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Preparation of a PLA–PEG block copolymer using a PLA derivative with a formyl terminal group and its application to nanoparticulate formulation

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Abstract

A novel poly(DL-lactic acid) (PLA) derivative with a diethoxy propanol ester at the end, named PLA-acetal, was synthesized by ring opening polymerization using DL-lactide and 3,3-diethoxy propanol. PLA-acetal was hydrolyzed to a PLA derivative with a formyl group, named PLA-aldehyde, by acid treatment. Reductive amination between PLA-aldehyde and methoxypolyethylene glycol amine (MeO-PEG(N)) gave the block copolymer (PLA–(MeO-PEG(N))). Nanoparticles were prepared by emulsification-solvent evaporation or solvent diffusion using PLA–(MeO-PEG(N)) or a conventional methoxypolyethylene glycol–PLA block copolymer, PLA–(MeO-PEG(O)). PLA–(MeO-PEG(N)) nanoparticles had a particle size of 60–340 nm, dependent on the preparative procedure, while PLA–(MeO-PEG(O)) nanoparticles prepared by solvent diffusion showed a particle size of 60 nm. The PLA–(MeO-PEG) nanoparticles with a smaller PEG introduction degree exhibited a more negative zeta potential. 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD) could be incorporated efficiently in PLA–(MeO-PEG(N)) nanoparticles. It is suggested that PLA-aldehyde should be useful as a functional intermediate for derivatization of PLA, and PLA–(MeO-PEG(N)) can be used for the preparation of PEG-coated PLA nanoparticles.

Keywords: PLA-aldehyde; PLA-acetal; Methoxypolyethylene glycol-PLA block copolymer; Nanoparticle; Reductive amination

1. Introduction

Biocompatible and biodegradable polymers have been playing an important role in medical and pharmaceutical fields. Poly(DL-lactic acid) (PLA) and poly(DL-lactic acid-co-glycolic acid) copolymer (PLGA) are clinically available as medical devices and drug carriers because of their biodegradability and biocompatibility (Gilding and Reed, 1979; Ogawa et al., 1988a,b). As they melt with heat treatment and are well soluble in organic solvents such as dichloromethane, they have been used to produce various dosage forms such

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as pellets, microcapsules, nanoparticles, etc. (Hirano et al., 1984; Birnbaum et al., 2000; Xinteng et al., 2002; Ozalp et al., 2002). Nanoparticles with PLA or PLGA as a core component have attracted much attention because they can be infused directly into a vein in order to deliver drugs to target sites and/or to control their release in the body. Simple PLA or PLGA nanoparticles are delivered mostly into liver and spleen possessing a reticuloendothelial system (Dunn et al., 1997). However, their pharmacokinetic profiles can be modified by changing particle size and surface properties. One way to modify the pharmacokinetic properties of PLA or PLGA nanoparticles is to use a surfactant (Dunn et al., 1997; Onishi et al., 2003). Polyethylene glycol–polypropylene glycol–polyethylene glycol block copolymer such as poloxamer is useful to enhance the systemic retention of nanoparticles with PLA as a core component. Furthermore, PLA derivatives have recently been developed to modify the characteristics of PLA or PLGA nanoparticles. Much attention has been paid to the polyethylene glycol–PLA block copolymer (PEG–PLA), which is a diblock copolymer with hydrophilic and hydrophobic blocks, because it allows the formation of a stable nanoparticulate suspension in an aqueous solvent, where PLA chains form the core and PEG chains are located outside (Gref et al., 1994; Bazile et al., 1995). The PEG shell prevents the interaction of PLA core with biomolecules, cells and tissues (Dunn et al., 1997; Mosqueira et al., 1999), and can suppress opsonization (Nguyen et al., 2003). Reportedly, PEG–PLA nanoparticles show a particle size of several dozen to a few hundred nanometers, and possess a hydrophilic and inactive surface of PEG, leading to a longer systemic circulation (Gref et al., 1994; Bazile et al., 1995; Panagi et al., 2001).

Thus, the surface modification of PLA or PLGA plays an essential role in the delivery of nanoparticles with PLA or PLGA as a core component. Furthermore, ligands bound specifically to the receptors are known to enable the active targeting of drugs. Galactose-bound macromolecules or liposomes have been utilized to specifically localize an incorporated drug to the liver, based on the specific recognition of the sugar by the asialoglycoprotein receptors of hepatic cells (Pimm et al., 1993; Nishikawa et al., 1995; Kawakami et al., 2001). This specific recognition is also a useful approach for other ligands such as antibodies, etc. (Noguchi et al., 1991; Maruyama, 2002). Surface modification of PEG–PLA has been actively studied by the Kataoka research group (Otsuka et al., 2000; Nagasaki et al., 2001; Yamamoto et al., 2001; Jule et al., 2003). They attached sugars, amino acids, peptides, etc. to the end of the PEG moiety of PEG–PLA. Sugar-installed PEG–PLA nanoparticles specifically interacted with the lectin. Introduction of amino acids or peptides changed a surface charge of PEG–PLA, which appeared to influence the pharmacokinetic behavior of the nanopaparticles. With such approaches, acetal-ended PEG–PLA is synthesized by sequential polymerization of ethylene oxide and DL-lactide. On the other hand, our present studies involves the synthesis of acetal-ended PLA (PLA-acetal), its conversion to PLA with a formyl terminal end and modification using reductive amination (Fig. 1). Namely, our approach was performed first by ring polymerization of DL-lactide using diethoxy propanol as an initiation molecule to obtain PLA-acetal, and subsequently PLA with a formyl group, called PLA-aldehyde, was prepared by acidic hydrolysis of PLA-acetal. PLA-aldehyde was reacted with the molecules having amino groups by reductive amination. PLA-aldehyde is considered to allow the direct modification of PLA. In the present study, a methoxypolyethylene glycol amine–PLA block copolymer (PLA–(MeO-PEG(N))) was prepared by reductive amination between PLA-aldehyde and methoxypolyethylene glycol amine, and used to prepare nanoparticles. The polymers and nanoparticles were characterized and evaluated in vitro.

