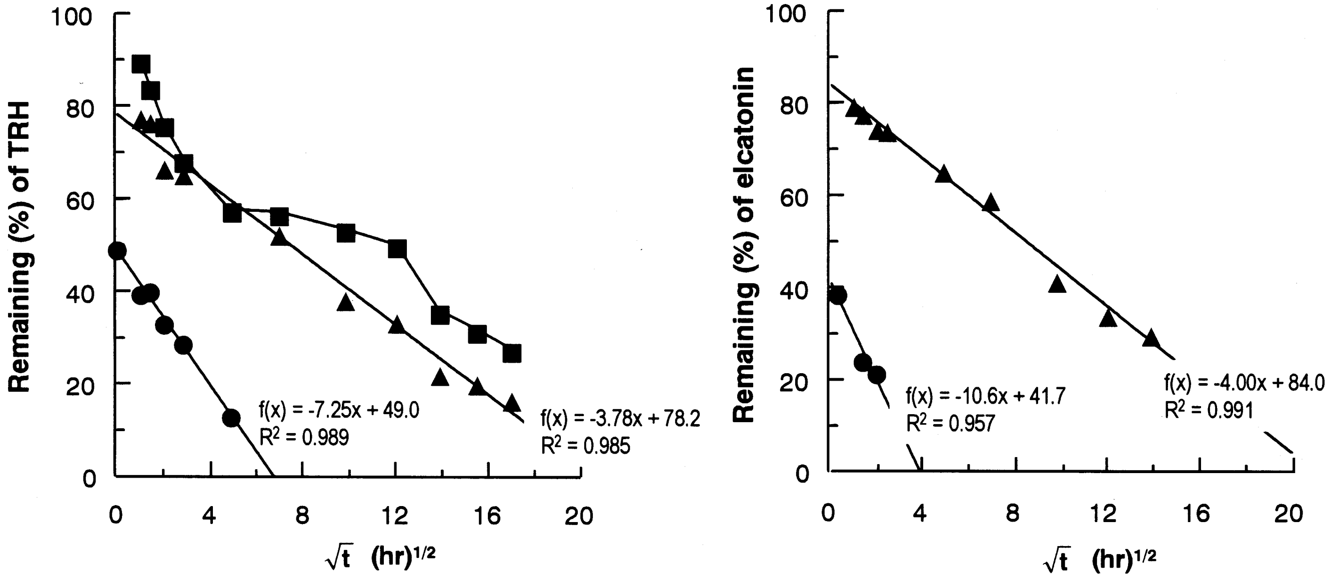

Fig. 6. Release profiles of TRH and Elcatonin from PLGA nanospheres in JP XII No. 2 solution. (●), emulsion solvent diffusion method in water; (▲), emulsion solvent diffusion method in oil; (■), phase separation method (the dissolution test of elcatonin from this method was not possible because drug content was too low).

3.2. Recoveries of nanospheres and encapsulation efficiency of drugs

The effects of the preparation method on nanosphere recovery, drug recovery and the drug content of nanospheres are shown in Table 1. The recoveries of nanospheres were dependent on the preparation method and were in the rank order of the emulsion solvent diffusion methods in water and oil, followed by the phase separation technique. These findings indicated that the precipitation of PLGA was almost completed in the aqueous phase. Precipitation of PLGA was more complete in the oil system than the coacervation of PLGA using the phase separation method. In the solvent diffusion method in oil, both peptides were more efficiently encapsulated in nanospheres compared with other methods since low levels of the hydrophilic drugs were found to partition into the oil phase. In the solvent diffusion method in water and the phase separation method, the encapsulation efficiency of the drug can be affected by the interaction of the drug with the polymer. In the phase separation method, when the polymer–drug interaction was low, the emulsion droplets of the drug were insufficiently covered with the coacervates of PLGA, producing nanospheres with thin polymer walls and low drug encapsulation efficiency.

