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In vitro and in vivo characterization of nanoparticles made of MeO-PEG amine/PLA block copolymer and PLA

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Abstract

The preparative method of a block copolymer of poly(DL-lactic acid) (PLA) and methoxypolyethylene glycol amine (MeO-PEG(N)), named PLA–(MeO-PEG), was refined. The degree of introduction of MeO-PEG(N) into PLA increased up to 55% (mol/mol) using a dichloromethane/methanol mixture (1:1, v/v) as a solvent at the reductive amination and taking all the fractions of the first peak in gel-chromatography. Plain and 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine (DiD)-loaded nanoparticles prepared using the PLA/PLA–(MeO-PEG) mixture of 45:55 (mol/mol) showed a mean size of 113 and 154 nm, respectively, and a positive zeta potential in water. DiD solution, i.v. administered, showed a lower plasma level and high distribution in liver, though DiD was distributed into the blood cells to a fair extent. Nanoparticles exhibited a higher plasma concentration of DiD than the DiD solution at 1 and 8 h, though DiD was distributed into the liver and spleen to a fair extent. Nanoparticles made of the PLA/PLA–(MeO-PEG) mixture of 44:55 (mol/mol) showed better plasma retention than those made of the PLA/PLA–(MeO-PEG) mixture of 64:36 (mol/mol). It is suggested that the PLA/PLA–(MeO-PEG) mixture nanoparticles with a higher PEG/PLA ratio should be useful as a carrier for the elevation of the plasma concentration of lipophilic drugs.

Keywords: PLA–(MeO-PEG) block copolymer; Nanoparticle; Biodistribution; DiD

1. Introduction

Recently, polymeric nanoparticles, liposomes, solid-lipid nanoparticles etc. have been extensively investigated to optimize medication with drugs including drug targeting (Yang et al., 1999; Nakanishi et al., 2001; Charrois and Allen, 2004; Brannon-Peppas and Blanchette, 2004; Shenoy et al., 2005; Abraham et al., 2005). Polymeric nanoparticles have attracted much attention because the chemical modification of their polymers is possible, leading to nanoparticles with various biological behaviors. Chemical modification of PLA or PLGA, being biocompatible and biodegradable, has been made actively in an attempt to modify the physicochemical and biological properties of the nanoparticles (Otsuka et al., 2000; Nagasaki et al., 2001; Yamamoto et al., 2001; Jule et al., 2003; Miura et al., 2004).

The particle size, electrical charge and surface structure affect the biological behaviors of micro- and nano-particulate dosage forms (Sugibayashi et al., 1979; Kanke et al., 1980; Unezaki et al., 1995; Tabata et al., 1998; Ishida et al., 1999). For nanoparticulate dosage forms, in particular, the surface structure is a very important factor determining biological fate in the body. Plain PLA or PLGA nanoparticles administered intravenously are delivered quickly and mostly into the liver and spleen via the reticuloendothelial system (Gref et al., 1994; Dunn et al., 1997; Mosqueira et al., 1999; Panagi et al., 2001). However, polyethylene glycol–polypropylene glycol–polyethylene glycol block copolymers such as poloxamer are useful for raising the systemic retention of nanoparticles with PLA as a core component (Dunn et al., 1997; Onishi et al., 2003; Machida et al., 2003). Polyethylene glycol–PLA block copolymer (PEG–PLA), which is a diblock copolymer with hydrophilic and hydrophobic blocks, has been reported to allow the formation of nanoparticules more stable in biological circumstances. In PEG–PLA nanoparticles, PLA chains form the core and PEG chains are located on the outer side. PEG–PLA nanoparticles vary in size from several dozen to a few hundred nanometers, and possess a hydrophilic

