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PREPARATION AND CHARACTERIZATION OF POLYLACTIDE-POLY(ETHYLENE GLYCOL)-POLYLACTIDE TRIBLOCK POLYMERS AND A PRELIMINARY *IN VIVO* EXAMINATION OF THE BLOOD CIRCULATION TIME FOR THE NANOPARTICLES MADE THEREFROM

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PREPARATION AND CHARACTERIZATION OF POLYLACTIDE-POLY(ETHYLENE GLYCOL)-POLYLACTIDE TRIBLOCK POLYMERS AND A PRELIMINARY *IN VIVO* EXAMINATION OF THE BLOOD CIRCULATION TIME FOR THE NANOPARTICLES MADE THEREFROM

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Key Words: Polylactide, Poly(ethylene Glycol), Triblock Polymers, Nanoparticles

ABSTRACT

Fractionated samples of polylactide-poly(ethylene glycol)-polylactide triblock copolymer were prepared and characterized. It was found that their molecular weights were related to the feed-ratios of monomer and initiator by the equation based on a sim-

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ple chain reaction model. Progestrone-containing nanoparti-cles prepared from the block copolymers were injected in rats to measure the retention of the medicine during their circulation in the blood. The results confirmed the general belief that the presence of hydrophilic groups in the particle is essential for extending the retention time and also showed that the initial concentration and retention half-life of progesterone are increased by using a fractionated base polymer for the particle.

INTRODUCTION

Polymeric nanoparticles are of interest to those who design injectable drug carriers which effectively deliver a medicine to a target organ or tissue site. A major issue in this design lies in how to prevent the nanoparticles from being trapped by the reticuloendothelic system (RES) as the Kupffer cells of the liver or the spleen macrophages [1]. The hydrophilicity of the particles is reported to play an essential role in depressing the trapping [2]. Gref *et al.* [3] synthesized a diblock copolymer consisting of poly (ethylene glycol) (PEG) and poly (lactide-co-glycolic acid) and showed that nanoparticles obtained by emulsifying its dichoromethane solution in water followed by evaporation of the solvent markedly enhances the blood circulation time of a medicine, i.e., greatly depresses trapping by RES. According to them, this result can be explained by an increase in surface hydrophilicity brought about by the migration of the PEG block to the particle surface during the emulsification. Their work has established a general belief that the trapping by RES can be reduced by nanoparticles made from a block copolymer consisting of hydrophilic and hydrophobic chains.

In this series of papers, we are concerned with relationship between typical molecular characteristics of a polylactide-poly(ethylene glycol)-poly-lactide triblock copolymer and the medicine retention of the nanoparticles made from it. The present paper focuses on the effect of molecular weight polydispersity and briefs a preliminary *in vivo* blood circulation experiment.

EXPERIMENTAL

Preparation and Characterization of Samples

Materials

L-lactide purchased from Boehringer had purity above 99.9% when examined by liquid chromatography, so we used it with no further purification.

PEG samples with molecular weight of 6000 (PEG#6000) and 20000 (PEG#20000) were purchased along with 1-decanol from Wako Chemical, Japan. Their polydispersity indexes M_w/M_n, where M_w and M_n are the weight-average and number-average molecular weights, respectively, were determined to be 1.2 by gel permeation chromatography. Tin (II) bis (2-ethylhexanoate) (SnOct) from Sigma Chemical was used as delivered.

Polymerization

Copolymerization of L-lactide with PEG or decanol were made by using the method of Leeslag and Pennings [4] after slight modifications.

In a typical experiment, prescribed amounts of the monomer L-lactide and an initiator alcohol PEG or decanol were mixed thoroughly, placed in a desiccator containing P_2O_5, and dried in vacuum for about 12 hours at room temperature. In a glove box purged by dry nitrogen, the mixture was transferred to a reactor vial that had been flame-dried for about 1 to 5 minutes, and a prescribed amount of chloroform containing 0.05 wt% SnOct was poured into the vial together with dry nitrogen. Then the vial was moved to an oil bath controlled at 130°C to get polymerization started. After 10 minutes, the reactant melted to the degree that it could be stirred vigorously by a motor equipped with an electric torquemeter. As the polymerization proceeded, the reactant became less transparent owing to crystallization of polylactide (PLA), solidified, and eventually was unable to be stirred. At this stage the reactant was annealed for about 400 minutes to allow the remaining monomers to react further, and then transferred to a water bath to stop the reaction.