3.3. Drug release properties of nanospheres

The drug release properties from nanospheres depended strongly on their preparation processes. The drug release profiles of nanospheres prepared by the emulsion solvent diffusion methods proved linear relationships for Higuchi plotting after the initial burst of drug with both TRH and elcatonin (Fig. 6). These findings indicated that the internal structure of the nanosphere was a polymeric matrix containing dispersed drug. The nanospheres prepared in an aqueous system immediately released almost all the drug upon dispersing, indicating a diffusion controlled release mechanism from the matrix. The dissolution profile of the nanospheres prepared in the oil system exhibited a burst of drug during the initial stage. During the later stage, drug release was continuous and slow, indicating that the drug release rate was determined by the diffusion of drug from the rigid matrix structure. The differences in drug release rate due to the preparation medium (e.g. water or oil) was a function of the differences in the particle diameter of the nanospheres (in water: ca. 250 nm, in oil: 700 nm). The drug release of nanospheres prepared with the phase separation

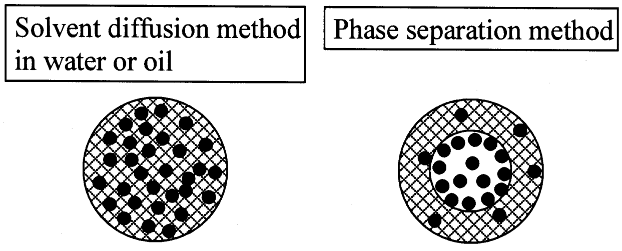

Fig. 7. Structure of nanospheres proposed, based on their methods of preparation and drug release profiles. (●), drug; meshed area: polymer matrix.

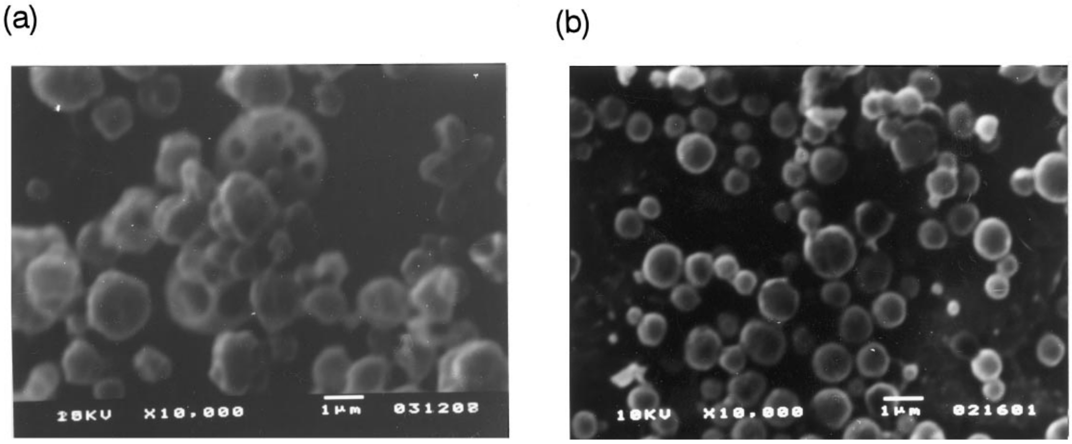

Fig. 8. The SEM of elcatonin containing nanospheres with elcatonin recovered after dissolution test (a), emulsion solvent diffusion method in oil; (b), phase separation method.

method behaved differently from those prepared with the emulsion solvent diffusion method. Their dissolution profiles did not follow the Higuchi equation (Fig. 6). After an initial burst, the drug release profile displayed a plateau for an extended period (about 120 h). Drug release was resumed at a later stage, after 144 h. The differences in the drug release behaviors suggested structural differences of the nanospheres, dependent on the method of preparation. In the solvent diffusion method in oil or in water, the drug might be dispersed evenly in the matrix of the polymer due to the coprecipitation of the polymer and the drug in the emulsion droplets as the organic solvent diffused into the outer phase. The nanospheres prepared by the phase separation method might construct a reservoir like structure, with the polymer precipitating at the surface of the drug solution droplets and producing a wall of the reservoir encapsulating the drug, as imaged in Fig. 7. Therefore, the drug particles adsorbed on the wall would be immediately released during the initial stage and the drug in the core of nanosphere would be released through the reservoir wall after an induction period, resulting in a triphase drug releasing pattern. The SEM of nanospheres with elcatonin prepared by the emulsion solvent diffusion method in oil (a) and the phase separation method (b) after dissolution test are shown in Fig. 8. These pictures support the above proposed mechanisms. Pores were not found on the particulate surface of the nanospheres prepared by the phase separation method, whereas nanospheres prepared by the emulsion solvent diffusion method in oil revealed many pores on the surfaces of the particle. It is suggested that the drug was released through water channels produced in the internal matrix structure of the nanospheres.

