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Poly(ethylene glycol)–poly(L-lactide) diblock copolymer prevents aggregation of poly(L-lactide) microspheres during ethylene oxide gas sterilization

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Abstract

Sterilization procedure is one of the most important obstacles in the clinical applications of biodegradable microspheres. The microspheres prepared with poly(α-hydroxy acid) were severely aggregated during ethylene oxide (EO) gas sterilization, and could not be used in clinical applications. In this study, the effects of EO gas sterilization on the poly(L-lactide) (PLLA) microspheres were analyzed by nuclear magnetic resonance spectroscopy (^{1}H-NMR), differential scanning calorimetry (DSC), gel permeation chromatography (GPC), scanning electron microscope (SEM) and size fractionation. The aggregation between the microspheres might be stimulated by high mobility of amorphous regions of PLLA on the microsphere surfaces since both water vapor and gas mixture can reduce glass transition temperature (T_g) of PLLA below the sterilization temperature. During EO gas sterilization, there were no changes in the molecular structure and the molecular weight of PLLA in microspheres, but there were changes in the crystallinity of PLLA in microspheres. In this study, poly(L-lactide)-poly(ethylene glycol) diblock copolymers (PLE) were blended with PLLA homopolymers in various ratios to design the microsphere suitable for EO gas sterilization. Aggregation of PLLA microspheres was markedly prevented when more than 4 wt% of PLE was blended in the microspheres. This inhibition effect on aggregation may be due to the increased initial crystallinity of the microspheres, which help to maintain the microsphere morphology during EO gas sterilization. © 2001 Published by Elsevier Science Ltd.

Keywords: EO gas sterilization; PLLA–PEG diblock copolymer; PLLA microsphere; Crystallinity

1. Introduction

Biodegradable microspheres that encapsulate therapeutic agents have been extensively studied in order to improve the stability, long-term therapy, and targeting of drugs [1]. The polymers of lactic and/or glycolic acid have been utilized as microsphere materials because of their versatile biodegradability and biocompatibility [2,3].

Microspheres for parenteral administration have to meet the pharmacopoeial requirements of sterility, which has been often neglected in the early designing of microspheres. Terminal sterilization is preferred to aseptic processing of microspheres in a clean room environment under Good Manufacturing Practice (GMP) conditions, if both sterility assurance and cost are considered. The common sterilization methods are steam, dry heat, ethylene oxide (EO) gas, and γ-irradiation [4,5]. Among these, dry heat and steam sterilizations are carried out at high temperature and can cause severe degradation and hydrolysis of the microspheres. In this respect, EO gas and γ-irradiation techniques have been preferred for the sterilization of biodegradable polymers.

Sterilization is one of the important procedures in producing biodegradable microspheres for the clinical applications. However, only a few reports have dealt in

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detail about the effects of sterilization procedure on the biodegradable microspheres composed of poly(α-hydroxy acid) [6–9]. Of those available, the efforts have been focused only on the γ-irradiation method, and little on EO gas sterilization. Apart from the reports that the poly(α-hydroxy acid) microspheres were aggregated during EO gas sterilization [10,11], a general lack of knowledge about EO gas sterilization might have contributed to the prevailing conception that it might be inappropriate for the sterilization of biodegradable microspheres. The aggregation of microspheres during EO gas sterilization, however, does make it difficult for microspheres to be injected through a syringe needle and cannot be used in clinical applications. The EO gas sterilization requires simpler equipment and lower cost than the γ-irradiation method; especially, EO gas sterilization does not affect the molecular weight of poly(α-hydroxy acid) unlike the γ-irradiation sterilization which significantly reduces it [12]. Therefore, it would be more advantageous to use EO gas sterilization if the aggregation problem is ameliorated.

In this study, we investigated the effects of EO gas sterilization on PLLA microspheres and tried to develop a microsphere system that would be suitable for EO gas sterilization by improving the aggregation problem. As the proposed method, PEG–PLLA diblock copolymers (PLE) were physically blended into PLLA homopolymers in various ratios for the microsphere preparation. The blending of hydrophilic polyethylene glycol (PEG) into biodegradable microspheres have received much interest because their physical properties and degradation characteristics can be easily tailored by simple blending in order to control the drug release rate from microspheres [13–18].

2. Materials and methods

2.1. Materials

Poly(L-lactide) (PLLA, Resomer L206, M_w 110,000) was purchased from Boehringer Ingelheim (Ingelheim, Germany). Poly(vinyl alcohol) (PVA, 98% hydrolyzed, average M_w 13,000–23,000), mono-methoxy PEG (MPEG, M_n 5000), L-lactide were obtained from Aldrich Chemical Co. (Milwaukee, WI). Stannous octoate were obtained from Sigma Chemical Co. (St. Louis, MO).

2.2. Synthesis and characterizations of PLLA–PEG diblock copolymer (PLE)

The PLLA–PEG diblock copolymer (PLE) was synthesized by solution polymerization as described by Stevels et al. [19]. L-lactide was recrystallized twice from ethyl acetate, and MPEG was dried at 60°C for 8 h under vacuum, prior to polymerization. L-lactide (10.2 g) and 2 g of MPEG were dissolved in 45 ml toluene at 70°C under nitrogen atmosphere. Stannous octoate (65 mg) was added to the mixture as an initiator. The mixture was refluxed for 24 h at 110°C to produce polymer, which was cooled and the solvent was removed under reduced pressure. The polymer was dried overnight at 40°C under vacuum. The dried polymer was dissolved in chloroform and precipitated using an excess of acetone/diethyl ether (1:4 v/v) mixture that was 10 times the volume of chloroform. The precipitated polymer was filtered and dried under vacuum. The polymer was reprecipitated using a methanol/hexane (4:1 v/v) mixture and finally dried under vacuum.