2. Materials and methods

2.1. Materials

Methoxypolyethylene glycol (MeO-PEG(O); MW 2000), 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 perchlorate (DiD) was purchased from Molecular Probes, Inc. (Oregon, USA). All other chemicals used were of reagent grade.

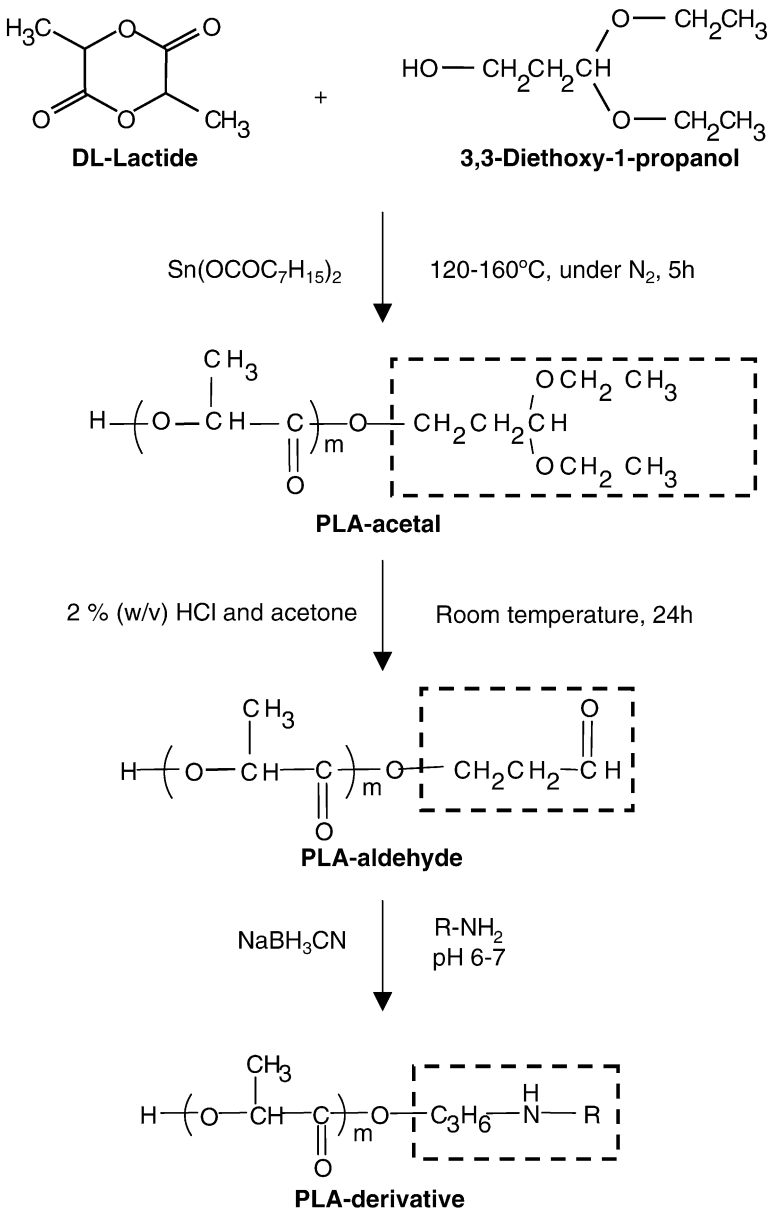

Fig. 1. Synthetic procedure of PLA-derivatives.

2.2. Synthesis of poly(DL-lactic acid) with a formyl group at the terminal end (PLA-aldehyde)

Acetal-ended poly(DL-lactic acid) (PLA-acetal) was synthesized with ring opening polymerization. Briefly, 6 g of DL-lactide purified by recrystallization from an ethyl acetate solution and 3,3-diethoxy-1-propanol (50, 100 or 150 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 120–160 °C with stirring. After the temperature of the mixture reached the set temperature, a toluene solution (5 ml) of stannous octoate (30 μg) was added to the reaction

mixture. 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. After evaporation of dichloromethane, the aqueous suspension was left at room temperature for 24 h. The precipitate was obtained by filtration and re-dissolved in dichloromethane. After drying the solution with anhydrous sodium sulfate overnight, it was filtered. The filtrate was evaporated to dryness to yield PLA-acetal.

Aldehyde-ended poly(DL-lactic acid) (PLA-aldehyde) was obtained by acidic hydrolysis of PLA-acetal. PLA-acetal (200 mg) was dissolved in 80 ml of acetone, and 2% (w/v) hydrochloric acid (30 ml) was added. The resultant suspension was stirred at room temperature for 24 h. After the removal of acetone by evaporation under reduced pressure, extraction from the remaining aqueous suspension with dichloromethane was performed. The entire organic phase was taken and dried on anhydrous sodium sulfate overnight. After filtration, the filtrate was evaporated to dryness to yield PLA-aldehyde.

2.3. Synthesis of methoxypolyethylene glycol amine-ploy(DL-lactic acid) block copolymer (PLA–(MeO-PEG(N))) and methoxy polyethylene glycol-poly(DL-lactic acid) block copolymer (PLA–(MeO-PEG(O)))

MeO-PEG(N) (300 mg), PLA-aldehyde (200 mg) and sodium cyanoborohydride (25 mg) were put in 25 ml of a tetrahydrofuran/methanol mixture (1:1, v/v), then the mixture was stirred at room temperature for 12 h, when the pH of the mixture was adjusted to pH 6–7 with 0.1N HCl aqueous solution and 0.1N NaOH aqueous solution. After that, organic solvents were evaporated, and the resultant suspension was lyophilized. To the residue was added chloroform, and the supernatant underwent gel permeation chromatography (GPC) to collect the fractions with a molecular weight larger than that of MeO-PEG(N). After the collected solution was evaporated, the residue was re-dissolved in acetone, and water was added to obtain a suspension. After evaporation of acetone from the suspension, the resultant aqueous suspension was washed with an ultrafilter membrane with an MW cut off of 10,000. After the remaining suspension was lyophilized, the product was dissolved in acetone. This acetone solution was suspended by addition of water, and the suspension was washed in the same manner as described above. PLA–(MeO-PEG(N)) was obtained by lyophilization of the remaining aqueous suspension.