4. Conclusion

A novel emulsion diffusion method in oil was demonstrated to improve the encapsulation efficiency of highly water soluble peptides, such as TRH and elcatonin, in matrix type PLGA nanospheres, when compared to microspheres prepared by a phase separation method in oil. The size distribution of nanospheres prepared in the oil system revealed a bimodal profile (average diameter: 700 nm), whereas a monodispersed one (average diameter: 250 nm) for the nanospheres prepared in the aqueous system. The nanospheres prepared by the emulsion solvent diffusion method in oil exhibited a biphasic drug release pattern with an initial burst and sustained release over 14 days, following the Higuchi rule. The drug release profiles of nanospheres prepared with the phase separation technique revealed a triphasic profile, with an initial burst, an induction period, and diffusion of drug through a wall of reservoir type nanosphere, extending the releasing period over 14 days. The dissolution properties and the SEMs of the nanospheres after dissolution demonstrated that nanospheres prepared using the emulsion solvent diffusion method and the phase separation technique were characterized by matrix and reservoir type structures, respectively.

Acknowledgements

A part of the present study was supported by a grant in aid for fundamental scientific research (Code number, B-2-08457595) from the Ministry of Education, Science and Culture, Japan.

References

[1] Y. Ogawa, H. Okada, M. Yamamoto, T. Shimamoto, 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) 1095–1103.

[2] H. Okada, Y. Doken, Y. Ogawa, Persistent suppression of the pituitary–gonadal system in female rats by 3-month depot injectable microspheres of leuprorelin acetate., J. Pharm. Sci. 85 (1996) 1044–1048.

[3] B. Seijo, E. Fattal, L. Roblot-Treupel, P. Couvreur, Design of nanoparticles of less than 50 nm diameter: preparation, characterization and drug loading, Int. J. Pharm. 62 (1990) 1–7.

[4] E. Allémann, R. Gurny, E. Doelker, F.S. Skinner, H. Schütz, Distribution, kinetics and elimination of radioactivity after intravenous and intramuscular injection of ^{14}C-savoxepine loaded poly(DL-lactic acid) nanospheres to rats, J. Control. Release 29 (1994) 97–104.

[5] T. Uchida, S. Martin, T.P. Foster, R.C. Wardley, S. Grimm, Dose and load studies for subcutaneous and oral delivery of poly(lactide-co-glycolide) microspheres containing ovalbumin, Pharm. Res. 11 (1994) 1009–1015.

[6] L. Marcheal-Heussler, H. Fessi, J.P. Devissaguet, M. Hoffman, P. Maincent, Colloidal drug delivery systems for the eye. A comparison of the efficacy of three different polymers: polyisobutylcyanoacrylate, polylactic-co-glycolic acid, poly-epsilon-caprolacton, S.T.P. Pharma Sci. 2 (1992) 98–104.

[7] A.T. Florence, A.M. Hillery, H. Nasir, P.U. Jani, Nanoparticles as carriers for oral peptide absorption: studies on particle uptake and fate, J. Control. Release 36 (1995) 39–46.

[8] T. Niwa, H. Takeuchi, T. Hino, N. Kunou, Y. Kawashima, 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. Control. Release 25 (1993) 89–98.

[9] T. Niwa, H. Takeuchi, T. Hino, N. Kunou, Y. Kawashima, 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.

[10] T. Niwa, H. Takeuchi, T. Hino, M. Nohara, Y. Kawashima, 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, Int. J. Pharm. 121 (1995) 45–54.[11] T. Niwa, H. Takeuchi, T. Hino, Y. Kawashima, Stabilization of lactide/glycolide copolymer (PLGA) nanospheres with peptide-drug by freeze-drying, Yakuzaigaku 55 (1995) 167–174.

[12] H. Fessi, F. Puisieux, J.Ph. Devissaguet, N. Ammoury, S. Benita, Nanocapsule formation by interfacial polymer deposition following solvent displacement, Int. J. Pharm. 55 (1989) R1–R4.