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International Journal of Pharmaceutics 185 (1999) 93–101

international journal of pharmaceutics

www.elsevier.com/locate/promis

Preparation of nanoparticles consisted of poly(L-lactide)–poly(ethylene glycol)–poly(L-lactide) and their evaluation in vitro

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Received 24 August 1998; received in revised form 3 March 1999; accepted 17 April 1999

Abstract

This study describes the preparation and the evaluation of biodegradable poly(L-lactide)–poly(ethylene glycol)–poly(L-lactide) copolymer (PLA–PEG–PLA) nanoparticles containing progesterone as a model drug. PLA and PLA–PEG–PLA copolymers, whose PEG content ranged from 5.2 to 25.8% (w/w), were polymerized in our laboratory. PEG with weight-average molecular weight (Mw) 6600 or 20 000 was introduced as a hydrophilic segment into a hydrophobic PLA homopolymer. A solvent evaporation method was used to prepare the nanoparticles. The drug trapping efficiencies were around 70% and the weight-averaged mean diameters of the nanoparticles were less than 335 nm. The amount of drug released increased as the PEG content and Mw of PLA–PEG–PLA copolymers increased and the total Mw of copolymers of nanoparticles decreased. The initial burst of drug release was reduced by removing the low Mw fraction from the polymer. During the release test, both the extent to which the copolymers were degraded and the size of the nanoparticles were increased slightly by increasing the content of PEG in the polymers. Drug release from the nanoparticles could potentially be controlled by changing the PEG content, PEG Mw and total Mw of the copolymer. The molecular weight distribution (Mw/Mn, Mn: number-average molecular weight) of copolymers was also an important factor for controlled release.

Keywords: Poly(L-lactide); Poly(ethylene glycol); Triblock copolymer; Progesterone; Nanoparticles; Release; In vitro; Fractionation

1. Introduction

Colloidal carriers are rapidly removed by the reticuloendothelial system (RES), because these particles are easily trapped by phagocytic cells. In recent years, various attempts have been made to

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0378-5173/99/$ - see front matter
PII: S0378-5173(99)00153-2

change this (Illum and Davis, 1984; Senior et al., 1991; Müller et al., 1992; Stolnik et al., 1994).

Stolnik et al. (1994) showed that poly(lactide-co-glycolide) (PLGA) nanospheres coated biodegradable poly(L-lactide)–poly(ethylene glycol) copolymer (PLA–PEG) were less susceptible to hepatic uptake than naked PLGA nanospheres. PEG-PLGA nanospheres (Gref et al., 1994), methoxy PEG-PLA nanoparticles (Bazile et al., 1995), and PLA-PEG nanoparticles (Verrecchia et al., 1995) remained in the circulation a long time. These characteristics of diblock copolymer nanoparticles are due to the reduction of protein adsorption owing to the formation of a hydrophilic layer and a low surface charge originating in PEG (Stolnik et al., 1994). On the other hand, it was supposed to be difficult for triblock copolymer nanoparticles to evade the RES, because their hydrophilic PEG domains like those PLA–PEG–PLA do not move freely. But we have reported that nanoparticles using this PLA–PEG–PLA triblock copolymer evaded capture by the RES and remained a long time in the circulation (Nakada et al., 1997). The half-life after injection of ^{3}H-progesterone loaded PLA–PEG–PLA nanoparticles was about twice that of ^{3}H-progesterone-loaded PLA nanoparticles and the ^{3}H-progesterone distribution to the liver and spleen after administration of ^{3}H-progesterone PLA–PEG–PLA nanoparticles was about half that of ^{3}H-progesterone PLA nanoparticles.

In this paper, we describe the characteristics of nanoparticles of this PLA–PEG–PLA triblock copolymer in vitro.

2. Materials and methods

2.1. Materials

Progesterone, as a model hydrophobic compound with a very short half-life in blood, was purchased from Sigma (MO, USA). Polyoxyethylene (20) sorbitan monooleate (Polysorbate 80) and PEG (weight-average molecular weight (Mw); 6600 and 20 000, respectively) were of reagent grade from Wako Pure Chemical Industries (Osaka, Japan). Polyvinyl alcohol (PVA-203, Kuraray, Tokyo, Japan) was used as supplied. L-Lactide was purchased from Boehringer Ingelheim (Germany).