It has been reported that hydroxy groups initiate ring-opening polymerization of L-lactide in the presence of SnOct, the polymer grows by a simple reaction mechanism, and the propagation is interrupted by chain transfer and ester-exchange [4, 5]. The interruption must be minimized to obtain polymers well-defined in molecular weight and low in polydispersity. Since it occurs more favorably as the reaction temperature is raised, it is advantageous for this purpose to lower the polymerization temperature. L-lactide melts at 98°C and begins decomposing at about 150°C, so that the polymerization has to be effected in the range between these two limits. However, since this range lies far below the crystallization point of PLA (ca 170°C), the reactant should gradually solidify as the polymerization proceeds, and the polymerization should stop before the monomers are consumed out. This final stage should come earlier at lower temperature, so that it is preferable to use a higher polymerization temperature in order to keep the monomers reacting longer. Thus, we have two oppo-

site requirements for the polymerization temperature for our system, and its choice becomes a matter of compromise.

After several preliminary experiments, we decided to choose 130°C as the polymerization temperature for all feed-ratios of PEG and lactide, with the catalyst concentration fixed at 0.05 wt% regardless of the hydroxy concentration of the initiator. Furthermore, we limited the annealing after solidification of the reactant to less than 500 minutes for the reason mentioned later. We also found it essential to dry the reactant and the vial thoroughly in order to prevent the formation of PLA homopolymer which is initiated by residual water.

Fractionation

A given crude copolymer sample, called f-0, was dissolved in chloroform at 1-3 wt% concentration, and a chloroform-methanol mixture was added slowly to it at 30°C under stirring until it got turbid. The solution turned almost transparent when warmed to 40-45°C, but reverted turbid upon slow cooling to 30°C or below and remained turbid even after standing for 12 hours. However, it got phase-separated when centrifuged at 7000 rpm for 0.5 to 2 hours. The gel phase taken out of the centrifuge tube was diluted with chloroform, and the polymer was precipitated by addition of methanol. A fraction of the polymer so obtained is hereafter called the first fraction and denoted by f-1. The supernatant phase was again subjected to further fractionation in the same way with for f-0 and the second fraction denoted by f-2 was precipitated from the new gel phase, and all the residue extracted from the new supernatant was called the third fraction f-3. For all crude samples thus fractionated the second fraction f-2 dominated about 80% of the total weight so that we regarded it as the main product from our polymerization experiment and used it for the molecular characterization described below.

Molecular Characterization

Gel permeation chromatograms for all samples including crude ones were obtained on a Toyo Soda HCL-802A analyzer equipped with a differential refractometer, and M_w and the polydispersity index M_w/M_n were calculated by the conventional methods. The instrument was calibrated with standard narrow-distribution polystyrene and PEG samples having known molecular weights. ^{1}H NMR measurements at 300 MHz were made in an about 10 wt% $CDCl_3$ solution at room temperature by use of a Brucker AM 300 spectrometer.

Preparation of Nanoparticles and *in-vivo* Experiment Materials

Progesterone was purchased from Sigma Chemical, and dichloromethane and HPLC grade methanol were from Wako Pure Chemical. Polyvinyl alcohol (PVA) from Kraray, Japan was used as supplied.

Making Nanoparticles

In this preliminary experiment, we used the method of Youxim [6] to make three kinds of nanoparticles containing progesterone from a PLA fraction (P-1), a crude PLA-PEG-PLA copolymer (P-2), and the second fraction from the latter (P-3). The process consisted of emulsifying an aqueous PVA by dropwise addition of 4 ml of dichloromethane containing 1 g/l progesterone and 10 g/l copolymer, followed by complete evaporation of the dichloromethane. Progesterone left unloaded in the particles was removed by gel permeation chromatography using a BioRod Ecomo-Pac 10DG. The mean diameter and trapping efficiency for progesterone of the particles were determined by the methods described previously [7].

In-vivo Experiment

A 0.5 wt% aqueous PVA dispersing progesterone-containing nanoparticles was injected into the tail vein of 5 week-old male ddYs, with the dose controlled to 1 mg per 1 kg of rat. The test animals were sacrificed after prescribed times and the progesterone concentrations in the plasma were determined by high pressure liquid chromatography.

RESULTS AND DISCUSSION

Polymer Characteristics

Table 1 shows some typical data for a series of the main products (the second fractions) at varying feed-ratios of L-lactide and PEG or 1-decanol.