PLA–(MeO-PEG(O)) was synthesized based on the method of Bazile et al. (1995). Briefly, DL-lactide (5 g), recrystallized from an ethyl acetate solution, and MeO-PEG(O) (0.84 g) were put in a three-neck flask, the inside of which was filled with nitrogen gas in advance, 15 ml of toluene was added, and the mixture was stirred and heated on an oil bath set at 120 °C with stirring. A toluene solution (5 ml) of stannous octoate (20 μg) was added to the reaction mixture, and the reflux was continued at 120 °C with stirring for 6 h. After that, the stirring was stopped, and the mixture was left at room temperature for 24 h. The solvent was evaporated at 70 °C under reduced pressure, 20 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. After evaporation of dichloromethane, the aqueous suspension was left at room temperature for 5 h. The precipitate was obtained by filtration, re-dissolved in dichloromethane, filtered, and the filtrate was evaporated to dryness to yield PLA–(MeO-PEG(O)).

2.4. ^{1}H-NMR and GPC studies

^{1}H-NMR spectra of PLA derivatives were measured using a JEOL JNM-GX270 spectrometer (Japan) to determine their chemical structures. Furthermore, the polymerization degree of PLA moieties in the PLA derivatives was calculated with the ^{1}H-NMR spectra by comparing the integrated intensity of the methine proton of the terminal lactic group of PLA to that of the methine proton of the inside lactic groups of PLA, which also allowed the calculation of the number-average molecular weight (MWn). GPC was performed to determine the molecular weights of PLA-derivatives, called MW_{GPC}, using standard markers with known molecular weight, and to make the polymer products purified. The GPC analysis was performed as follows:

A Shimadzu LC-6AD equipped with the refractive index detector Shimazu RID-10A was used with chloroform as a mobile phase. MW_{GPC} were examined using a GPC K-800 column (8 mm in inner diameter × 300 mm in length; Shodex, Japan), and the flow rate of the mobile phase was 1 ml/min. The preparative separation of the polymers was performed using a column of GPC K-2003 (20 mm in inner diameter × 300 mm in length; Shodex, Japan), and the mobile phase was flowed at 3.5 ml/min. Several polystyrenes (PSs) of known MW (Showa Denko K.K., Japan) were used as standard markers.

2.5. Preparation of nanoparticles by emulsification-solvent evaporation

PLA–(MeO-PEG(N)) (30 mg) was dissolved in 2 ml of dichloromethane, and put in 20 ml of water. The mixture was stirred with a vortex mixer for 30 min, and sonicated at 45 kHz (100 W) for 5 min using an ultrasonicator, VS-100III SUNPAR (Iuchiseieido, Japan), to obtain an emulsion. The resultant emulsion was stirred at room temperature for 2 h to give an aqueous suspension of the particles.

2.6. Preparation of nanoparticles by solvent diffusion

As for the preparation by solvent diffusion, three different methods were performed as follows: (A) PLA–(MeO-PEG(N)) or PLA–(MeO-PEG(O)) (30 mg) was dissolved in a mixture of 10 ml of acetone and 5 ml of ethanol, and an equi-volume of water was added gradually. The organic solvent was evaporated at 18 °C under reduced pressure to give an aqueous suspension of particles. (B) 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. The aqueous suspension of particles was obtained by evaporation at 18 °C under reduced pressure. (C) PLA–(MeO-PEG(N)) or PLA–(MeO-PEG(O)) (30 mg) was dissolved in the mixture of 2 ml of acetone and 1 ml of ethanol, and dropped gradually into 5 ml of water stirred with a vortex mixer, and sonicated at 45 KHz (100 W) for 1 min. The organic solvent was evaporated at 18 °C under reduced pressure to obtain an aqueous suspension of particles.

2.7. Particle characteristics

The particle size, its distribution and zeta potential of the particles were measured using an ELS-800 dynamic light scattering apparatus (Otsuka Electronic Co., Ltd., Japan). Namely, the aqueous suspension of particles was diluted adequately with water, and analyzed with this apparatus.

2.8. Preparation and characterization of DiD-loaded nanoparticles

Nanoparticles containing DiD were prepared according to the modified solvent diffusion method B. Briefly, 35 mg of PLA–(MeO-PEG(N)) 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), when 0.45% (w/v) sodium chloride was used as an elution solvent.

A specified volume of the obtained suspension of DiD-loaded nanoparticles was dried. The residue was dissolved in dichloromethane, and measured spectrophotometrically at 644 nm to determine the amount of DiD, which also provided the recovery of DiD. In addition, the residue was dissolved in $CDCl_3$, a specified amount of 4′-methoxyacetophenone was added as a standard, and the ^{1}H-NMR spectrum of the supernatant of the mixture was measured. The polymer amount was determined from comparison of the integrated intensities between the methine proton of PLA–(MeO-PEG(N)) and the methyl proton of the acetyl group of 4′-methoxyacetophenone, which also allowed the calculation of the recovery of PLA–(MeO-PEG(N)). The incorporation efficiency of DiD was calculated as the ratio of the observed DiD content to its ideal content. The particle size was measured as stated above.