2.2. Synthesis of polymer

2.2.1. Polymerization

Bulk polymerization of L-lactide initiated by the hydroxyl moiety of PEG was carried out according to Leenslag and Pennings' method (Leenslag and Pennings, 1987) with minor modification. Prescribed amounts of an initiater and L-lactide were mixed thoroughly, placed in a desiccator containing P_2O_5, and dried in a vacuum for about 12 h at room temperature. The feeding composition is shown in Table 1.

In a glovebox purged by dry nitrogen, the dried mixture was transferred into a reactor vial which had been frame-dried for 1–5 min beforehand. A prescribed amount of 50% (w/w) Tin(II)bis(2-ethylhexanoate) chloroform solution was added and then dry nitrogen was poured into the vial. The vial was submerged in an oil bath controlled

Table 1
Feeding composition

Lot	Feeding (% (w/w))			SnOct (% (w/w))
	PEG or 1-decanol		L-Lactide	
A	1-Decanol (Mw)	0.11	99.8	0.05
B	PEG (6600)	5.0	95.0	0.05
C	PEG (6600)	10.0	90.0	0.05
C-1	PEG (6600)	10.0	90.0	0.05
D	PEG (6600)	6.0	94.0	0.67
E	PEG (20 000)	13.0	87.0	0.05

Table 2
Properties of PLA homopolymers and PLA–PEG–PLA copolymers

Lot	PEG Mw	Mw	Mw/Mn	PEG contents (% (w/w))
A	0	129 000	1.9	0
B	6600	147 000	2.0	5.2
C	6600	119 000	1.6	10.3
C-1	6600	83 000	11.7	10.5
D	6600	60 000	1.1	25.8
E	20 000	121 000	1.3	15.8

at 135°C. After about 10 min, the reactants became molten and then were stirred vigorously by a motor with an alectrical torquemeter. As the polymerization proceeded, the reactant become less transparent due to crystallization of polylactide, until finally the torque loaded the stirring motor to the point where it could not turn even at a slow rotation of 15 rpm. To react any residual monomer which might be present, the reaction product was annealed at 135°C for about 400 min once the stirring had stopped, and then the reactor vial was cooled by submerging in a water bath to stop the reaction. We denote the polymer samples in which the reaction was initiated by decanol and PEG as PLA and PLA–PEG–PLA based on the presumed structure, respectively.

2.2.2. Fractionation and purification

Most polymerized products were fractionated and purified as follows. A given product was dissolved in chloroform at a concentration of 1–3 wt.%. A chloroform–methanol mixture (50–70% methanol in volume) or methanol was slowly added to the solution with stirring at 30°C until the solution became turbid. The turbid solution was warmed to 40–45°C until it had become almost transparent. It was then slowly cooled to 30°C or lower with stirring until it had become turbid again. The solution was aged for about 12 h at this temperature, but, no complete phase separation took place. When centrifuged at 7000 rpm for 0.5–2 h at the same temperature, however, phase separation occurred. The gel phase was removed from the centrifuge tube, and the polymer was reprecipitated from a chloroform solution into methanol. The supernatant phase was subject to further precipitation. By repeating this procedure, we could divide each sample into two or three parts. Mostly, the second or first fraction dominated in weight (morally 95% or more) and this fraction was regarded as the principal component of the polymerization product.

The weight-average molecular weight (Mw) and the molecular weight distribution (Mw/Mn, where Mn is the number-average molecular weight) of PLA–PEG–PLA were measured by Gel permeation chromatography (GPC). GPC was performed using a model L-7200 GPC system (Hitachi, Tokyo) and a refractive index meter (detector, RI-8010, Tosoh, Tokyo, Japan) at a flow rate of 1.0 ml/min at 35°C. PLA or PLA–PEG–PLA dissolved in 100 μl of chloroform was separated on a GMHXL column (TSK–GEL, Tosoh, Tokyo). The Mw was calibrated by the use of standard polystylene. The PEG content of the polymers was measured by NMR (AM300, Bruker, Germany). The composition and the properties of the PLA–PEG–PLA used in this study are summarized in Table 2. All other chemicals were of reagent grade.