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and inactive surface of PEG, leading to a longer systemic circulation (Gref et al., 1994; Bazile et al., 1995; Yamamoto et al., 2001; Panagi et al., 2001; Avgoustakis et al., 2003; Miura et al., 2004). The PEG shell prevents PLA core from interacting with biomolecules, cells and tissues (Gref et al., 1994; Bazile et al., 1995; Mosqueira et al., 1999; Panagi et al., 2001), and suppresses opsonization (Nguyen et al., 2003). Recently, Kataoka et al. have reported various kinds of PEG–PLA nanoparticles with modified surface (Otsuka et al., 2000; Nagasaki et al., 2001; Yamamoto et al., 2001; Jule et al., 2003), in which sugars, amino acids, peptides etc. are attached to the PEG terminal of PEG–PLA. In these derivatives, an acetal-ended PEG-PLA was synthesized by sequential polymerization of ethylene oxide and DL-lactide, and utilized for further modification. On the other hand, we developed a novel approach to obtain PLA derivatives (Sasatsu et al., 2005). Our method involves the synthesis of acetal-ended PLA (PLA-acetal), its conversion to PLA with a formyl terminal end (PLA-aldehyde), and the modification of the formyl group using reductive amination. Namely, ring polymerization of DL-lactide using diethoxypropanol as an initiator was performed to obtain PLA-acetal. PLA-acetal was subsequently hydrolyzed to PLA with a formyl group, then PLA-aldehyde was reacted with the molecules having amino groups by reductive amination. As this approach allows the modification of PLA directly, various kinds of PLA derivatives are expected to be produced. A block copolymer of PLA and methoxypolyethylene glycol amine (MeO-PEG(N)), called PLA–(MeO-PEG), was reported previously to be prepared by reductive amination between PLA-aldehyde and MeO-PEG(N), but the degree of introduction of MeO-PEG(N) into PLA was not necessarily reproducible or extensive (Sasatsu et al., 2005). In the present study, the preparative conditions of PLA–(MeO-PEG) were refined to improve the introduction of MeO-PEG(N) into PLA. Further, as the present PLA–(MeO-PEG) has a secondary amino group, different from the conventional block copolymer made of PLA and methoxypolyethylene glycol (Gref et al., 1994; Bazile et al., 1995; Quellec et al., 1998), the nanoparticles prepared here were characterized in vitro and in vivo. A lipophilic fluorescent dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine (DiD) perchlorate was used as a model drug. As DiD is a very lipophilic, it is considered possible to evaluate the incorporation properties of the nanoparticles for lipophilic drugs. Further, since DiD seems to be incorporated stably in the core of PLA or PLA–PEG nanoparticle and little diffused to the outer aqueous solution (Mosqueira et al.), the pharmacokinetic features of the nanoparticles may markedly affect the biodistribution of DiD. The present study has dealt with the loading properties of the PLA/PLA–(MeO-PEG) mixture nanoparticles for DiD and the effect of the nanoparticles on the biodistribution of DiD.

2. Materials and methods

2.1. Materials

Methoxypolyethylene glycol amine (MeO-PEG(N); MW 2000), 3,3-diethoxy-1-propanol and stannous octoate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). DL-Lactide was obtained from Tokyo Kasei Kogyo Co., Ltd. (Japan). 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine (DiD) perchlorate was purchased from Molecular Probes, Inc. (OR, USA). All other chemicals used were of reagent grade.

2.2. Animal

Male ddY mice (6–7 weeks old, 30 g) were purchased from Tokyo Laboratory Animals Science Co., Ltd (Japan), and soon used for animal experiments. They were kept on the breeding diet MF (Oriental Yeast, Japan) with water ad libitum at room temperature maintained at $23 \pm 1\,^{\circ}C$ and a relative humidity of $60 \pm 5\%$. The experimental protocol was approved by the Committee on Animal Research of Hoshi University, Tokyo, Japan, and the animal experiments were performed in compliance with Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, Japan.

2.3. Polymer synthesis

Acetal-ended poly(DL-lactic acid) (PLA-acetal) was synthesized with ring opening polymerization, and aldehyde-ended poly(DL-lactic acid) (PLA-aldehyde) was obtained by acidic hydrolysis of PLA-acetal according to a previous paper (Sasatsu et al., 2005). Briefly, 6 g of DL-lactide purified by recrystallization from an ethyl acetate solution and 3,3-diethoxy-1-propanol (50 mg) were placed in a three-neck flask, the inside of which was filled with nitrogen gas in advance, 45 ml of toluene was added, and the mixture was stirred and heated on an oil bath set at 160 °C with stirring. After the mixture reached a set temperature, a toluene solution (5 ml) of stannous octoate (30 μg) was added. Then, the reflux was continued at the set temperature with stirring for 5 h. After that, the stirring was stopped, and the mixture was left at room temperature for 24 h. The solvent was evaporated at 60 °C under reduced pressure, 120 ml of dichloromethane was added to the residue, and the mixture was filtrated with a glass filter. The filtrate was put into 200 ml of water stirred at 60 °C, and the dichloromethane was evaporated. The precipitate was obtained by filtration, re-dissolved in dichloromethane, and dried on anhydrous sodium sulfate. After the sodium sulfate was removed by filtration, the filtrate was evaporated to dryness to yield PLA-acetal. Although the product contained PLA-acetal and PLA, the product was named PLA-acetal(*n*), in which *n* was a lot number. The product was used in the following experiment without further purification.