3. Results

3.1. Preparation of acetal-ended PLA (PLA-acetal) and aldehyde-ended PLA (PLA-aldehyde)

Although the direct attachment of acetaldehyde diethylacetal to PLA was attempted by the reaction

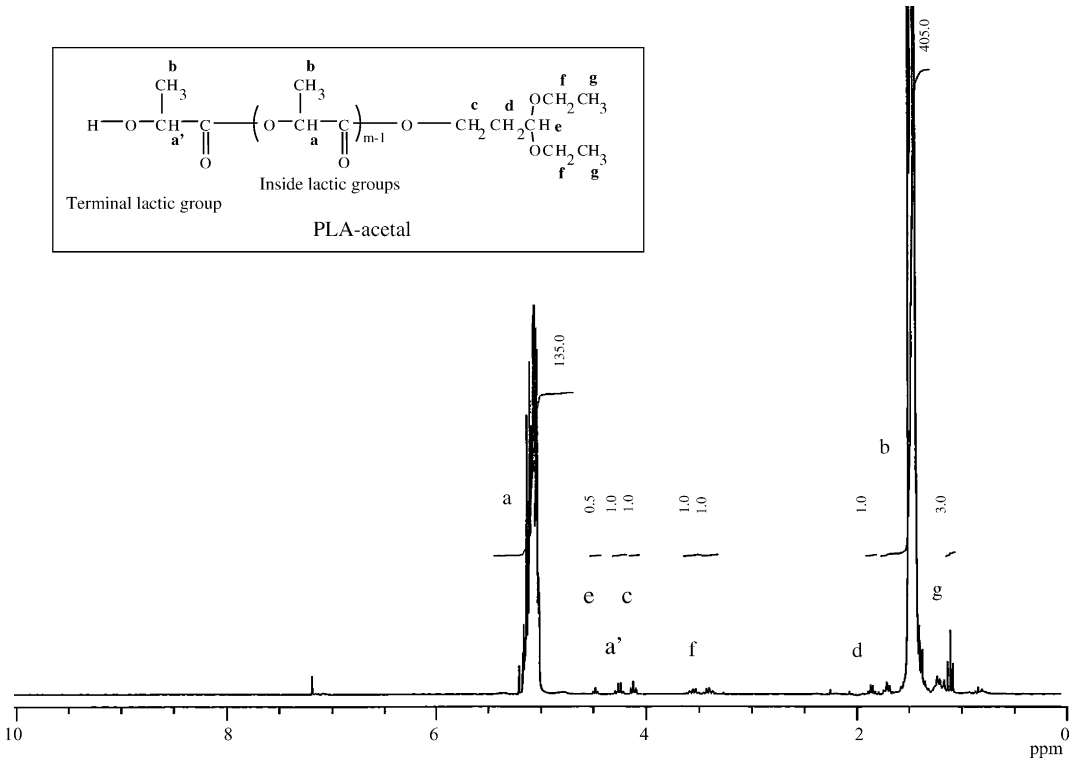

Fig. 2. Representative ^{1}H-NMR spectra of PLA-acetal. The spectrum is for the product obtained with the formulation 4.

of acetaldehyde diethylacetal bromide with PLA in dimethylformamide in the existence of potassium carbonate, the degree of introduction of acetaldehyde diethyacetal was less than 10% (mol/mol).Therefore, ring opening polymerization was performed to prepare acetal-ended PLA (PLA-acetal). In the present study, ring polymerization of DL-lactide was executed using 3,3-diethoxy-1-propanol as an initiation molecule and stannous octoate as a catalytic agent. The representative ^{1}H-NMR spectrum of the PLA-acetal is shown in Fig. 2. The introduction degree of 3,3-diethoxy-1-propanol was obtained based on the ^{1}H-NMR spectrum of PLA-acetal. That is, the introduction degree of 3,3-diethoxy-1-propanol to PLA in PLA-acetal was calculated by comparison of the integrated intensity of the methylene protons of the ethoxy group of 3,3-diethoxy-1-propanol with that of the methine proton of the terminal lactic group of PLA (Fig. 2). The acetal end group was well introduced to PLA as shown in Table 1.The degree of polymerization and the introduction degree of the acetal end were hardly affected by the addition ratio of 3,3-diethoxy-1-propanol to DL-lactide,

Table 1
Structural characteristics of PLA-acetal produced under various preparative conditions

Formulation	DL-lactide (g)	3,3-Diethoxy-1–propanol (g)	Reaction temperature (°C)	Polymerization degree of PLA moiety[a]	Diethoxy/PLA[a] (%, mol/mol)
1	6	0.05	120	68–71	22–45
2	6	0.1	120	58–67	42
3	6	0.15	120	67–82	40
4	6	0.05	140	123–135	49–54
5	6	0.05	160	179–244	70–85

[a] The values were determined by ^{1}H-NMR measurement in $CDCl_3$. The results are expressed as the range given by some experiments (n = 2–3).

but by the reaction temperature (Table 1). Namely, the degree of polymerization and introduction degree of the acetal end group were raised with the increase in the reaction temperature.

Although hydrolysis of PLA-acetal was attempted using a diluted hydrochloric acid aqueous solution as a hydrolytic solvent, the transformation from acetal to aldehyde was insufficient. The conversion from acetal to aldehyde proceeded well using a mixture of acetone and 2% (w/v) hydrochloric aqueous solution as a hydrolytic solvent. In this reaction, the signals of e, f and g in Fig. 1 disappeared, and formyl proton was generated at 9.74 ppm (singlet) in $CDCl_3$. The polymerization degree of PLA in PLA-aldehyde was similar to that of PLA in PLA-acetal. The conversion degree from PLA-acetal to PLA-aldehyde was approximately 80% (mol/mol) from comparison between the integrated intensity of the formyl proton and that of the methine proton of the terminal lactic group of PLA (data not shown), although the conversion degree slightly varied.