2.3. Preparation of nanoparticles containing progesterone

A solvent evaporation method (Verrecchia et al., 1995) was used to prepare the progesterone-loaded nanoparticles. Progesterone (4 mg) and PLA or PLA–PEG–PLA (40 mg) were dissolved in 4 ml of dichloromethane. The solution was loaded into a 10 ml syringe attached to a tubelcrine needle and emulsified into a polyvinyl alcohol solution (0.5% (w/v), 16 ml) with stirring using a homogenizer (Polytron, Kinematica AG, Littau, Switzerland) for 2 min at room tempera-

ture. After homogenization, the emulsion was sonicated (Sonifier 250, Branson Ultrasonics, CT, USA) for 5 min. Next, the dichloromethane in the emulsion was evaporated under reduced pressure, and nanoparticles were obtained as suspension. Free progesterone was removed by gel permeation chromatography (GPC) using an Econo-Pac Cartridge 10DG column (Bio-Rad Laboratories, CA, USA) and pH 7.2 phosphate buffer containing 0.5% (w/v) polyvinyl alcohol and 1 M sodium chloride. The nanoparticle samples were kept at −30°C until needed for the experiments.

The size distributions and the mean diameters of nanoparticles were measured with an electrophoretic light scattering spectrophotometer (ELS-800 Ohtsuka Electronics, Osaka, Japan). The weight-average size measured by ELS-800 was adopted as the mean diameter.

2.4. Determination of trapping efficiency of progesterone in nanoparticles

The amount of progesterone in the PLA–PEG–PLA nanoparticles was determined as follows. A 0.5 ml suspension of progesterone-loaded nanoparticles was added to 5 ml of acetone, shaken for 10 min and sonicated for 20 min. The dispersion was filtrated through a membrane filter (pore size 0.2 μm, Gelman Sciences, USA). The progesterone concentration in the clear solution was determined by HPLC. For HPLC, a reversed-phase Inertsil ODS column (150 × 4.6 mm i.d., particle size 5 μm, GL Sciences Inc., Japan) was used, and the column temperature was maintained at 35°C. The mobile phase was a mixture of methanol and water (75:25). The flow-rate was 1.2 ml/min and the absorbance of the eluate at 254 nm was monitored. The HPLC system consisted of an LC-6A flow pump, a model SIL-6A autoinjector, a model SPD-6A UV detector and a model C-R6A integrator (Shimadzu Corporation, Kyoto, Japan).

The trapping efficiency for progesterone of the PLA-PEG-PLA nanoparticles was calculated as follows;

$$\text{Trapping efficiency } (\%) = A/B \times 100$$

where A is the amount of progesterone in the nanoparticle suspension after gel permeation chromatography, and B is the amount of progesterone in the nanoparticle suspension before gel permeation chromatography.

2.5. In vitro release test

The release of progesterone from the nanoparticles was examined as follows. To a 1 ml progesterone nanoparticles suspension was added 19 ml of medium in a screw-capped tube. The tubes were placed in an air bath maintained at 37°C and stirred with a stirring bar at low or high speed (low speed; 750 rpm, high speed; 1300 rpm). A pH 7.4 phosphate buffer containing 0–0.1% (w/v) Polysorbate 80 aqueous solution was used as medium for the release test. At given time intervals, the aliquots were centrifuged at 100 000 × g for 10 min at 10°C (CP-56G, Hitachi Koki, Tokyo, Japan) and the amount of progesterone in the supernatants was measured by HPLC.

In a preliminary study, the total amount of progesterone in the whole suspension after the release test was measured, and it was confirmed that progesterone was stable during the release test and extracted from nanoparticles.

To examine the degradation behavior of the PLA and PLA–PEG–PLA chain in the nanoparticles during the drug release test, nanoparticle suspensions were added to the releasing medium, and aliquots were taken at suitable intervals to measure the Mw change of the PLA and PLA–PEG–PLA with GPC as described Section 2.2. GPC samples were prepared as follows. The precipitate after twice being ultracentrifuged (100 000 × g, 10 min) was dried under reduced pressure at 35°C for 10 h. The dried samples were dissolved in chloroform and used for GPC.