PLA-aldehyde was obtained by acidic hydrolysis of PLA-acetal. PLA-acetal(*n*) (200 mg) was dissolved in 80 ml of acetone, and 2% (w/v) hydrochloric acid (30 ml) was added. The mixture was stirred at room temperature for 24 h, and acetone was removed by evaporation to precipitate the product. After the supernatant was removed, the precipitate was dissolved in 80 ml of acetone. Then, 2% (w/v) hydrochloric acid (30 ml) was added to the solution, and the stirring and precipitation were repeated twice more in the same way. The final precipitate was washed with water, and dried in vacuo to yield PLA-aldehyde. Although the product contained PLA-aldehyde and PLA, the mixture was named PLA-aldehyde(*n*), in which *n* was a lot num-

ber. The product was used in the following experiment without further purification.

Methoxypolyethylene glycol amine-poly(DL-lactic acid) block copolymer (PLA–(MeO-PEG)) was obtained by reductive amination between PLA-aldehyde and MeO-PEG(N). Namely, PLA-aldehyde(*n*) (200 mg), MeO-PEG(N) (300 mg) and sodium cyanoborohydride (25 mg) were put in 25 ml of a mixture of dichloromethane and methanol (1:1, v/v), and the mixture was stirred at room temperature for 24 h, when the pH of the mixture was adjusted to 6–7 with 0.1N hydrochloric acid aqueous solution and 0.1N sodium hydroxide aqueous solution. After organic solvents were evaporated, the resultant suspension was lyophilized. Chloroform was added to the product, and the supernatant was subjected to gel permeation chromatography (GPC). In the separation by GPC, three kinds of ways of separating the product were applied (Fig. 1). In one separation, the fractions of the start point to the peak top in the first peak were collected (A in Fig. 1). In the other cases, the fractions of the start point to the half point of the decreasing curve and those of the start point to the end point were gathered for the first peak (B and C, respectively, in Fig. 1). The fractions collected were evaporated, then the residue was dissolved in acetone. The solution was suspended by addition of water, and acetone was evaporated. Then, the suspension was washed by ultrafiltration using a membrane with a MW cut off of 10,000. This sequential operation of dissolution, precipitation and ultrafiltration was repeated twice more. The final aqueous suspension was lyophilized to yield PLA–(MeO-PEG). Although the product contained PLA–(MeO-PEG) and PLA, the product was named PLA–(MeO-PEG)(*n*), in which *n* was a lot number. The PLA–(MeO-PEG)(*n*), composed of PLA–(MeO-PEG) and PLA, was used in the following experiment without further purification.

2.4. ^{1}H NMR spectroscopy and gel-permeation chromatography

Chemical structures of PLA derivatives were confirmed from ^{1}H NMR spectra obtained with a JEOL JNM-GX270 spectrometer (JEOL, Japan). The degree of polymerization of the PLA moiety in the PLA derivatives was calculated from the ^{1}H NMR spectra by comparing the integrated intensity of the methine proton of the terminal lactic group of the PLA chain to that of the methine proton of the inside lactic groups of the PLA chain. The number-average molecular weight (MWn) was also calculated using the degree of polymerization. The proportion of the diethylacetal, formyl or MeO-PEG(N) group introduced into PLA was calculated based on the ^{1}H NMR spectrum. That is, the extent of the introduction was calculated by comparing the integrated intensity of the specific proton of each moiety with that of the methine proton of the terminal lactic group of the PLA chain.

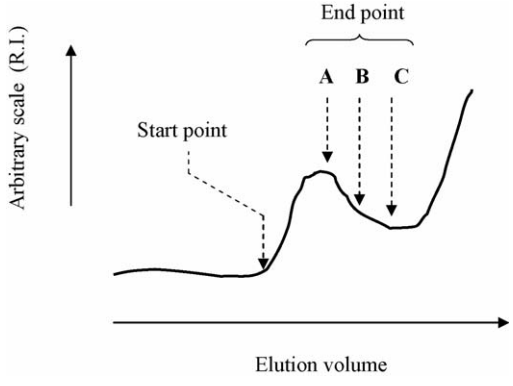

Fig. 1. Separation manner of the product after reductive amination between PLA-aldehyde and MeO-PEG(N). The starting point for collection is the same, but the end point for the fractions collected are different (A, B and C).

GPC was performed at room temperature to purify the polymer products. As reported previously (Sasatsu et al., 2005), the GPC system using a column of GPC K-800 (8 mm in inner diameter × 300 mm in length; Shodex, Japan) or GPC K-2003 (20 mm in inner diameter × 300 mm in length; Shodex, Japan) with chloroform as an elution solvent was useful for the purification of PLA derivatives. A Shimadzu LC-6AD equipped with a refractive index detector (Shimazu RID-10A) was used, when the flow rates of the elution solvent were 1 and 3.5 ml/min for GPC K-800 and GPC K-2003, respectively. Several polystyrenes (PSs) of known MW (Showa Denko K.K., Japan) were used as standard markers for MW.