3.2. Preparation and characteristics of PLA–(MeO-PEG(N)) and PLA–(MeO-PEG(O))

PLA–(MeO-PEG(N)) was prepared by the formation of a Schiff's base between PLA-aldehyde and MeO-PEG(N), followed by the reduction with sodium cyanoborohydride. Since the formulation 5 in Table 1 exhibited a higher introduction degree of 3,3-diethoxy-1-propanol to PLA, PLA-aldehyde derived from PLA-acetal obtained with the formulation 5 was used for the preparation of all the PLA–(MeO-PEG(N))s. The introduction degree of MeO-PEG(N) to PLA was calculated based on the ^{1}H-NMR spectrum. That is, it was calculated by comparing the integrated intensity of MeO-PEG(N) methylene protons with that of the methine proton of the terminal lactic group of PLA. The representative GPC elution profiles of PLA-acetal and PLA–(MeO-PEG(N)) derived from the same PLA-acetal are shown in Fig. 3. For PLA-acetal and PLA–(MeO-PEG(N)) shown in Fig. 3, the molecular weight from GPC (MW_{GPC}) was calculated using the calibration curve given by the standard markers, in which the relationship between log MW and retention time for the standard markers was linear. The MW_{GPC} values were parallel to the MWn values (Table 2). The PLA–(MeO-PEG(N)) described in Fig. 3 and Table 2 and PLA–(MeO-PEG(O)) were compared with regard to the introduction degree or reaction efficiency (Table 3). This PLA–(MeO-PEG(N)) showed the fairly high introduction degree of MeO-PEG(N), that is 39% (mol/mol), which was larger than those of the other lots of PLA–(MeO-PEG(N)) (see Tables 4 and 5). However,

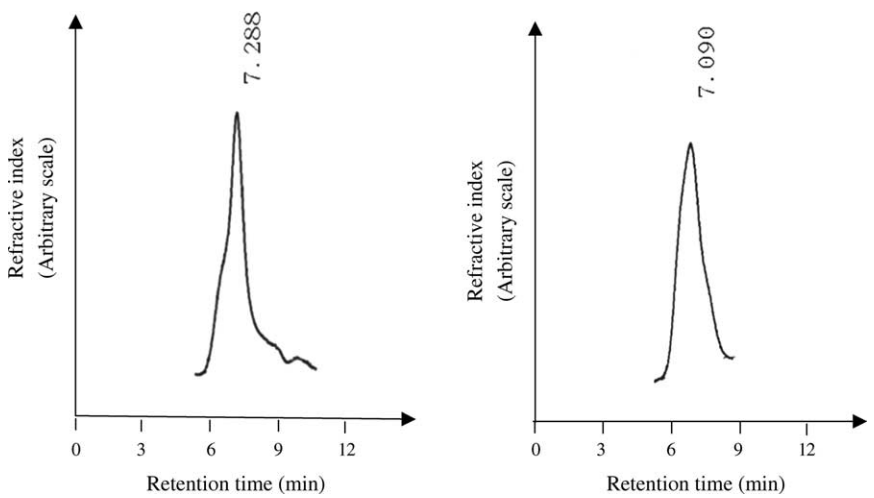

Fig. 3. Gel permeation chromatograms of PLA-acetal (A) and PLA–(MeO-PEG(N)) (B). Retention time of PS standard markers (min): PS(MW 2.86×10^4) 6.86; PS(MW 1.09×10^4) 7.83; PS(MW 3×10^3) 9.14. The PLA-acetal (A) was obtained with the formulation 5 (Table 1). The PLA–(MeO-PEG(N)) (B) was prepared using PLA-aldehyde (51% (mol/mol) formyl/PLA), derived form the PLA-acetal (A).

Table 2
Molecular weight characteristics of PLA-acetal and PLA–(MeO-PEG(N))

PLA derivative[a]	MW_{GPC}[b]	MWn[c]	MW_{GPC}/MWn
PLA-acetal	22900	13100	1.7
PLA–(MeO-PEG(N))[d]	25300	15800	1.6

[a] The derivatives are the polymers shown in Fig. 3.
[b] Determined by GPC.
[c] Determined by ^{1}H-NMR spectra in $CDCl_3$.
[d] This product was synthesized using PLA-aldehyde (51% (mol/mol) formyl/PLA).

Table 3
Introduction degree of Me-PEG(N) or MeO-PEG(O) to PLA

PLA derivative	PEG/PLA[a] (%, mol/mol)	Reaction efficiency[a] (%)
PLA–(MeO-PEG(N))[b]	39	76
PLA–(MeO-PEG(O))	73	73

[a] Determined by ^{1}H-NMR spectra in $CDCl_3$.
[b] This product was the polymer shown in Fig. 3 and Table 2, and synthesized using PLA-aldehyde (51% (mol/mol) formyl/PLA).

PLA–(MeO-PEG(O)) showed a much higher introduction degree of MeO-PEG(O) of 73% (mol/mol).

3.3. Particle characteristics of PLA–(MeO-PEG(N)) and PLA–(MeO-PEG(O)) nanoparticles

PLA–(MeO-PEG(N)) varied to some extent in molecular weight and introduction degrees of MeO-PEG(N) among the lots obtained. In the preparation of the nanoparticles, PLA–(MeO-PEG(N))s with different molecular weights and distinct introduction degrees of MeO-PEG(N) were used (Tables 4 and 5). An aqueous suspension of PLA–(MeO-PEG(N)) particles was prepared by the emulsification-solvent evaporation method or different solvent diffusion methods. The results are shown in Table 4. The particle size of the nanoparticles produced by the emulsification-solvent evaporation ranged from 300–400 nm. The diameter of the particles obtained from solvent diffusion method A was approximately 240 nm. On the other hand, the particle size was 100–140 nm when the particles were made by solvent diffusion method B. Solvent diffusion method C, in which an acetone–water mixed solvent and sonication process were applied, gave the smallest nanoparticles with a size of 50–80 nm. The particle size of PLA–(MeO-PEG(N)) nanoparticles depended on the preparation method rather than MW or composition of PEG/PLA. PLA–(MeO-PEG(O)) nanoparticles, prepared by solvent diffusion methods A and C, showed almost the same particle size of approximately 60 nm in both methods (Table 4).

Zeta potentials were compared among the nanoparticles with a similar particle size of approximately 60 nm. As shown in Table 5, all the nanoparticles exhibited negative zeta potential values, which were different depending on the polymer species. The nanoparticles prepared using a polymer with the smaller introduction degree of MeO-PEG showed a more negative zeta potential.