Figure 1 illustrates the annealing time dependence of M_w for samples a5 and a10. It is seen that even after 400 hours annealing, M_w keeps increasing, i.e., no complete consumption of lactide monomers is yet attained. We observed that the reactant changed color and M_w for some samples began decreasing after annealing over 500 hours. This was the reason why we decided to stop annealing at or less than 500 hours in all cases.

TABLE 1. Feed Compositions and Results from Molecular Characterizations

Sample	Feed Ratio/ wt%			SnOct	$M_w\ 10^{-4}$	M_w/M_n	PEG wt%
Code	PEG or 1-decanol		L-lactide	/ wt%			from NMR
a03	PEG#6000	0.3	99.7	0.05	110	1.15	0.40
a1	PEG#6000	1.0	99.0	0.065	50.1	1.50	
a5	PEG#6000	5.0	95.0	0.05	14.7	1.82	5.2
a6	PEG#6000	6.0	94.0	0.67	6.6	1.14	
a10	PEG#6000	10.0	90.0	0.05	11.9	1.62	10.3
a30	PEG#6000	30.0	70.0	0.05	3.7	1.37	
a40	PEG#6000	40.0	60.0	0.05	2.9	1.58	39.56
a49	PEG#6000	49.0	51.0	0.05	2.9	1.09	
a70	PEG#6000	70.0	30.0	0.05	2.3	1.12	
b13	PEG#20000	13	87	0.05	12.1	1.25	
b30	PEG#20000	30	70	0.05	5.3	1.11	
b49	PEG#20000	49	51	0.05	5.5	1.08	
b70	PEG#20000	70	30	0.05	4.3	1.43	
c005	decanol	0.05	99.9	0.05	10.8	1.30	
c01	decanol	0.11	99.8	0.05	12.9	1.51	
c03	decanol	0.31	99.6	0.05	7.9	1.21	
c15	decanol	1.5	98.5	0.05	1.7	1.40	
c41	decanol	4.1	95.9	0.05	0.8	1.30	

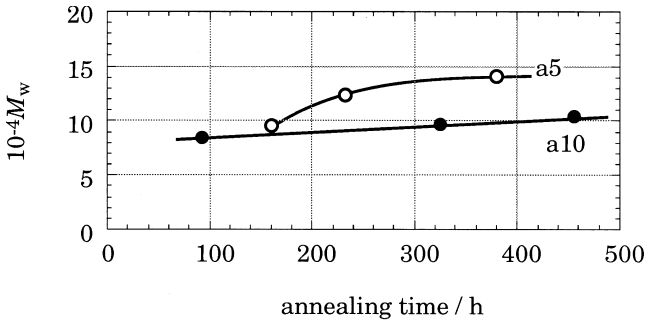

Figure 1. Anneal time dependence of the molecular weight in poly(ethylene glycol initiated polymerization of L-lactide for samples a5 and a10 (feed ratios of PEG#6000 and 5 and 10 wt%, respectively). The annealing started after the reactant was solidified due to crystallization of polylactide.

Figure 2 shows ^{1}H NMR spectra for samples a03 and a40. The peaks around 4.2 ppm for a40 are assignable to the methylene protons in the PEG repeat units linked to the lactide moiety [8], evidencing that lactide starts polymerizing from the hydroxy end groups of PEG. As the PEG feed-ratio was lowered below 10 wt%, these peaks got hardly measurable, probably due to the decrease in the number of PEG-PLA linkages relative to that of PEG units. However, we did not take this phenomenon to indicate the termination of the polymerization reaction.

In Figure 2, each peak at 3.65 ppm is due to the methylene protons in the PEG repeat units not directly adjacent to the chain ends. Remarkably, this peak for a03 is observable even at as low a PEG feed-ratio as 0.3 wt%, indicating that it is very sensitive to the presence of PEG. Its relative intensities for four samples, given in the last column in Table 1, are close to the corresponding feed-ratios of PEG, suggesting that the feed-ratio may be identified with the composition ratio for all samples. The peaks other than those above can be assigned to the polylactide moiety [8].