2.5. Preparation of plain and DiD-loaded nanoparticles

In the present study, plain nanoparticles (nanoparticles not containing DiD) and DiD-loaded nanoparticles were prepared using PLA–(MeO-PEG)(n). The plain nanoparticles were prepared by the solvent diffusion method as follows. PLA–(MeO-PEG)(n) (30 mg) was dissolved in a mixture of 2 ml of acetone and 1 ml of ethanol, and dropped gradually into 5 ml of water stirred gently. Then, an aqueous suspension of particles was obtained by evaporation of the organic solvents at 18 °C under reduced pressure. DiD-loaded nanoparticles were prepared as follows. PLA–(MeO-PEG)(n) (30 mg) and 1 mg of DiD were dissolved in a mixture of 1 ml of acetone and 0.5 ml of ethanol, then dripped into 2.5 ml of water stirred gently. The organic solvent was evaporated at 18 °C under reduced pressure to obtain a suspension of nanoparticles. The nanoparticles were separated from free DiD by gel-filtration with a Sephadex G-50 column (2.5 cm × 15 cm) using 0.45% (w/v) NaCl as an elution solvent. The eluted nanoparticles were characterized for the drug content and encapsulation efficiency. Namely, a specified volume of the obtained suspension of DiD-loaded nanoparticles was dried. The residue was dissolved in acetone, and the supernatant was measured spectrophotometrically at 644 nm to determine the amount of DiD contained. Further, after the residue was dissolved in $CDCl_3$, a specified amount of 4′-methoxyacetophenone was added, and the supernatant of the mixture was checked by ^{1}H NMR spectroscopy. The amount of polymer was determined by comparing the integrated intensities of the methine proton of the PLA chain and the methyl proton of the acetyl group of 4′-methoxyacetophenone, which also gave the recovery of the polymer (PLA and PLA–(MeO-PEG)) in the nanoparticles. The incorporation efficiency of DiD was calculated as the ratio of the observed DiD content to its ideal content.

2.6. In vitro characterization of nanoparticles

The particle size and zeta potential of the nanoparticles were measured using an ELS-800 dynamic light scattering apparatus (Otsuka Electronic Co., Ltd, Japan) after the aqueous suspension of nanoparticles was diluted adequately with water. The morphological examination of nanoparticles was done by transmission electron microscopy (TEM) using a JEOL JEM-1011 transmission electron microscope (JEOL, Japan). Observations were made after the nanoparticles were stained using a 3% (w/v) uranyl acetate aqueous solution and placed on copper grids.

Furthermore, release of DiD from the nanoparticles was investigated in vitro. Namely, suspensions with three different particle concentrations of DiD-loaded nanoparticles, 0.84, 2.1 and 4.2 mg/ml, were incubated in a mixture of PBS and EtOH (7:3, v/v) at 60 strokes per min at 37 °C. At 1, 5 and 24 h after incubation, the samples were taken and centrifuged at 40,000 rpm for 20 min. The supernatant was adequately diluted, and the absorbance was measured at 644 nm using a Beckman DU-640 spectrophotometer to determine the amount of DiD released.

2.7. Biodistribution experiments

DiD-loaded nanoparticles (18 μg DiD equiv.) suspended in 0.3 ml of 0.45% (w/v) NaCl, and DiD solution (18 μg DiD equiv.) in 0.04 ml of a mixture of DMSO and saline (10:1, v/v) were injected intravenously as a bolus via the tail vein into mice. At 1, 8 and 24 h after the injection, the mice were sacrificed by dislocation of the cervical vertebrae, and blood was taken from the heart using a heparinized syringe, and the kidney, spleen, lung and liver were excised. After the plasma was obtained by centrifugation of the blood at 3000 rpm for 10 min, 1 ml of acetone was added to 0.1 ml of plasma. After the mixture was stirred with a vortex mixer for 3 min, the supernatant was separated by centrifugation of the mixture at 3000 rpm for 15 min. As for each organ excised, after it was wiped with filter paper, it was put in saline of the same weight, and the mixture was homogenized using a glass homogenizer with a Teflon pestle. Then, the homogenate was diluted 5.5-fold (v/v) by addition of acetone, and the resultant mixture was stirred with a vortex mixer for 3 min. The mixture was centrifuged at 3000 rpm for 15 min to obtain the supernatant. The supernatant was diluted adequately and measured fluorometrically using a JASCO FP-777 spectrofluorometer (JASCO, Japan) with excitation and emission wavelengths of 644 and 667 nm, respectively. The recovery experiments were performed by adding specified amounts of DiD-loaded nanoparticles to fresh plasma or organs, preparing the sample in the same manner as in the tested sample, and measuring the concentration of the supernatant. The amounts calculated from the concentration of the supernatant were used directly as the distributed amounts because the recovery of DiD in plasma and the organs tested was almost complete.