3.4. Preparation and particle characteristics of PLA–(MeO-PEG(N)) nanoparticles containing DiD

PLA–(MeO-PEG(N)) nanoparticles containing DiD as a model drug were prepared using the modified

Table 4
Particle characteristics of PLA–(MeO-PEG) nanoparticles produced by various polymer species and different preparative methods

Nanoparticles	Polymer	MWn of polymer	PEG/PLA (%, mol/mol)	Method	Mean diameter[a] (nm)
NP-N1	PLA–(MeO-PEG(N))	17600	33	Emulsification-evaporation	337.8 ± 48.2
NP-N2	PLA–(MeO-PEG(N))	27900	30	Solvent diffusion A	244.7 ± 9.2
NP-O1	PLA–(MeO-PEG(O))	10100	73	Solvent diffusion A	58.5 ± 0.4
NP-N3	PLA–(MeO-PEG(N))	17600	31	Solvent diffusion B	107.7 ± 3.6
NP-N4	PLA–(MeO-PEG(N))	19000	25	Solvent diffusion B	134.8 ± 4.4
NP-N5	PLA–(MeO-PEG(N))	27900	30	Solvent diffusion C	59.4 ± 0.3
NP-N6	PLA–(MeO-PEG(N))	19000	25	Solvent diffusion C	71.5 ± 0.6
NP-O2	PLA–(MeO-PEG(O))	10100	73	Solvent diffusion C	58.2 ± 1.1

[a] The results are expressed as the mean ± S.D. ($n = 3$).

Table 5
Comparison of electric surface potential of PLA–(MeO-PEG) derivative nanoparticles

Nanoparticles	Polymer	MWn of polymer	PEG/PLA (%, mol/mol)	Mean diameter (nm)	Zeta-potential[a] (mV)
NP-O2	PLA–(MeO-PEG(O))	10100	73	58.2	−13.2
NP-N5	PLA–(MeO-PEG(N))	27900	30	59.4	−22.2
NP-N7	PLA–(MeO-PEG(N))	17900	24	62.3	−43.2

[a] The results are expressed as the mean (n = 1–2).

Table 6
Particle characteristics of DiD-containing PLA–(MeO-PEG(N)) nanoparticles obtained by the modified solvent diffusion method B

Mean diameter (nm)	DiD content (%, w/w)	Recovery of polymer (%, w/w)	Recovery of (%, w/w)	Incorporation efficiency (%)
218.5 ± 2.1	2.7 ± 0.3	55.8 ± 2.0	52.0 ± 4.0	96.1 ± 10.3

All the results are expressed as the mean ± S.D. (n = 3).

solvent diffusion method B. This preparative method was employed because the solvent diffusion method B gave the nanoparticles with the mean particle size of 100–140 nm (Table 4), which had been reported as the best size for passive targeting to solid tumors in polyethylene glycol-coated liposomes (Ishida et al., 1999). As a result, DiD-containing PLA–(MeO-PEG(N)) nanoparticles showed the mean particle diameter of 218.5 ± 2.1 nm (Table 6). The recovery of polymer was 55.8 ± 2.0% (w/w), the DiD content was 2.7 ± 0.3% (w/w), and the recovery of DiD was 52.0 ± 4.0% (w/w). The incorporation efficiency of DiD was 96.1 ± 10.3% (w/w).

4. Discussion

PLA derivatives have been attracting much interest since PLA–PEG diblock copolymer (Gref et al., 1994; Bazile et al., 1995), poly(L-lactide)–PEG–poly(L-lactide) triblock copolymer (Ah et al., 2001) and PLGA–PEG–PLGA triblock copolymer (Jeong et al., 2004) were found to form a stable nanoparticulate aqueous suspension. PLA is often used as a drug carrier because of its biocompatible and biodegradable characteristics. However, simple PLA nanoparticles are subjected to rapid systemic clearance because of their lipophilic and opsonized characteristics (Gref et al., 1994; Bazile et al., 1995; Dunn et al., 1997; Mosqueira et al., 1999; Panagi et al., 2001; Nguyen et al., 2003). On the other hand, the surface modification of PLA nanoparticles can change the pharmacokinetic properties of PLA based on the characteristics of surface moieties. In the nanoparticles prepared with the block copolymers stated above, the surface PEG prevents the particles from interacting with complements or the cellular surface, leading to a good retention of the particles in the systemic circulation (Gref et al., 1994; Bazile et al., 1995; Panagi et al., 2001). These properties of the nanoparticles prepared with hydrophobic polymer–PEG block copolymers have been applied to the passive targeting of drugs to diseased areas such as solid tumors, based on the enhanced permeability and retention (EPR) effect (Matsumura and Maeda, 1986; Luo and Prestwich, 2002). The efficacy of an antitumor drug has been reported to be improved extensively using PEG–PLA nanoparticles as a drug carrier (Miura et al., 2004).

Recently, further modification of PLA–PEG has been attempted in order to obtain nanoparticles with better functionality. Macromolecular or liposomal carries have already been modified with chemical moieties such as sugars, antibodies, etc. Such modifications change the pharmacokinetic characteristics of macromolecule–drug conjugates and drug-containing liposomes. Recently, further chemical modification of PLA–PEG has been found to modify the biological characteristics of the polymeric nanoparticles (Otsuka et al., 2000; Nagasaki et al., 2001; Yamamoto et al., 2001; Jule et al., 2003). The present study focused on the development of a method to easily obtain various PLA derivatives. Since the aldehyde-ended PLA (PLA-

aldehyde) was considered to be very useful due to high reactivity, its preparation was attempted to realize the simple modification of PLA.

PLA-acetal was not readily obtained by the direct coupling of acetaldehyde diethylacetal bromide with PLA, while ring opening polymerization of DL-lactide using 3,3-diethoxy-1-propanol as an initiation molecule gave the introduction degree of 30–85% (mol/mol). A reaction temperature of 160 °C was the best for the introduction of the acetal end to PLA and achievement of higher polymerization degree of PLA. The higher temperature appeared to enhance polymerization. A derivatization could not be achieved completely, and the introduction degree of the acetal end to PLA and polymerization degree of PLA varied to some extent among the lots obtained. The coexisting polymer in the product was possibly PLA itself, which was considered to be formed by the cleavage of DL-lactide or the existence of a marginal water.