Figure 3 compares GPC curves for sample a10 before and after fractionation. Two shoulders assignable to L-lactide monomer and oligomer are seen in the curve for the crude sample, but disappear in that for the fractionated one. If the areas under each curve right and left to $M_w = 1000$ are denoted by P_m and P_s,

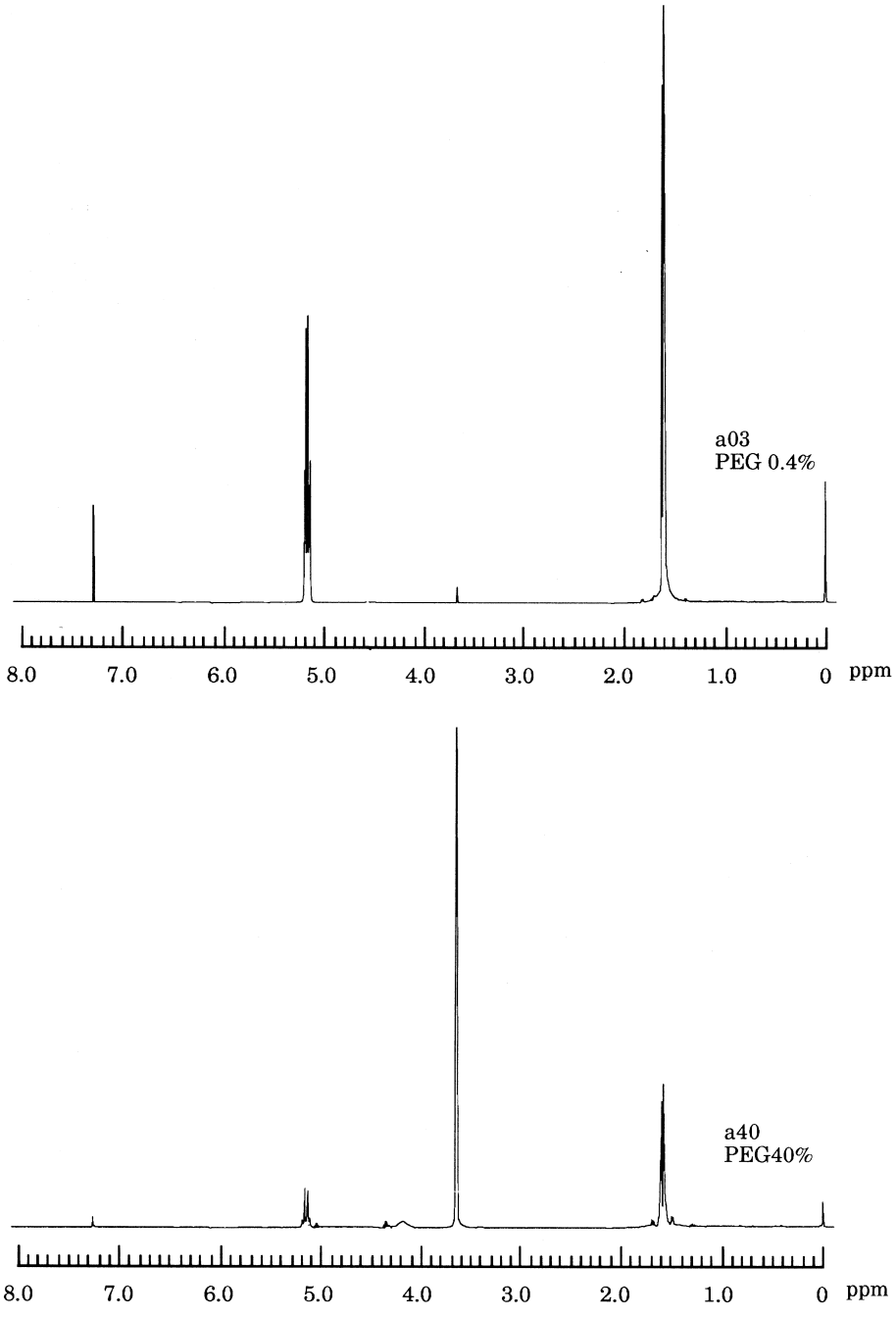

Figure 2. Proton NMR spectra for polylactide-poly(ethylene glycol)-polylactide triblockpolymers obtained with polyethylene glycol feed-ratios of 0.4 wt% (upper) and 40 wt% (lower).