2.8. Partition of DiD between plasma and blood cells

The blood was taken in the same manner as in the biodistribution study. The DiD solution (0.01 ml) in the mixture of dimethyl sulfoxide and saline (10:1, v/v) containing 0.225, 0.45 and 2.25 μg were added to 0.5 ml of the blood, and mixed with a vortex mixer for 10 s. The mixture was incubated for 5 min at 37 °C, then centrifuged at 3000 rpm for 10 min. The supernatant, plasma, was treated in the same way as stated above, and the DiD concentration in plasma was determined fluorometrically in the same manner as described above.

3. Results and discussion

3.1. Preparation and characteristics of PLA–(MeO-PEG)

As to PLA-aldehyde(n) (Table 1), the MWn and formyl ratio were obtained from ^{1}H NMR spectra ($CDCl_3$), in which relative integrated intensities for the specific protons, appearing at 4.32 (q, $J = 7$ Hz), 5.05–5.30 (m) and 9.72 (s), were 1/99/0.70, 1/186/0.60, 1/154/0.55 and 1/221/0.55 for PLA-aldehyde(1), PLA-aldehyde(2), PLA-aldehyde(3) and PLA-aldehyde(4), respectively. Furthermore, the MWn and introduction degree of MeO-PEG(N) to PLA for PLA–(MeO-PEG)(n) were calculated based on ^{1}H NMR spectra ($CDCl_3$), in which relative integrated intensities for the specific protons, appearing at 3.61 (s), 4.32 (q, $J = 7$ Hz) and 5.03–5.30 (m), were 43/1/223, 54/1/363, 65/1/159 and 99/1/216 for PLA–(MeO-PEG)(1), PLA–(MeO-PEG)(2), PLA–(MeO-PEG)(3) and PLA–(MeO-PEG)(4), respectively (Table 1).

As reported previously, PLA–(MeO-PEG) was prepared by the formation of a Schiff's base between PLA-aldehyde and MeO-PEG(N) and subsequent reductive amination with sodium cyanoborohydride (Sasatsu et al., 2005). However, the portion of MeO-PEG(N) introduced into PLA was not reproducible or high, which were considered due to the reaction condition and

Table 1
Characteristics of the products obtained by reductive amination of PLA-aldehyde and MeO-PEG amine

Product	PLA-aldehyde used		Condition		PLA–(MeO-PEG) produced	
	MWn	Formyl ratio (%, mol/mol)	Solvent	Separation manner of first peak[a]	MWn	PEG/PLA (%, mol/mol)
PLA–(MeO-PEG)(1)	7900	70	THF-MeOH	A	17900	24
PLA–(MeO-PEG)(2)	13500	60	THF-MeOH	A	23300	30
PLA–(MeO-PEG)(3)	11100	55	CH_2Cl_2-MeOH	B	14400	36
PLA–(MeO-PEG)(4)	15900	55	CH_2Cl_2-MeOH	C	17400	55

[a] A, B and C represent the fractions from the start point to the end points A, B and C were collected.

separation manner. Therefore, the preparation conditions and separation way were refined. In the previous reductive amination, the mixture of tetrahydrofuran and methanol (1:1, v/v) was used as a solvent, and the pH was adjusted using 0.1N hydrochloric acid aqueous solution and 0.1N sodium hydroxide aqueous solution, and the separation was performed by collecting the fractions with very high molecular weight in order to separate the product completely from the second peak of MeO-PEG(N) (Fig. 1A). However, in this condition, it was not easy to maintain the pH at 6–7, sometimes leading to the degradation of the polymer. The introduction degree of MeO-PEG(N) to PLA was low for the formyl group. As the pH adjustment is important for the reductive amination to proceed (Borch et al., 1971), the unstable condition might disturb the reaction. Further, it might cause the low introduction degree of MeO-PEG(N) to PLA that the separation in GPC was set to collect the fractions with higher molecular weight. As shown in Table 1, the separation with the end point A in Fig. 1 presented the high molecular weight for PLA–(MeO-PEG) and PLA.