The synthesized PLE was analyzed using ^{1}H-NMR (JEOL, JNM-LA 300 WB FT-NMR, Tokyo, Japan). Since the molecular weight of PEG block was already known, the number average molecular weight of PLE was calculated by the ratio of peak areas between the PLLA block and the PEG block in the ^{1}H-NMR spectrum. The molecular weight distribution of the PLE diblock copolymers was determined by gel permeation chromatography (GPC, Waters Co., Milford, MA). GPC measurements were carried out with three Waters Styragel columns (HR 1, HR 3, and HR 4), which were serially connected, and the elution rate of tetrahydrofuran was 1 ml/min. The columns were calibrated with polystyrene standards, and the internal and column temperatures were kept constant at 35°C. Thermal properties of PLE were characterized by DSC (Model 2010, TA Instruments Inc., New Castle, DE), and the temperature was raised from −65 to 190°C at a scanning rate of 10°C/min.

2.3. Preparation and sterilization of microspheres

Microspheres were prepared by solvent evaporation technique in oil-in-water emulsion. Both PLLA (3.75 g) and PLE (0, 2, 4, 8 wt% based on PLLA weight) were dissolved in 75 ml of dichloromethane. This polymer solution was injected into 600 ml of aqueous solution containing 2 w/v% of PVA while mixing vigorously by a homogenizer (Ultra-Turrax T25, Janke and Kunkel IKA-Work, Staufen, Germany) at 8000 rpm. After homogenization for 10 min, the solution was gently stirred for 2 h at 40°C with a magnetic stirrer in order to evaporate dichloromethane. The microspheres were collected by a centrifuge at 15,000 rpm for 10 min. The obtained microspheres were washed with distilled water and freeze-dried.

EO gas sterilization was carried out using a 3M ster-Vac 5XL (3M, St. Paul, MN). Microspheres were sterilized by the warm cycle at 55°C or the cool cycle at 37°C. Both cycles consisted of 3 steps, such as preconditioning, gas exposure and fresh air purging. The precondition step establishes chamber vacuum, temperature and humidity. For humidification of the chamber, steam was injected 3 times for 33 min (cool cycle) and 4 times for 25.5 min

(warm cycle). Prior to gas exposure step, initial vacuum was allowed to reach 170 mbar and humidity was set above 30%. In the gas exposure step, approximate duration of EO gas exposure for the cool cycle and the warm cycles were 220 and 100 min, respectively. The pressure in the sterilizer chamber was maintained at 700 ± 5 mbar during the initial gas exposure step and at 620 ± 5 mbar during the final gas exposure step. And the residual EO gas in the sterilized samples was removed by the final fresh air purge for over 8 h at the selected cycle temperatures. The sterilized microspheres were sealed in glass vials and stored at 4°C before use.

2.4. Characterizations of the microspheres

The morphologies of the microspheres before and after EO gas sterilization were observed by scanning electron microscope (SEM, JSM-5800, JEOL, Tokyo, Japan). Thermal analysis of microspheres was performed with DSC. All the samples were scanned from 0 to 200°C at a heating rate of 10°C/min.

The degree of crystallinity, X_c, of microspheres was calculated as

$$X_c\ (\%) = 100 \times (\Delta H_m + \Delta H_c)/\Delta H_m^0,$$

where ΔH_m is the measured enthalpy of melting, ΔH_c is the measured enthalpy of recrystallization, and ΔH_m^0 is the enthalpy of melting for 100% crystalline polymer (for PLLA, $\Delta H_m^0 = 100$ J/g [20]).

Size distribution of the prepared microspheres were measured using a F-1000 Universal Fractionator (FFFractionation, LLC., SLC, UT). The carrier liquids used included 0.05% sodium dodecyl sulfate and 0.02% sodium azide. To calibrate the system, polystyrene latex spheres were used. Standard sieves of 150, 250, 500, 710, 1000, 2000, and 4750 μm in mesh size were also used to characterize aggregation of microspheres during EO gas sterilization. Sterilized microspheres were passed through the sieves, and collected fractions were weighed.

In an annealing experiment, recrystallization was induced by heating PLLA microspheres at three different temperature conditions. The first condition was heating the microspheres at 60°C for 1 h, and the second condition was heating the microspheres at 85°C for 5 min. The third condition was heating the microspheres at 60°C for 5 min, after which the temperature was increased up to 85°C at the rate of 1.3°C/min. An additional heating at 85°C for 5 min followed. These samples were compared with untreated microspheres. All samples were heated under vacuum to exclude other effects such as hydrolytic degradation by water vapor or gases in air.