PLA-aldehyde was prepared by acidic hydrolysis of PLA-acetal. The simple hydrolysis of PLA-acetal with hydrochloric acid aqueous solution did not give PLA-aldehyde well. This was considered due to the less water-soluble property of PLA-acetal. On the other hand, when PLA-acetal was suspended in the acetone–hydrochloric acid aqueous solution, the formyl group was generated efficiently without degradation of PLA. The observed conversion degree from PLA-acetal to PLA-aldehyde was approximately 80% (mol/mol) of the complete conversion degree calculated, though the conversion degree slightly varied. This incomplete conversion suggested that PLA-acetal might slightly remain after the reaction or that the cleavage of the ester bond between 3,3-diethoxy-1-propanol and the carboxyl group of PLA might happen to some extent. The polymerization degree of PLA in PLA-aldehyde was similar to that of PLA in the original PLA-acetal (Fig. 1), suggesting that PLA-aldehyde should not degrade in the conversion from PLA-acetal to PLA-aldehyde.

MeO-PEG(N) was introduced to PLA-aldehyde by reductive amination, when the pH for the reaction was set at 6–7 according to Borch et al. (1971). As shown in Table 1, the formulation 5 exhibited a higher introduction degree of 3,3-diethoxy-1-propanol to PLA than the other formulations did. Therefore, for all the PLA–(MeO-PEG(N))s, the reductive amination was performed between MeO-PEG(N) and PLA-aldehyde derived from PLA-acetal obtained with the formulation 5. With the present method for the preparation and purification of PLA–(MeO-PEG(N)), it could be completely separated from MeO-PEG(N). Since MW_{GPC} calculated by GPC paralleled MWn, GPC was useful for the separation and calculation of the molecular weight of the PLA derivatives (Fig. 3, Table 2). The introduction degree of MeO-PEG(N) to PLA was 25–40% (mol/mol). The maximum reaction efficiency in the reductive amination was nearly 80% (mol/mol) (Table 3). The incomplete introduction of MeO-PEG(N) to PLA suggested the possibility that the formyl group might slightly remain, the aldehyde itself partially might undergo reduction or the ester bond attaching a formyl end group might be cleaved partially. On the other hand, PLA–(MeO-PEG(O)) obtained by ring opening polymerization using Me-PEG(O) as an initiation molecule exhibited the introduction degree of 70–80% (mol/mol). As this reaction is completed in one step, a high substitution degree was considered to be achieved (Table 3).

The nanoparticles obtained using PLA–(MeO-PEG(N)) showed different particle characteristics dependent on the preparative methods. Also, the particle size tended to be influenced by the preparative method rather than by the molecular weight or introduction degree of MeO-PEG(N) (Table 4). The nanoparticles prepared by the emulsification-solvent evaporation method exhibited a larger particle size. Also, the particle diameter was larger in the precipitation from acetone solution by the addition of water (solvent diffusion method A). On the other hand, PLA–(MeO-PEG(O)) nanoparticles showed a smaller particle size even when they were prepared with solvent diffusion method A. Bazile et al. reported that the nanoparticles produced with a mixture of PLA–(MeO-PEG(O)) and PLA exhibited a larger particle size with the increase in the ratio of PLA to PLA–(MeO-PEG(O)) (Bazile et al., 1995). Therefore, since PLA–(MeO-PEG(N)) had a lower introduction degree of MeO-PEG than PLA–(MeO-PEG(O)) did, the former nanoparticles were considered to be larger than the latter nanoparticles. These phenomena appeared to be associated with the surface density of PEG moieties. With the solvent diffusion methods B and C, the PLA–(MeO-PEG(N)) nanoparticles were smaller, and sonication made the particle size smaller. On the other hand,

the particle size of PLA–(MeO-PEG(O)) nanoparticles produced by solvent diffusion method C did not differ from that of the particles obtained by solvent diffusion method A. PLA–(MeO-PEG(N)) nanoparticles with a lower PEG density might be affected more easily by the preparative method. As PLA–(MeO-PEG(O)) nanoparticles exhibited a small particle size even in solvent diffusion method A, the sonication in solvent diffusion method C might little affect particle size.

The zeta potentials were compared among the nanoparticles with a similar particle size of approximately 60 nm. Those potentials were different among the nanoparticles. The PLA–(MeO-PEG(O)) nanoparticles showed a higher zeta potential (Table 5). This was considered to be because carboxyl end groups were highly substituted by MeO-PEG(O), that is, the negative charge was masked to a great extent by that ester bond. In PLA–(MeO-PEG(N)) nanoparticles, the nanoparticles with a higher introduction degree of MeO-PEG(N) showed a higher zeta potential. Although the introduction of MeO-PEG(N) causes a positive charge, the low introduction degree was considered not to raise the zeta potential of PLA–(MeO-PEG(N)) nanoparticles beyond that of the PLA–(MeO-PEG(O)) nanoparticles.

As to the preparation of DiD-containing PLA–(MeO-PEG(N)) nanoparticles, the solvent diffusion method B was applied because this method gave the PLA–(MeO-PEG(N)) nanoparticles with a particle diameter of 100–140 nm (Table 4), adequate for passive targeting to solid tumors (Ishida et al., 1999). As a result, the particle size of the DiD-containing PLA–(MeO-PEG(N)) nanoparticles obtained was larger than that of the PLA–(MeO-PEG(N)) nanoparticles without DiD. This particle size of the DiD-containing PLA–(MeO-PEG(N)) nanoparticles (approximately 220 nm) was fairly good for the passive targeting to solid tumors, though it might not be the best (Ishida et al., 1999). The incorporation of DiD appeared to make the particles bigger. Therefore, when incorporating drugs to the nanoparticles, the attention must be paid to the change in the particle size caused by the incorporation. The lipophilic dye DiD could be incorporated in PLA–(MeO-PEG(N)) nanoparticles at a high incorporation efficiency (Table 6). The recoveries of polymer and DiD in the nanoparticles were relatively high (Table 6). These suggested that the PLA–(MeO-PEG(N)) nanoparticles could be well loaded with a lipophilic drug.