On the other hand, when a mixture of dichloromethane and methanol (1:1, v/v) was used as a new solvent system for the reductive amination, the pH of the reaction mixture could be adjusted more easily and stably to 6–7 using 0.1N hydrochloric acid aqueous solution and 0.1N sodium hydroxide aqueous solution. Further, the separation manner in GPC was changed as shown with the end points B and C in Fig. 1. The introduction degrees of MeO-PEG(N) to PLA were 36 and 55%, respectively. The molecular weight of each product was consistent with that of the PLA-aldehyde. The difference in MeO-PEG(N) substitution between PLA–(MeO-PEG)(3) and PLA–(MeO-PEG)(4) was considered to be due to the separation manner. As considered it together with the results in PLA–(MeO-PEG)(1) and PLA–(MeO-PEG)(2), PLA–(MeO-PEG) contained in the first peak appeared to have a smaller molecular weight than PLA coexisting there. When the production of PLA–(MeO-PEG)(4) was repeated ($n = 2$), the product showed almost the same characteristics for MeO-PEG(N) substitution and molecular weight. Thus, the reaction condition and polymer separation in PLA–(MeO-PEG)(4) were regarded as the best condition, in which the recovery of PLA in the conversion from PLA-aldehyde(4) to PLA–(MeO-PEG) was approximately 50% (w/w).

3.2. *In vitro characteristics of plain and DiD-loaded nanoparticles*

Plain nanoparticles were prepared with the solvent diffusion method using PLA–(MeO-PEG)(1, 2 or 4) as a drug carrier. Their sizes were studied by the dynamic light scattering method.

Table 2
Physicochemical characteristics of plain nanoparticles

Nanoparticles	PLA–(MeO-PEG)(n)	Mean diameter[a] (nm)	Zeta-potential[a] (mV)
NP1	PLA–(MeO-PEG)(1)	62.3	−43.2
NP2	PLA–(MeO-PEG)(2)	59.4	−22.2
NP3	PLA–(MeO-PEG)(4)	112.1	22.8

[a] These are shown as the mean value ($n = 2$).

Their mean particle sizes and zeta potentials are shown in Table 2. The nanoparticles prepared with PLA–(MeO-PEG)(4) exhibited larger size of more than 100 nm. As the molecular weight was distributed more widely in PLA–(MeO-PEG)(4), the particle size might be greater. As to a zeta potential, since PLA–(MeO-PEG)(4) possessed more secondary amino groups of positive charge, that is, 55% (mol/mol), its nanoparticles were considered to show positive zeta potentials.

The particle characteristics of DiD-loaded nanoparticles are shown in Table 3. The DiD-loaded nanoparticles showed a larger size than the plain nanoparticles. DiD was incorporated into the nanoparticles efficiently. The zeta potential was measured only for NP5, and it was 5.2 mV. After the DiD-loaded nanoparticles were dissolved in $CDCl_3$ and measured for the ^1H NMR spectrum, the ratio of PLA–(MeO-PEG) to PLA decreased to a small extent than that in PLA–(MeO-PEG)(4) (data not shown). Change in the particle size and content of PLA–(MeO-PEG) were considered to cause the difference in zeta potential between plain and DiD-loaded nanoparticles.

TEM micrographs of non-loaded and DiD-loaded nanoparticles prepared using PLA–(MeO-PEG)(3) are shown in Fig. 2. As to the DiD-loaded nanoparticles, the particle size observed in TEM was almost consistent with that obtained in dynamic light scattering. Further, the plain nanoparticles showed 150 nm in the measurement by dynamic light scattering, which was similar to the result in Fig. 2.

The release of DiD from the nanoparticles was examined in vitro by the incubation of NP5 in a mixture of PBS and EtOH (7:3, v/v) at 37 °C, in which the concentration given by the complete release was much lower than the solubility. At 1, 4 and 24 h after incubation, the amount dissolved was less than 1% (w/w) under each set of conditions (data not shown), and the nanoparticles precipitated after ultracentrifugation almost completely retained the blue color within them. Thus, DiD was well retained in the nanoparticles. As DiD is highly hydrophobic, it was considered to be incorporated stably in the PLA core of the nanoparticles. Further, DiD incorporated in nanoparticles made of conventional PEG–PLA appears not to transfer to cells easily

Table 3
Physicochemical characteristics of DiD-loaded nanoparticles

Nanoparticles	PLA–(MeO-PEG)(n)	Drug content (%, w/w)	Encapsulation efficiency (%)	Mean diameter (nm)
NP4	PLA–(MeO-PEG)(3)	3.2 ± 0.3	99.2 ± 9.6	188.6 ± 13.6
NP5	PLA–(MeO-PEG)(4)	2.9 ± 0.0	95.3 ± 1.2	154.3 ± 3.1

The results are shown as the mean ± S.D. ($n = 3$).