3. Results and discussion

The synthesized PLE was analyzed by ^{1}H-NMR, GPC and DSC. The ^{1}H-NMR spectrum of PLE showed resonances at 1.58 ppm (CH_3 doublet) and at 5.19 ppm (–CH quartet), which were shown to belong to PLLA block in Fig. 1. The signal at 3.65 ppm (–O–CH_2–CH_2 singlet) is characteristic of methylene units in the MPEG block. The number average molecular weight of the final product was calculated as 32,500 Da from the NMR data, and the molecular weight distribution (M_w/M_n) was deter-

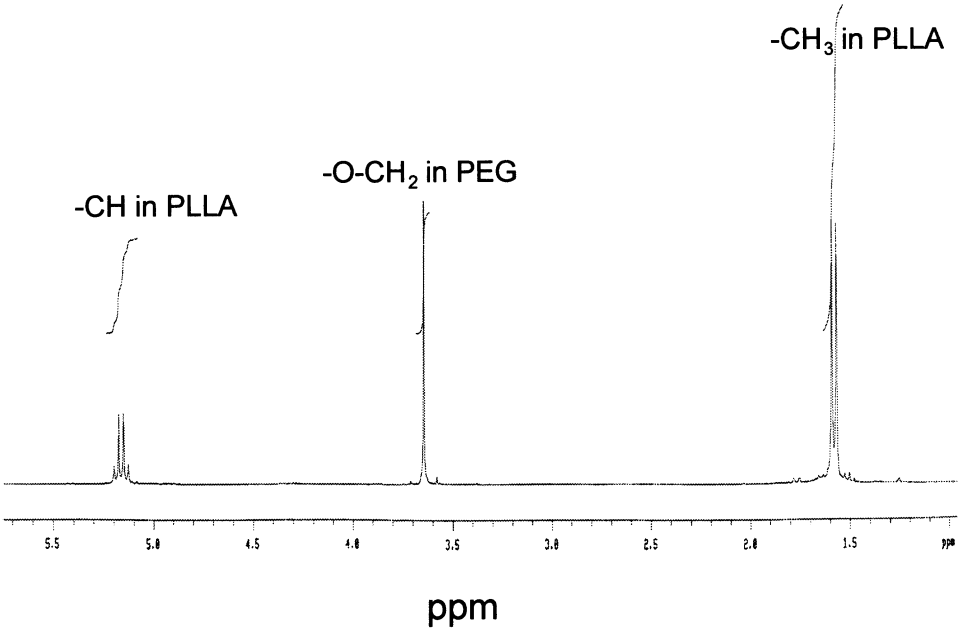

Fig. 1. ^{1}H-NMR spectrum of PLE copolymer.

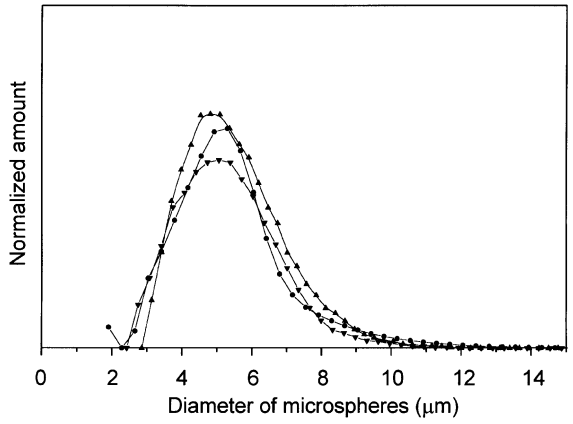

Fig. 2. Size distributions of PLLA microspheres containing PLE: (●) PLE0 MS, (▲) PLE4 MS, (▼) PLE8 MS.

mined as 1.46 in the GPC experiment. The DSC thermal spectrum showed the melting temperature of PLLA block of PLE to be 169°C. A broad peak from 18 to 40°C represents both the glass transition temperature of PLLA segment and the melting temperature of PEG segment. These data confirmed that PLE diblock copolymer was successfully synthesized.

The synthesized PLE diblock copolymers were blended with PLLA homopolymers and prepared as microspheres. The microspheres had spherical shape and smooth surface when they contained PLE below 8 wt%. The size and size distribution of microspheres were not changed by PLE content. The size distribution of microspheres was found in the range of 2–10 μm (Fig. 2). The PLLA microspheres (PLE0 MS) were not dispersed well in PBS, and they could be dispersed well only in the presence of surfactants in the aqueous solution. PLLA microspheres that contained PLE, on the other hand, were dispersed well in PBS without adding any surfactants. It was also found that the higher the PLE content, the better the dispersion of the microspheres in PBS. This may be due to the hydrophilic PEG segment of PLE contained in the PLLA microspheres.

To evaluate the miscibility of PLE with PLLA in the microsphere, the first heating curve of the microspheres in the DSC measurement was analyzed (Table 1). Both glass transition temperature (T_g) and cold crystallization temperature (T_{cc}) of the polymers were decreased by blending 8 wt% PLE (PLE8 MS) as shown in Fig. 3. As a result, the crystallization of PLLA was enhanced during microsphere preparation, resulting in higher ΔH_m value of PLLA block. In other words, the degrees of crystallinity of polymers increased with the increase of PLE content in the microspheres. The initial degree of crystallinity of PLLA microspheres (PLE0 MS) was 33.0%. This value was increased by 4.6, 11.7, and 12.9% in the PLE2 MS (microspheres that contained 2 wt% PLE), PLE4 MS (microspheres that contained 4 wt% PLE), and PLE8 MS (microspheres that contained 8 wt% PLE), respectively. Yue et al. reported that T_g was decreased and the degree of crystallinity was increased when PEG polymers were mixed well with PLLA homopolymers in microspheres [14]. Therefore, it could be concluded that PLE molecules were blended well with the PLLA homopolymers in microspheres when the microspheres contained less than 8 wt% of PLE.