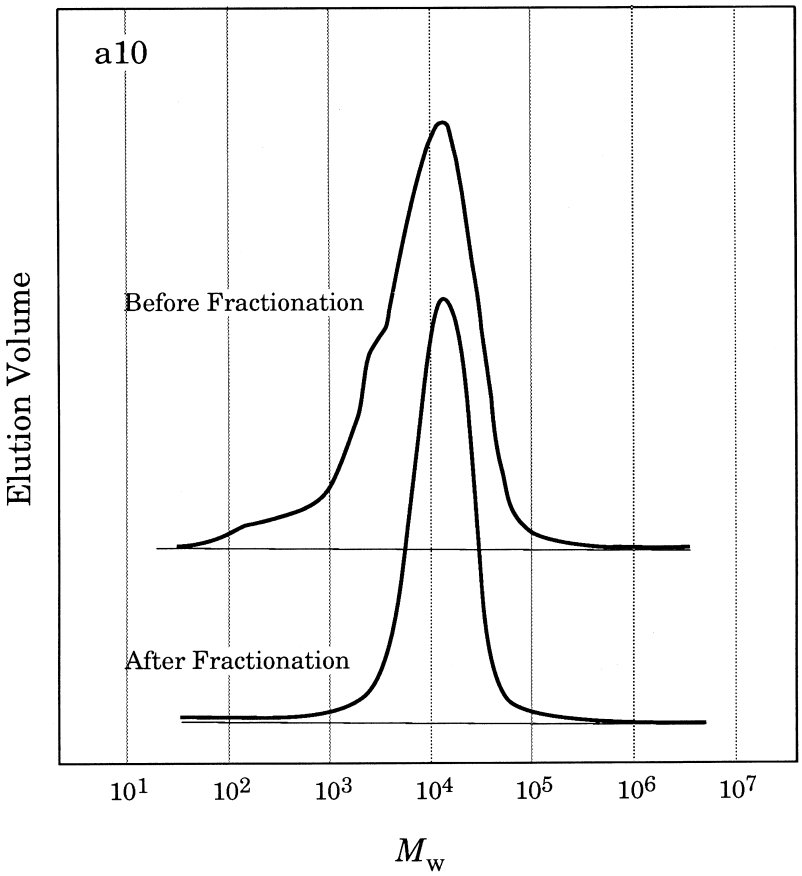

Figure 3. Gel permeation chromatograms after and before fractionation for sample a10.

respectively, we may regard P_s/P_m as a measure of the amount of residual monomers in the sample. In Figure 3, this ratio is 0.08 and 0 for the crude and fractionated samples, respectively.

Figure 4 illustrates for sample a1 how the weight-average molecular weight, polydispersity index, P_s/P_m, and PEG content varied as the sample was fractionated. It is seen that, as is usually the case, M_w decreases monotonically with fractionation, but the accompanying change in polydispersity index is not regular. While P_s/P_m is zero for f-1 and f-2, it is higher for f-3 than for f-0. This implies that the residual monomer in f-0 is concentrated in f-3. The PEG content

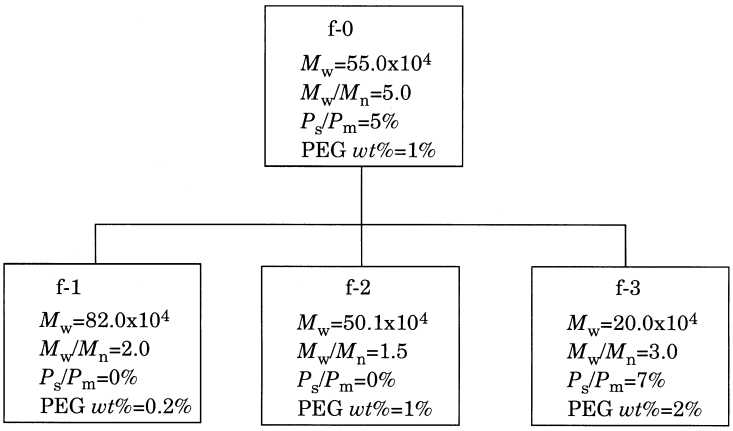

Figure 4. Fractionation effects on the characteristics of sample a1. F-0 denotes the crude sample, and f-1, f-2, and f-3, the first, second, and third fraction, respectively.

is lower in f-1 than in f-0, and higher in f-3 than in f-2. Thus, PLA homopolymers or copolymers relatively rich in PLA were retained in the gel phase in the first fractionation, while PEG homopolymers or copolymers relatively rich in PEG were purged to the supernatant phase in the second fractionation. This behavior is consistent with the fact that chloroform is a good solvent for both PEG and PLA, but methanol is a poor solvent for PLA but a good solvent for PEG. Both f-0 and f-2 have equal PEG contents, which implies that even if by-products such as PLA homopolymers and PLA-PEG diblock copolymers were produced, they were effectively expelled from the main product by fractionation.