3. Results and discussion

3.1. Characterization of the nanoparticles

Progesterone nanoparticles were prepared by a solvent evaporation method. Values for the mean diameter of distribution and the drug trapping

Table 3
Characterization of progesterone-loaded PLA and PLA–PEG–PLA nanoparticles

Preparation no.	Lot of polymer	Diameter and size distribution[a] (nm)	Drug trapping efficiency[b] (%)
A-NP	A	264 ± 78	71
B-NP	B	220 ± 92	65
C-NP	C	223 ± 102	74
C-1-NP	C-1	335 ± 120	67
D-NP	D	193 ± 87	70
E-NP	E	324 ± 131	65

[a] Diameters and size distribution were measured with an electrophoretic light scattering spectrophotometer ($n = 1$).
[b] Mean ($n = 3$).

efficiency of each preparations are summarized in Table 3. A typical size distribution graph based on weight is presented in Fig. 1.

The nanoparticles had a mean diameter of 193–335 nm and trapping efficiencies of 65–74%. Both the mean diameter and the trapping efficiency were independent of the polymer composition in this study.

3.2. Drug release behavior of PLA–PEG–PLA nanoparticles

3.2.1. Effect of the release test conditions

The drug release behavior of nanoparticles C-NP (PEG content, 10.3%; Mw, 119 000) containing progesterone was evaluated in various media (Fig. 2).

In the Polysorbate 80 free solution, there was a lag in the initial release. The lag time was shortened by increasing the Polysorbate 80 concentration. The lag time in 0, 0.01 and 0.1% Polysorbate 80 was 24, 6 and 3 h, respectively. The release enhancement by Polysorbate 80 may be due to surfactant.

Next, the release behavior of progesterone from C-NP was tested at different speeds of stirring in 0.01% Polysorbate 80 (Fig. 3). The release was remarkably faster at high speed than at low speed. In addition, the release at high speed showed a biphasic pattern, an initial burst phase followed by a slow release phase.

This biphasic pattern resembled the pattern for PLGA nanoparticles reported by Niwa et al. (1993), who supposed that the initial burst was due to the rapid release of drugs deposited on the surface and in the water channels inside of the nanospheres. At a high stirring speed, the biphasic pattern became more obvious.

Since the drug release was enhanced by the use of surfactant and the increase of stirring speed, it was supposed that the release is influenced by both the wettability of the nanoparticle surface and the water permeability of the nanoparticles. Therefore, we synthesized various PLA–PEG–PLA triblock copolymers and studied the effect of the polymer composition on the drug release. We chose triblock type copolymers as a matrix of nanoparticles since we supposed that the hydrophilic PEG fraction was introduced not only on the surface but also to the inside of the nanoparticles, which made the aqueous channels.

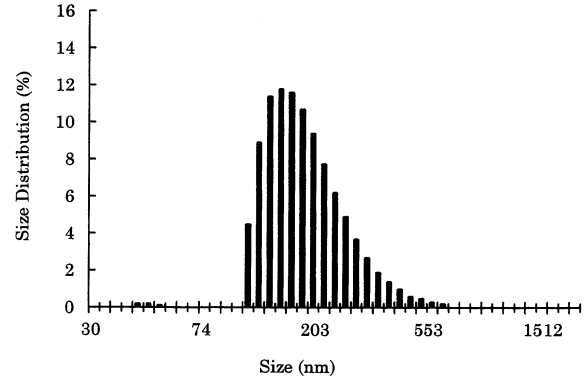

Fig. 1. Size distribution based on weight (C-NP).