Table 1
Changes in the thermal properties of PLLA during EO gas sterilization

Samples	Sterilization condition	T_g	T_{cc}	T_m	ΔH (J/g)[a]
PLE0 MS	Before sterilization	56.0	86.0	176.0	33.0
	Sterilization at 37°C	54.4	85.5	174.8	35.5
	Sterilization at 55°C	57.8	—[b]	175.4	52.2
PLE2 MS	Before sterilization	54.6	85.7	177.0	37.6
	Sterilization at 37°C	53.4	84.4	176.3	40.2
	Sterilization at 55°C	56.8	—[b]	176.5	50.2
PLE4 MS	Before sterilization	51.5	85.0	176.7	44.7
	Sterilization at 37°C	54.5	84.7	177.3	45.5
	Sterilization at 55°C	57.2	—[b]	176.0	47.0
PLE8 MS	Before sterilization	49.7	84.1	177.0	45.9
	Sterilization at 37°C	52.3	86.2	176.1	47.6
	Sterilization at 55°C	54.9	—[b]	176.0	48.6

[a] $\Delta H = \Delta H_c + \Delta H_m$.
[b] not detected.

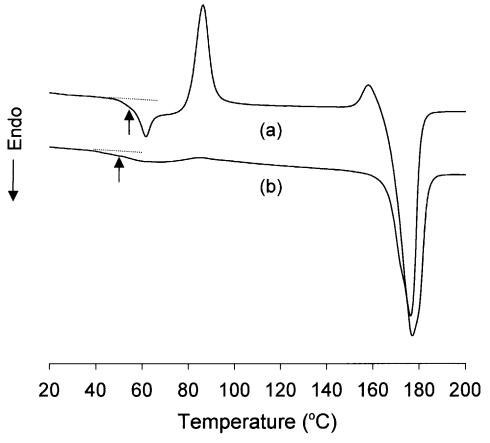

Fig. 3. DSC themograms of microspheres: (a) PLE0 MS, (b) PLE8 MS. Arrow indicates the glass transition temperature.

When PLLA microspheres were sterilized by EO gas at 55°C (warm cycle), the microspheres became severely aggregated. The PLLA microspheres was connected with each other by fusion on their surfaces and they formed large aggregates after sterilization (Fig. 4(a) and (b)). These aggregates were not separated even by a vortex at a high speed, and they could not be injected through a syringe needle. Surface fusion and severe aggregation were also shown in PLE2 MS as shown in Fig. 4(c) and (d). On the other hand, the surface fusion between