Simple Chain Reaction Model

Following the reported information about the polymerization of L-lactide, [4, 5, 9] we assume a simple chain reaction (SCR) model for the growth of lactide chains from the hydroxy end groups of a PEG chain. This model assumes that the polymerization is induced by a single initiation mechanism and proceeds at a single propagation rate, regardless of the kind of alcohol and with no side reactions such as chain transfer, ester exchange, and cage effect taking place. It allows us to predict that the PLA-PEG-PLA block copolymer produced

should be monodisperse in molecular weight (and in composition too) and have PLA chains with equal lengths and its molecular weight, M_{poly}, is given by

$$M_{poly} = M_a + \frac{m_l}{m_a} M_l \quad (1)$$

Here, M_a and M_l are the molecular weights of PEG and lactide, respectively, and m_a and m_l the moles of fed PEG and lactide, respectively. Equation 1 may be rewritten

$$\frac{M_{poly}}{M_a} = 1 + \frac{W_l}{W_a} \quad (2)$$

with W_l and W_a denoting the weight fractions of fed lactide and PEG.

Figure 5 shows a test of Equation 2 with our experimental results, with M M_{poly} replaced by M_w. The data points for fractionated samples are fitted fairly well by the calculated curve, but those for unfractionated ones are not. Thus,

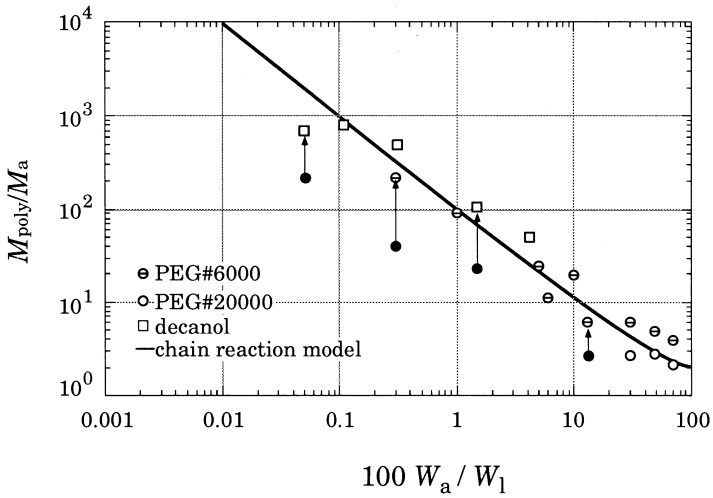

Figure 5. The feed-ratio dependence of the molecular weight for the main products (fractionated samples) with different initiators. The solid line shows theoretical values calculated from Equation 2. For comparison, the molecular weights before fractionation for the same samples are presented with filled circles and the arrows show change in molecular weight by fractionation.

the main products satisfy the feed-ratio dependence of M_{poly} predicted by the SCR model. Comparison of the third and last columns in Table 1 also substantiates this conclusion. However, the data in the seventh column of Table 1 show the failure of the main products to obey another prediction of the SCR model, giving M_w/M_n significantly larger than unity except for a few. It is surprising that nonetheless, our main products follows Equation 2 fairly well. As mentioned in the experimental section, PLA crystallizes during the polymerization with PEG. This process alters the nature of the reactant system and should deviate the chain propagation reaction from the ideal one as assumed by the SCR model. Probably, it is mainly responsible for broadening the molecular weight distribution of our main products. Large polydispersity indexes of the crude samples are undoubtedly due to the presence of monomers left unreacted. We have left for future work the prediction concerning the lengths of PLA blocks in the copolymers.

Characteristics of Nanoparticles and Blood Circulation Test

Table 2 gives some relevant data for the three nanoparticles prepared. A very large polydispersity index of crude sample a10 suggests considerable contamination with by-products as well as monomers and oligomers. For the three particles the mean diameter and the trapping efficiency appear to have little correlation to the polydispersity index and PEG content of the base copolymer.