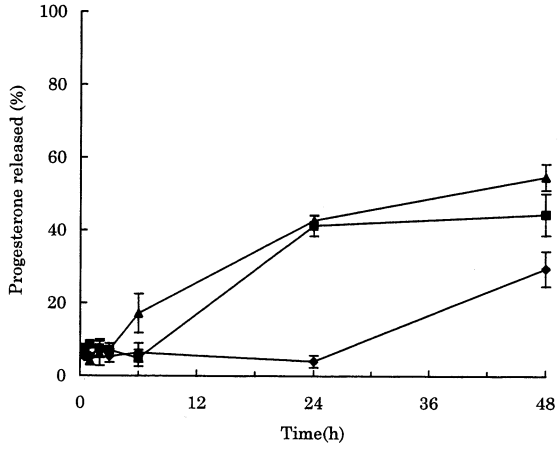

Fig. 2. In vitro release of progesterone from PLA–PEG–PLA nanoparticles (C-NP) in various media at low speed. Each point and bar represent the mean ± S.D. of three samples. Media are 0.1% (w/w) Polysorbate 80 (▲), 0.01% (w/w) Polysorbate 80 (■) and 50 mM phosphate buffer (pH 7.4) (◆). Polysorbate 80 is solved with 50 mM phosphate buffer (pH 7.4).

3.2.2. Effect of polymer composition

The release of progesterone from nanoparticles consisting of various copolymers was investigated in 0.01% Polysorbate 80 (Fig. 4). The drug release from copolymer B-NP (PEG content; 5.2%) was slow and almost the same as from homopolymer A-NP. The percent drug release of A-NP and B-NP in 24 h was 40 and 44%, respectively. The initial release from copolymer C-NP (PEG content; 10.3%) was faster than that from A-NP and B-NP. The percent drug release for C-NP in 24 h was 77%, about twice that for A-NP and B-NP.

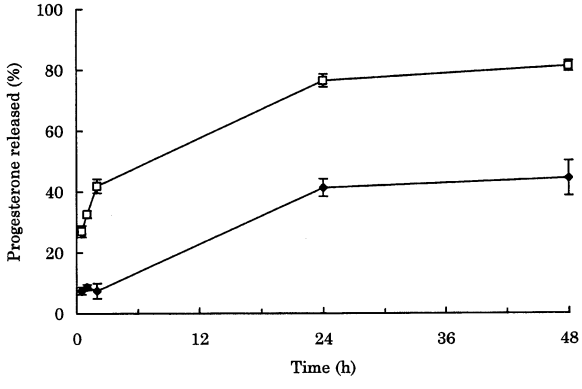

Fig. 3. In vitro release of progesterone from PLA–PEG–PLA nanoparticles (C-NP) under various conditions. Each point and bar represent the mean ± S.D. of three samples. Media are 0.01% (w/w) Polysorbate 80 at high speed (□), 0.01% (w/w) Polysorbate 80 at low speed (◆). Polysorbate 80 is solved with 50 mM phosphate buffer (pH 7.4).

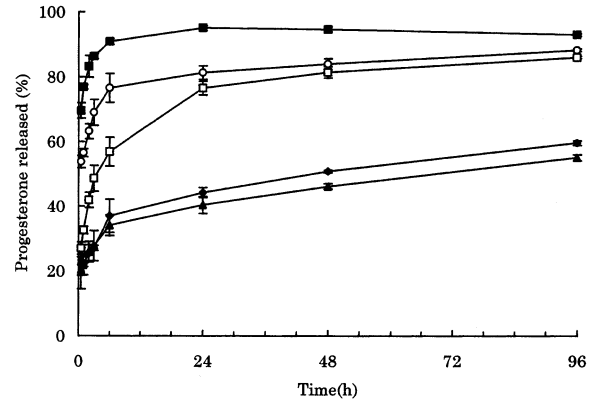

Fig. 4. In vitro release of progesterone from PLA–PEG–PLA nanoparticles with various amounts of PEG in their polymers in 50 mM phosphate buffer (pH 7.4) containing 0.01% (w/v) Polysorbate 80 at high speed. Each point and bar represent the mean ± S.D. of three samples. A-NP (◆), B-NP (▲),C-NP (□), D-NP (■), E-NP (○).

By comparing the drug release from C-NP, D-NP and E-NP, whose PEG contents were 10.3, 25.8 and 15.8%, respectively, it was found that the drug release increased as the PEG content increase. The release from D-NP was the fastest among all nanoparticles in this study, with 95% of the drug released within 24 h. Since the total Mw of copolymer D was the lowest in this study, it seemed that not only the high PEG content but also the low total Mw enhanced the drug release. Niwa et al. (1993) concluded that the molecular weight of polymer was a factor in controlling drug release.