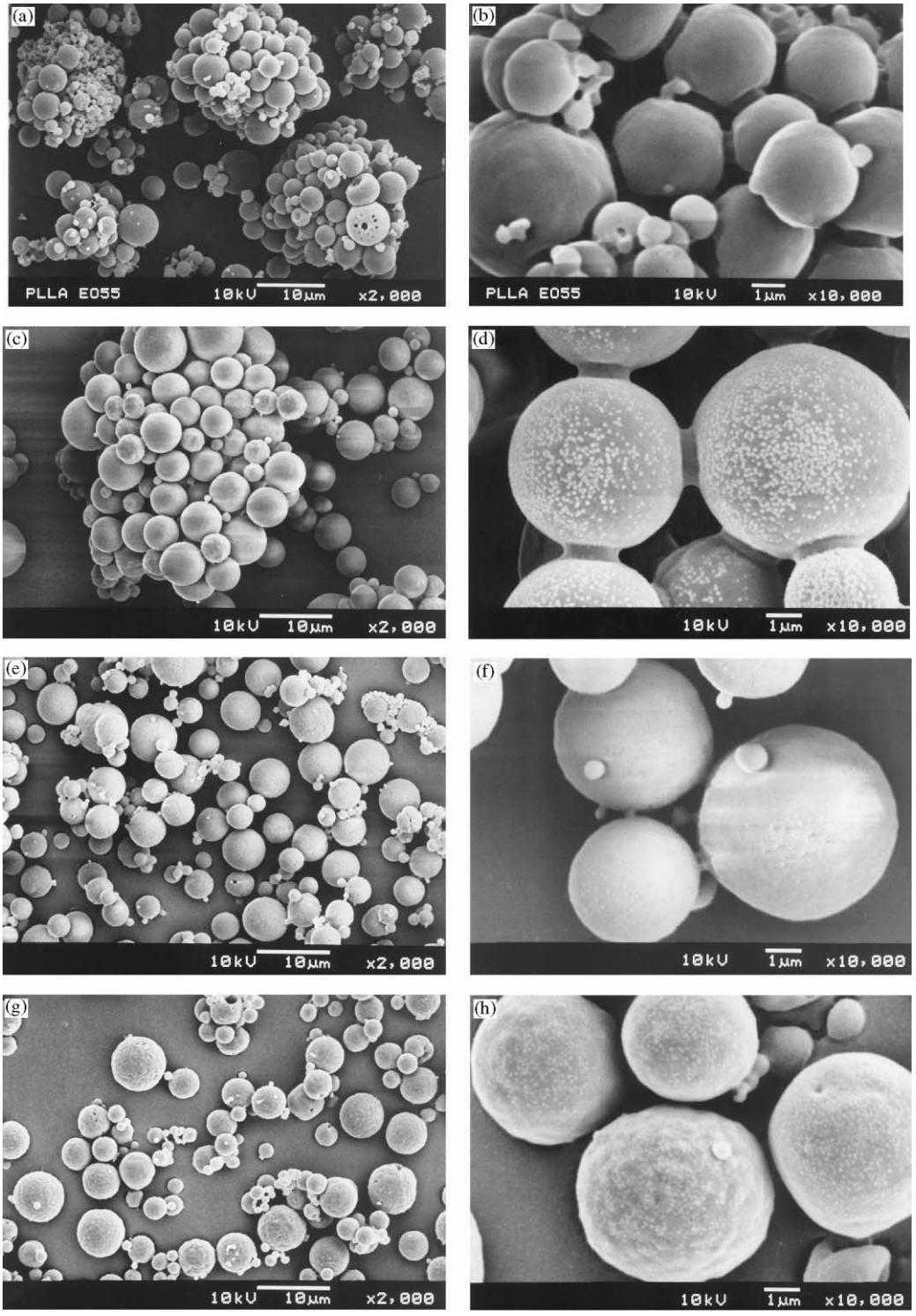

Fig. 4. Formation of the microsphere aggregates by EO gas sterilization at 55°C: (a), (b) PLE0 MS, (c), (d) PLE2 MS, (e), (f) PLE4 MS, (g), (h) PLE8 MS.

microspheres during sterilization was markedly reduced in PLE4 MS and PLE8 MS (Fig. 4(e)–(h)).

The aggregation of microspheres was quantitatively analyzed by the size distribution of microsphere aggregates as shown in Fig. 5. For PLE0 MS, 55% of PLLA microspheres formed as large aggregates with more than 710 μm diameter, and 8.5% of PLLA microspheres formed as large aggregates with diameters ranging from 150 to 710 μm. For PLE2 MS, 61% of microspheres had diameters of over 150 μm after sterilization. The weight

fraction of microsphere aggregates having diameters of over 710 μm was decreased by 10% when compared to PLE0 MS. For PLE4 MS, however, portion of the aggregates having diameters of over 150 μm was decreased to 12.6%. Particles sized over 710 μm in diameter were only 6.4%. Most of the sterilized microspheres were well dispersed in the aqueous solution without any surfactants, and such suspension of the microspheres could be easily injected through a syringe needle. It was observed that, for PLE8 MS, the morphology of microspheres was not changed by the EO gas sterilization. The weight fraction of the aggregates having a diameter of over 150 μm was only 6.5%. Furthermore, the aggregates sized over 710 μm in diameters were not formed during sterilization. Therefore, when PLLA microspheres contained more than 4 wt% of PLE, the microspheres were not aggregated after EO gas sterilization and dispersed well in the aqueous solution without any surfactants. Also, such microspheres could be injected well into the body through a syringe needle.

The aggregation phenomena of PLLA microspheres was also observed during the cool cycle condition at 37°C in EO gas sterilization. As shown in Fig. 5, the reducing effect of PLE copolymers, which were contained in PLLA microspheres, on the aggregation in the cool cycle sterilization was similar to that of the warm cycle. As shown in Fig. 6, severe fusion between microspheres took place in PLE0 MS and PLE2 MS, although the surface fusion was markedly reduced in PLE4 MS and PLE8 MS. Therefore, the aggregation of PLLA microspheres in EO gas sterilization could not be avoided by lowering the temperature.

Changes in the PLLA microsphere property during EO gas sterilization were analyzed by NMR, GPC and DSC. During EO gas sterilization, there were no changes in the molecular structure and the molecular weight of PLLA in microspheres, but there were changes in the crystallinity of PLLA in microspheres. In fact, crystallinity was the only property that was changed, as shown in Fig. 7. When the microspheres were sterilized at 55°C, crystallinity was highly increased by 19.2 and 12.6% for PLE0 MS and PLE2 MS, respectively. In contrast, crystallinity increased only by 2.3 and 2.7% for PLE4 MS and PLE8 MS, respectively.

The large changes in the crystallinity of PLE0 MS and PLE2 MS indicate that the polymer chain mobility was