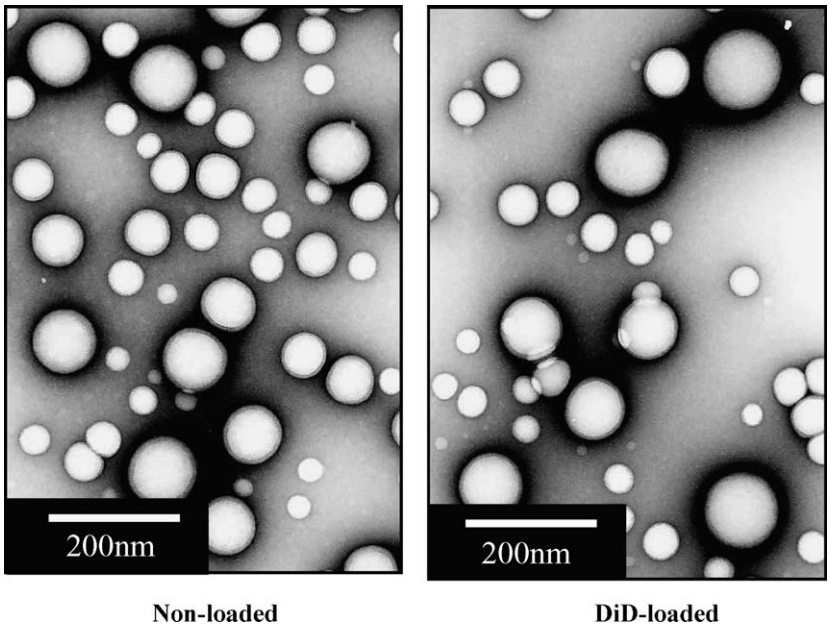

Fig. 2. TEM micrographs of non-loaded and DiD-loaded PLA–(MeO-PEG) nanoparticles. PLA–(MeO-PEG)(3) was used for the preparation of both the nanoparticles. The length of the white bar is 200 nm.

(Mosqueira et al., 1999). These suggested that the distribution of DiD should reflect the biodistribution of the nanoparticles themselves to a fair extent.

3.3. Biodistribution of DiD after i.v. administration

DiD solution and the DiD-loaded nanoparticles (NP4 and NP5) were used for the biodistribution experiments. The biodisposition profiles of the total (free plus incorporated) amount of DiD in plasma and major organs were compared among these formulations after the bolus i.v. injection at a dose of 0.6 mg DiD equiv./kg in mice (Fig. 3). At 1 and 8 h after the administration, NP5 showed approximately 10 and 5 times higher plasma levels of DiD than the DiD solution, respectively. The plasma levels of DiD were approximately 8 and 4 times greater in NP4 than in the DiD solution at 1 and 8 h after administration, respectively. At 24 h after administration, both nanoparticles exhibited a slightly lower plasma level of DiD than the DiD solution. These indicated that both the nanoparticles raised the plasma retention of DiD despite their positive zeta potential. Furthermore, it was found that the nanoparticles prepared with a higher PEG/PLA ratio (NP5) exhibited higher plasma retention of DiD. This was considered to be because the higher density of PEG on the nanoparticulate surface prevents the nanoparticles from their association with cells or biomacromolecules as having been reported by Mosqueira et al. (1999) and Nguyen et al. (2003). It is suggested that the nanoparticles made of the mixture of PLA–(MeO-PEG) and PLA at a high PEG/PLA ratio should be more useful for high plasma retention. After the administration of the DiD solution, DiD tended to be highly accumulated in the liver. On the other hand, after administration of the nanoparticles, DiD showed a tendency to be distributed in both the spleen and liver.

As DiD was hydrophobic, its partition to blood cells which were separated from plasma was considered to take place. As shown in Fig. 4, when the blood concentration of DiD was low,