The drug release from E-NP was about 20% higher than from C-NP in 6 h. Although the PEG content of E-NP (15.8%) was almost the same as that of C-NP (10.3%), the PEG Mw of copolymer E (20 000) was three times that of copolymer C (6600). Therefore, it was supposed that the initial release was greater the higher the PEG Mw.

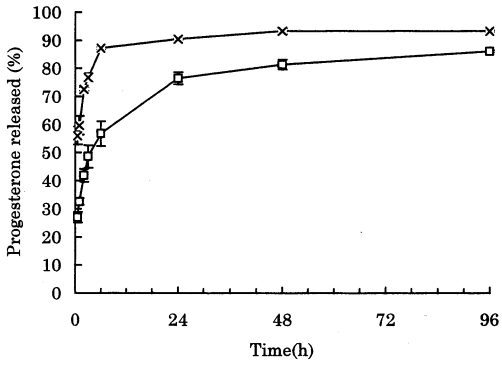

Fig. 5. In vitro release of progesterone from nanoparticles consisting of fractionated and unfractionated PLA–PEG–PLA containing 10% (w/w) PEG in 50 mM phosphate buffer (pH 7.4) containing 0.01% (w/v) Polysorbate 80 at high speed. Each point and bar represent the mean ± S.D. of three samples. C-NP (□), C-1-NP (X).

3.2.3. Effect of polymer fractionation

The fractionation of the polymer influenced the drug dissolution behavior especially in the early phase, as shown in Fig. 5. Gel permeation chromatograms of C-1 (before fractionation) and C (after fractionation) are presented in Fig. 6. The low molecular weight fraction of C copolymer was remarkably less than that of the unfractionated C-1 copolymer.

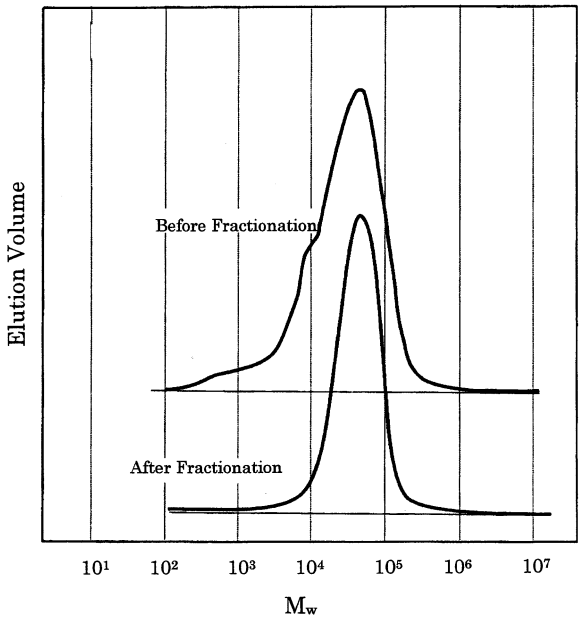

Fig. 6. Gel permeation chromatograms before (C-1) and after (C) fractionation.

The percent drug release from the fractionated copolymer C-NP and the unfractionated copolymer C-1-NP in the initial 0.5 h was 27 and 56%, respectively. The initial burst for C-NP was obviously lower than for C-1-NP. As shown in Table 1, since the monodispersity (Mw/Mn) and the total Mw differed between C-NP and C-1-NP, these two factors may explain why the release from C-1-NP was faster than from C-NP. It is supposed that the broad Mw/Mn, in other words, the oligomers of C-1-NP, caused the burst. Although it may not be always valid in ultra-thin layers such as surface of nanoparticles, the elimination of the low molecular weight fraction in polymer-bulk may prevent the burst. It is supposed that the low molecular fraction easily escapes from nanoparticles during the release test especially in the initial phase. In the case of the microsphere, it is known that the heptamer or smaller oligomers of lactic acid caused an increase in the initial burst (Okada et al., 1994). Regarding the PLA–PEG–PLA used in this study, it is supposed that the removed fraction in Fig. 5 consisted of heptamer or smaller oligomers of lactic acid and oligomers combined with PEG. On the removal of these oligomers, PLA–PEG–PLA nanoparticles may restrain the burst. On the other hand, Ogawa et al. (1988) showed the total Mw affects the drug release from microcapsules. Therefore, the monodispersity (Mw/Mn), in other words, the existence of the oligomers and total Mw are likely important parameters of drug release.