Figure 6 shows for the three particles and free progesterone how the progesterone concentration decreased during the circulation in the blood, and Table 3 gives the half-lives of the decrease estimated from the slope of the straight line fitted to the indicated data points by the least-squares method. It is seen that the retention time of progesterone during blood circulation markedly increases when the medicine is loaded in these nanoparticles. However, the half-

TABLE 2. Typical Characteristics of the Base Polymers and the Nanoparticles

Nanoparticle	Mean Diameter / nm	Trapping Efficiency / %	Base Polymer	$10^{-4}M_w$	M_w/M_n	PEGwt%
P-1	205	65	c01 (fraction)	12.9	1.51	0
P-2	152	64	a10 (crude)	8.3	11.7	10.5
P-3	197	49	a10 (fraction)	11.9	1.62	10.3

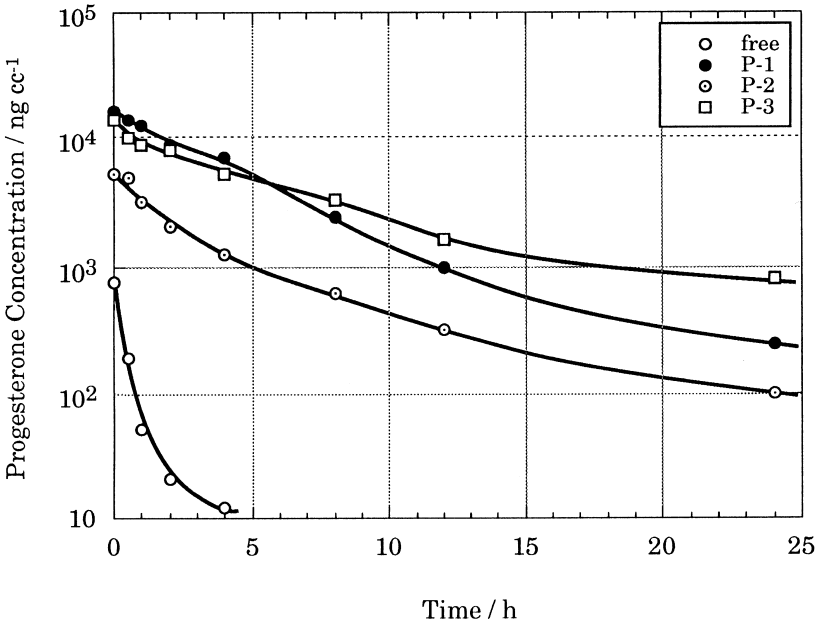

Figure 6. Decay of progesterone concentration with time during blood circulation after administrating rates for the nanoparticles P-1, P-2, and P-3 and progesterone alone.

life for P-1 containing no PEG is significantly shorter than those for P-2 and P-3 containing about 10 wt% PEG. This difference is consistent with the finding of Gref *et al*. [3] as referred to in the Introduction. It is also observed that, despite both have the same PEG content, the half-life for the fractionated sample P-3 is shorter by about 2 hours than that for the crude sample P-2. This result suggests that the molecular weight polydispersity of the base polymer for the nanoparticle has something to do with the drug retention of the particle. Similar results are being accumulated in our laboratory and will be reported in due time.

TABLE 3. Half-Lives of Progesterone Retention in Blood Circulation

Sample	Half Life / h
progesterone alone	2.1
P-1	3.9
P-2	6.0
P-3	7.9

The initial progesterone concentration is higher for P-1 and P-3 than for P-2., which indicates that the initial burst rate of the particle is slower for the first two than for the last, and appears to be ascribed to the fact that the base polymers for P-1 and P-3 were fractionated samples but that for P-2 was a crude one. In this connection it may be worth mentioning that the presence of low-molecular weight compounds in bulk polymers weakens mechanical strength.

It can be seen from Figure 6 that when compared after the circulation of one day, P-3 has the progesterone concentration higher than P-2 by one order and P-1 by a half order. This marked difference suggests that the reduced polydispersity of the base polymer for the particle serves to decrease the frequency of injection needed to keep a drug concentration in the blood. For example, if 10^3 mg/cc is a critical concentration for drug-efficiency, only one time injection a day will be enough with P-3, while three or four time injection will be needed with P-2 and two time one with P-1.

CONCLUSION

In summary, the present study confirms that the incorporation of hydrophilic chains into a nanoparticle greatly enhances the drug retention time during the circulation in the blood, and the initial concentration and retention half-life of the drug have definite correlation with the molecular weight polydispersity of the base polymer for the particle.

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