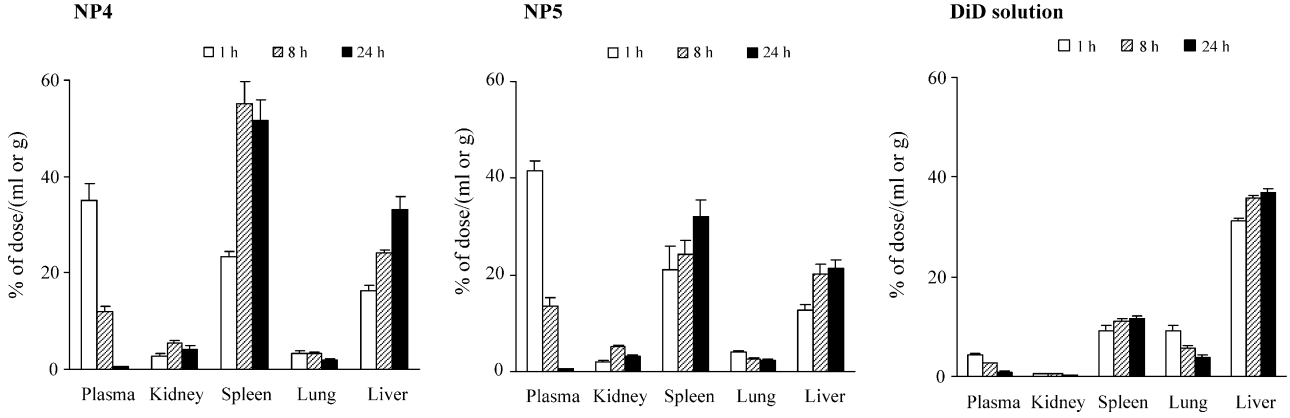

Fig. 3. Tissue distribution of DiD after bolus i.v. injection of DiD-loaded nanoparticles and DiD solution at a dose of 0.6 mg DiD equiv./kg in mice. The results are expressed as the mean ± S.E. ($n = 4$).

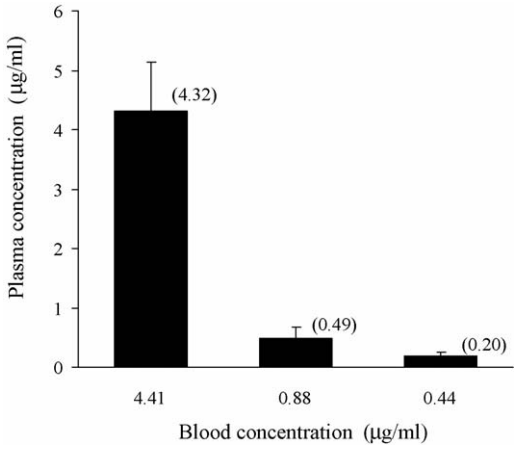

Fig. 4. Partition of DiD between plasma and blood cells. The DiD solution (0.01 ml) in the mixture of dimethyl sulfoxide and saline (10:1, v/v) containing 0.225, 0.45 and 2.25 μg were added to 0.5 ml of the blood, mixed and incubated at 37 °C, then the plasma was separated by centrifugation. The results are expressed as the mean ± S.E. ($n = 3$). Each value in parentheses represents the mean plasma concentration.

the partition of DiD to the blood cells increased. Therefore, DiD was more distributed into blood cells in the low plasma concentration. Considering that the ratio of the volume of blood cells to that of plasma is 3:5 and that murine blood volume is 77.8 ml/kg, Fig. 3 indicated that the greater part of DiD administered was considered to be recovered in the tissues or organs used in the experiments. Even though the partition of DiD between blood cells and plasma was take into account, the nanoparticles exhibited much higher blood concentration of DiD than the DiD solution at 1 h after administration. The nanoparticles showed a fairly high plasma concentration, which was similar to the characteristics of other systemically long-circulating delivery systems. Thus, it is suggested that these nanoparticles should be useful as a carrier for a drug delivery system elevating a plasma drug concentration, which may allow a passive targeting in inflammatory diseased sites such as solid tumor due to the EPR effect (Matsumura and Maeda, 1986).

In this study, DiD was used to make clear the effect of the nanoparticles on the biodistribution of drugs because DiD could be stably incorporated into the nanoparticles and little released due to its high lipophility. These results were considered to be informative for lipophilic drugs but not to be extrapolated directly to all real drugs, because the drug release would depend on their physicochemical properties. For example, nanoparticles made of conventional PEG–PLA showed a fairly fast drug release for some drugs (Gref et al., 1994; Miura et al., 2004). Therefore, further examination will be needed in the application of the present nanoparticles to other compounds or drugs.

4. Conclusion

The synthetic procedure for PLA–(MeO-PEG) could be refined. It was obtained more easily and reproducibly by using a new solvent system, a mixture of dichloromethane and methanol (1:1, v/v), in a reductive amination between PLA-aldehyde and MeO-PEG(N) and by collecting the whole first peak in the GPC separation. The DiD-loaded nanoparticles prepared using the mixture of PLA–(MeO-PEG) and PLA (55:45 (mol/mol)) had a size of 154 nm and a little positive zeta potential. The nanoparticles exhibited a better systemic retention of DiD as compared with the DiD solution. These in vitro and in vivo results suggested that the nanoparticles made of PLA–(MeO-PEG) and PLA at a high PEG/PLA ratio should be useful as a carrier for a drug delivery system elevating a plasma concentration.

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