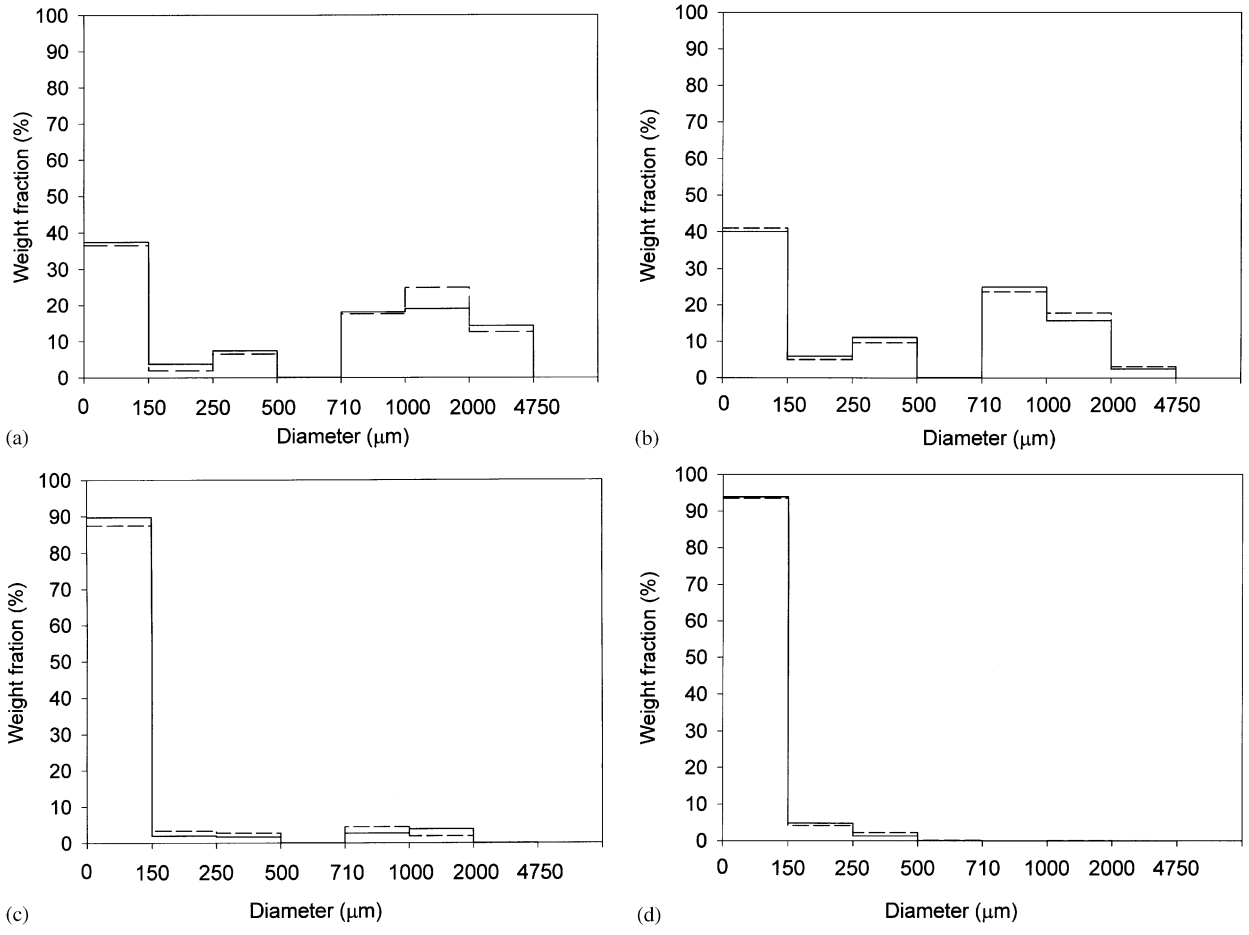

Fig. 5. Size distribution of the microsphere aggregates: (a) PLE0 MS, (b) PLE2 MS, (c) PLE4 MS, (d) PLE8 MS. (— —) at 55°C, (——) at 37°C.