3.3. The change in the Mw of the copolymer and mean size of nanoparticles during the release test

The Mw losses of copolymer C (PEG content; 10.3%) and D (PEG content; 25.8%) after 96 h of release were 11 and 25%, respectively, although no Mw loss of homopolymer A was observed, as shown in Fig. 7.

No change in the mean diameter of homopolymer A-NP was observed while the mean diameter of C-NP decreased slightly, as shown in Table 4.

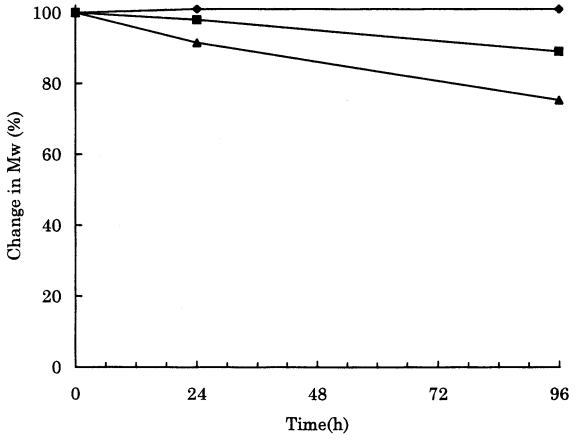

Fig. 7. Molecular weight loss of PLA–PEG–PLA copolymer during the release test in 50 mM phosphate buffer (pH 7.4) containing 0.01% (w/v) Polysorbate 80 at high speed. A-NP (◆), C-NP (■) and D-NP (▲).

The difference of the Mw loss and the mean diameter among the two PLA–PEG–PLA copolymers and homopolymer PLA suggests that the PEG segment enhance a slightly the decomposition of polymers during the release test.

As shown in Fig. 4, the release from nanoparticles showed a biphasic pattern in this study: a burst of drug release at the initial stage. It seems that Mw loss (Fig. 7) reflecting polymer decomposition does not contribute to the drug release since only slight Mw loss was observed during the initial main release period. Since there was no Mw loss nor size decrease for A-NP, it is supposed that the drug release from A-NP is caused by water permeation and drug diffusion through the PLA matrix. In the case of copolymer particles, since both the Mw loss and diameter decrease were slight, the reason why the drug release from PLA–PEG–PLA nanoparticles containing more than 10% PEG was faster than from homopolymer nanoparticles may be not only due to the enhancement of water permeation and drug diffusion through the polymer matrix brought about by the hydrophilic PEG segment but also due to the erosion of nanoparticles. However, the erosion of nanoparticles seemed to little affect the release because most of the drug was released at the initial stage in which only slight Mw loss was observed. That drug release would require only the partial hydrolysis of the macromolecular carrier as suggested by Peracchia et al. (1997) seems to support our result.

4. Conclusion

In conclusion, we studied progesterone-loaded PLA–PEG–PLA nanoparticles whose release appears to be controlled by the hydrophilic segments introduced into the hydrophobic parent PLA polymer. The amount of drug released was greater with the higher PEG content and Mw, and the lower total Mw of the polymers. Therefore, the drug release from nanoparticles was potentially controlled by PEG content, PEG Mw, and total Mw of the copolymer. Mw/Mn was also an important parameter in the control of drug release. We have previously confirmed that these nanoparticles possess the ability to evade capture by the reticuloendothelial system (Nakada et al., 1997). Studies into the preparation of nanoparticles which can release a drug at a suitable rate into the circulation are awaited.

Table 4
Mean size of progesterone-loaded PLA–PEG–PLA nanoparticles before and after the release test

Preparation no.	Lot of polymer	Diameter and size distribution[a] (nm)	
		Before the release test	After the release test
A-NP	A	248 ± 31	245 ± 72
C-NP	C	224 ± 85	201 ± 74

[a] Diameters and size distribution were measured with an electrophoretic light scattering spectrophotometer ($n = 1$).

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