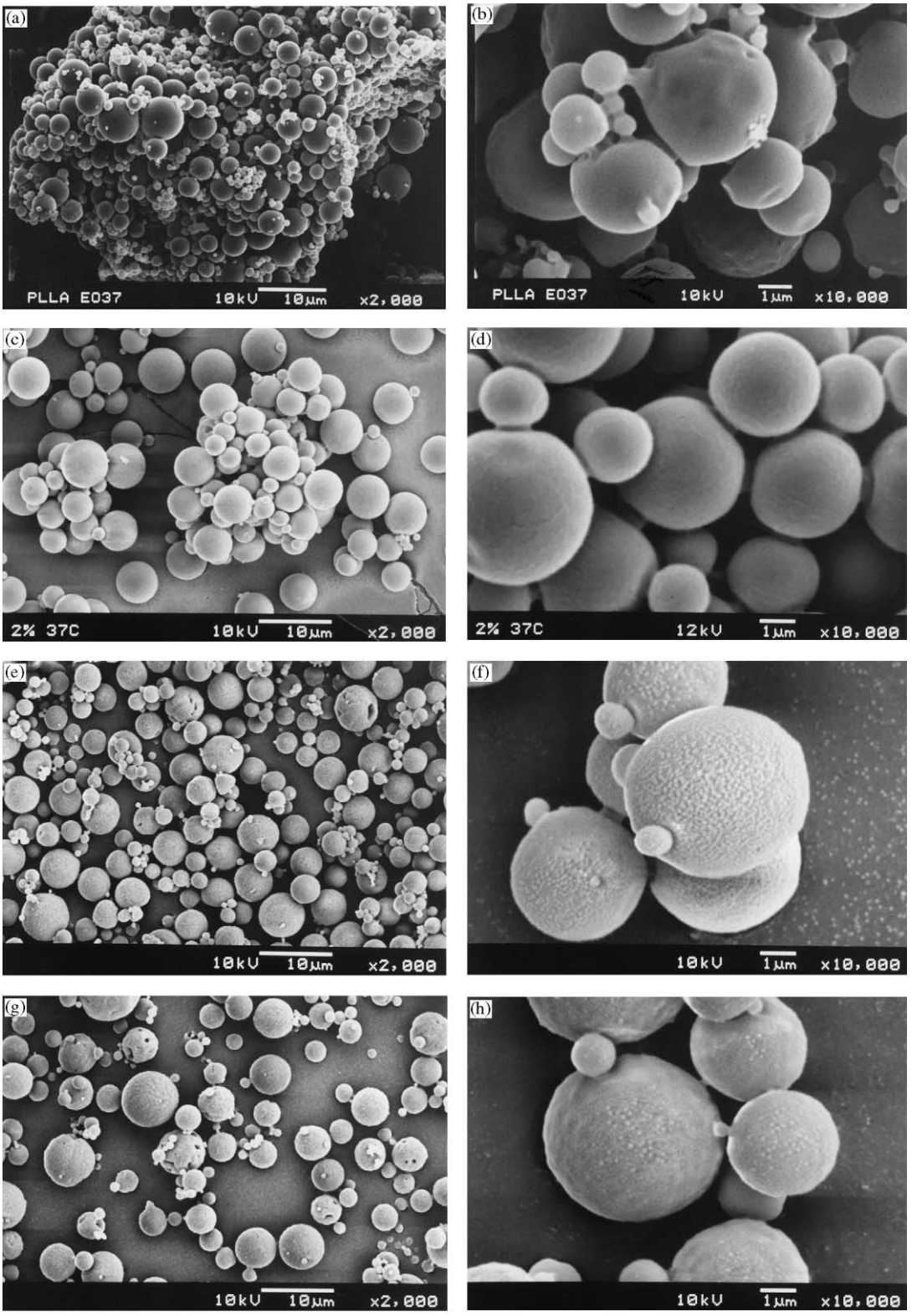

Fig. 6. Formation of the microsphere aggregates by EO gas sterilization at 37°C: (a), (b) PLE0 MS, (c), (d) PLE2 MS, (e), (f) PLE4 MS, (g), (h) PLE8 MS.

increased in both inner and outer amorphous parts of microspheres during the sterilization. The fusion between the microsphere surfaces might have been stimulated by this high mobility. Since the sterilization was carried out at 55°C, which is lower than T_m of PLLA microspheres (180°C), fusion of the microspheres could only be due to

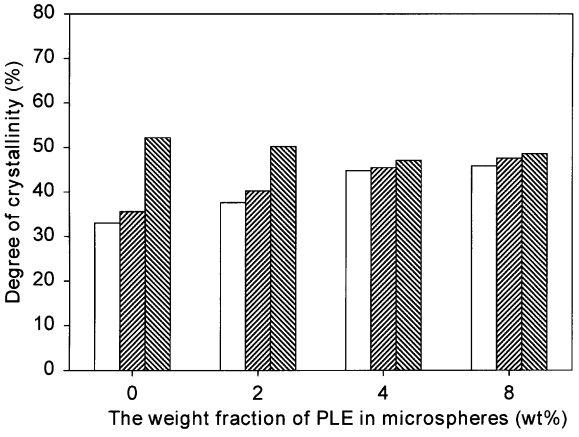

Fig. 7. Change of crystallinity of PLLA microspheres containing PLE during EO gas sterilization: (□) before sterilization, (▨) EO gas sterilization at 37°C, (■) EO gas sterilization at 55°C.

the amorphous region of microspheres. The mobility of amorphous region is mainly related with T_g of microspheres. During the EO gas sterilization, EO gas and freon gas were mixed in the ratio of 12/88 at 30% humidity. Since hydration of the microspheres and gas absorption could change T_g, both water vapor and gas mixture can stimulate fusion on the microsphere surface by lowering T_g below the sterilization temperature. It was reported that water acts as a plasticizer in microspheres, thereby decreasing T_g by about 8°C [21].

For PLE4 MS and PLE8 MS, aggregation was markedly inhibited. This may be due to the high initial crystallinity, since the only difference between PLE2 MS and PLE4 MS was the initial degrees of crystallinity. The initial crystallinity of microspheres increased when the blending ratio of PLE in the microspheres was increased, as mentioned above. This means that the initial crystallinities of PLE4 and PLE8 were high enough to limit aggregation.

When the microspheres were sterilized at 37°C, the degree of crystallinity was increased by 2.5 and 2.6% for PLE0 MS and PLE2 MS, respectively. The crystallinities of PLE4 MS and PLE8 MS, on the other hand, were not changed. For PLE0 MS and PLE2 MS, in spite of small changes in their crystallinities, they were severely aggregated by fusion of their surfaces during the sterilization (Fig. 6(a)–(d)). The small changes in their crystallinities may be due to the fact that the sterilization temperature was not high enough to increase mobility of the amorphous parts of microspheres. Both the small changes in the

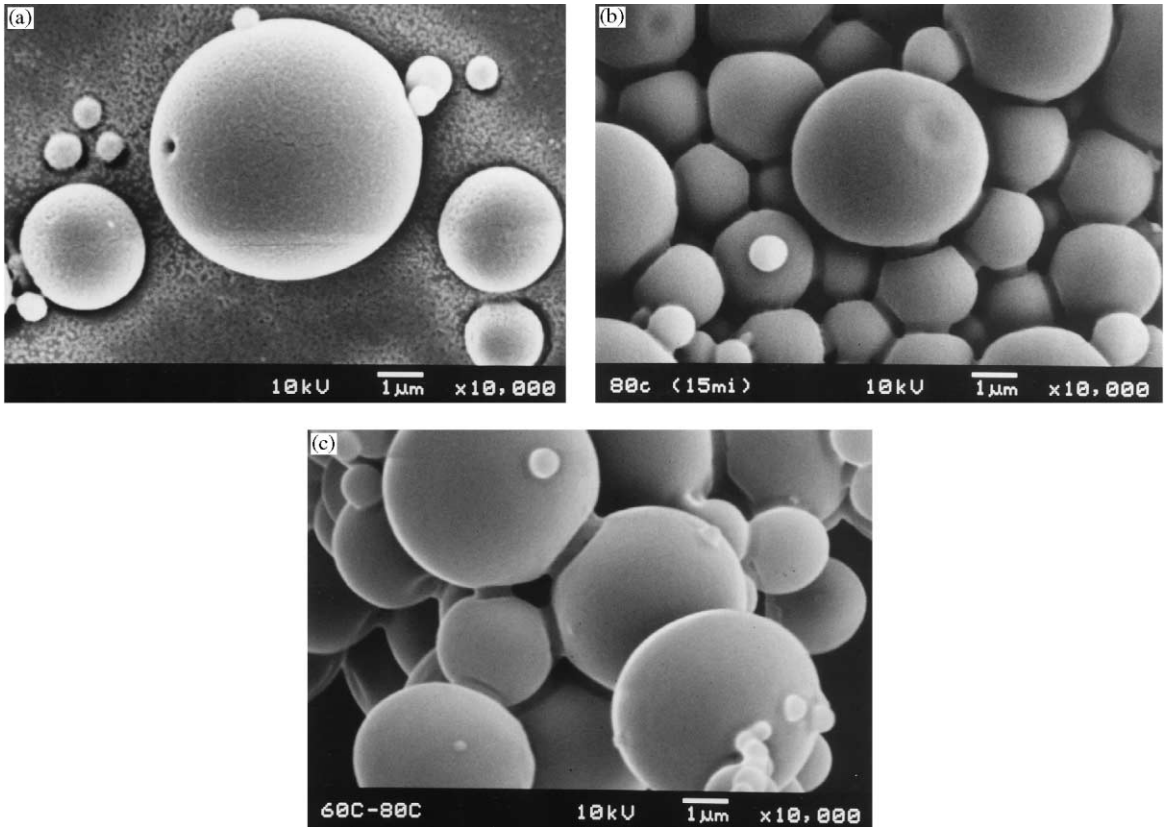

Fig. 8. Formation of the microsphere aggregates during the annealing process: (a) PLLA microspheres were heated at 60°C for 1 h, (b) PLLA microspheres were heated at 85°C for 5 min, (c) PLLA microspheres were heated at 60°C for 5 min, after which the temperature was allowed to reach 85°C at the rate of 1.3°C/min. Additional heating followed at 85°C for 5 min.

crystallinity and surface fusion, therefore, indicate that rearrangement of polymer chains is constrained only on the surface of the microspheres. Since the outer part of organic droplets during microsphere preparation is in contact with aqueous solution, precipitation in the outer part of microsphere is faster than in the inner part. That is, the organic solvent in the inner part of the droplet suspension gets slowly evaporated, taking longer time for crystallization in the inner part. Since DSC measurement cannot make distinction between the surface layer and the bulk crystallinities, the difference in the crystallinity between the inner and the outer parts of microspheres cannot be confirmed. However, the surface layer of PLE0 MS may be more susceptible to aggregation.

Annealing experiments were performed to evaluate whether PLLA microspheres could be aggregated in spite of the small changes in crystallinity (Fig. 8). In this experiment, PLLA microspheres were heated at three different temperature conditions to induce recrystallization. In the first condition where PLLA microspheres were heated at 60°C for 1 h, crystallinity was not changed and aggregation of microspheres did not occur. However, in the second condition where microspheres were heated at 85°C for 5 min, the crystallinity of microspheres was increased by 2.0%. The third condition had microspheres first heated at 60°C for 5 min, after which the temperature was allowed to reach 85°C at the rate of 1.3°C/min. Additional heating then followed at 85°C for 5 min. In this case, we found that the crystallinity of microspheres increased by 6.3%. Under the second and the third conditions, we observed severe aggregation of microspheres, although changes in crystallinity were small. Therefore, we found that the microsphere surface is highly susceptible to aggregation even at moderate conditions of cool cycle when the initial crystallinity of microsphere material is low.

4. Conclusions

When PLLA microspheres were sterilized by EO gas, the microspheres became severely aggregated, and this aggregation could not be avoided by lowering the temperature. The fusion between the amorphous regions of microsphere surfaces might have been stimulated by this high mobility since both water vapor and gas mixture can stimulate fusion on the microsphere surface by lowering T_g below the sterilization temperature. This aggregation was greatly improved by simple blending with PLE. Large aggregates of microspheres almost disappeared when PLE was blended above 4 wt% in PLLA microspheres. This inhibition of aggregation may be due to the increased initial crystallinity of microspheres, which helps to maintain the microsphere morphology during sterilization. Blending method with PLE, therefore, could be considered as a simple one for the preparation of microspheres suitable for EO gas sterilization.

Acknowledgements

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