Although the data are not presented here, the present PLA-aldehyde was applied to the introduction of a fluorescent hydrazine derivative or polyethylene glycol diamine. These molecules were introduced well to PLA-aldehyde (data not shown). The conjugate between PLA and fluorescent hydrazine derivative could be prepared by simple mixing, and was considered to be useful for the analysis of the in vivo behavior of PLA itself due to the existence of the fluorescent marker. The formation of the PLA derivative by the reductive amination between PLA-aldehyde and polyethylene glycol diamine suggested that PLA-aldehyde should be available for the preparation of other block copolymers. Thus, it is suggested that the present approach should be useful for the development of PLA derivatives as carriers for drug delivery systems or for the analysis of in vivo behaviors of PLA-based nanoparticles.

5. Conclusion

A novel acetal-ended PLA (PLA-acetal) could be prepared efficiently with conventional ring opening polymerization of DL-lactide. PLA-acetal was well converted to aldehyde-ended PLA (PLA-aldehyde). PLA-aldehyde was available to introduce molecules possessing amino groups to PLA by reductive amination. PLA–(MeO-PEG(N)), obtained by reductive amination between PLA-aldehyde and MeO-PEG(N), gave the nanoparticles of 60–340 nm, the size of which was dependent on the preparative method. The PLA–(MeO-PEG(N)) nanoparticles could be loaded efficiently with DiD, and showed a fairly good particle size for passive targeting. These results demonstrated that PLA-aldehyde should be useful for the derivatization of PLA and that PLA–(MeO-PEG(N)) could be utilized for the preparation of polymeric nanoparticulate dosage forms.

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References

Ah, Y.C., Choi, Y., Kim, S.Y., Kim, S.H., Lee, K.S., Byun, Y., 2001. Effects of ethylene oxide gas sterilization on physical properties of poly(L-lactide)–poly(ethylene glycol)–poly(L-lactide) microspheres. J. Biomater. Sci. Polym. Ed. 12, 783–799.

Bazile, D., Prud'homme, C., Bassoullet, M.T., Marlard, M., Spenlehauer, G., Veillard, M., 1995. Stealth Me.PEG–PLA nanoparticles avoid uptake by the mononuclear phagocytes system. J. Pharm. Sci. 84, 493–498.

Birnbaum, D.T., Kosmala, J.D., Henthorn, D.B., Brannon-Peppas, L., 2000. Controlled release of beta-estradiol from PLAGA microparticles: the effect of organic phase solvent on encapsulation and release. J. Contr. Release 65, 375–387.

Borch, R.F., Bernstein, M.D., Durst, H.D., 1971. The cyanohydridoborate anion as a selective reducing agent. J. Am. Chem. Soc. 93, 2897–2904.

Dunn, S.E., Coombes, A.G.A., Garnett, M.C., Davis, S.S., Davies, M.C., Illum, L., 1997. In vitro cell interaction and in vivo biodistribution of poly(lactide-co-glycolide) nanospheres surface modified by poloxamer and poloxamine copolymers. J. Contr. Release 44, 65–76.

Gilding, D.K., Reed, A.M., 1979. Biodegradable polymers for use in surgery, polyglycolic/poly(lactic acid) homo- and copolymers 20, 1459–1464.

Gref, R., Minamitake, Y., Peracchia, M.T., Trubetskoy, V., Torchilin, V., Langer, R., 1994. Biodegradable long-circulating polymeric nanospheres. Science 263, 1600–1603.

Hirano, M., Sakatoku, M., Yamashita, R., Iwa, T., 1984. Studies on the new long-acting anticancer preparation, 5-fluorouracil-polyglycolic acid composite. Jpn. J. Cancer Chemother. 13, 1569–1572.

Ishida, O., Maruyama, K., Sasaki, K., Iwatsuru, M., 1999. Size-dependent extravasation and interstitial localization of polyethyleneglycol liposomes in solid tumor-bearing mice. Int. J. Pharm. 190, 49–56.

Jeong, J.H., Kim, S.W., Park, T.G., 2004. Biodegradable triblock copolymer of PLGA–PEG–PLGA enhances gene transfection efficiency. Pharm. Res. 21, 50–54.

Jule, E., Nagasaki, Y., Kataoka, K., 2003. Lactose-installed poly(ethylene glycol)–poly(DL-lactide) block copolymer micelles exhibit fast-rate binding and high affinity toward a protein bed simulating a cell surface. A surface plasmon resonance study. Bioconjug. Chem. 14, 177–186.

Kawakami, S., Munakata, C., Fumoto, S., Yamashita, F., Hashida, M., 2001. Novel galactosylated liposomes for hepatocyte-selective targeting of lipophilic drugs. J. Pharm. Sci. 90, 105–113.

Luo, Y., Prestwich, G.D., 2002. Cancer-targeted polymeric drugs. Curr. Cancer Drug Targets 2, 209–226.

Maruyama, K., 2002. PEG-immunoliposome. Biosci. Rep. 22, 251–266.

Matsumura, Y., Maeda, H., 1986. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. Cancer Res. 46, 6387–6392.

Miura, H., Onishi, H., Sasatsu, M., Machida, Y., 2004. Antitumor characteristics of methoxypolyethylene glycol-poly(DL-lactic acid) nanoparticles containing camptothecin. J. Contr. Release 97, 101–113.

Mosqueira, V.C., Legrand, P., Gref, R., Heurtault, B., Appel, M., Barratt, G., 1999. Interactions between a macrophage cell line (J774A1) and surface-modified poly(DL-lactide) nanocapsules bearing poly(ethylene glycol). J. Drug Target. 7, 65–78.

Nagasaki, Y., Yasugi, K., Yamamoto, Y., Harada, A., Kataoka, K., 2001. Sugar-installed block copolymer micelles: their preparation and specific interaction with lectin molecules. Biomacromolecules 2, 1067–1070.

Nguyen, C.A., Allemann, E., Schwach, G., Doelker, E., Gurny, R., 2003. Cell interaction studies of PLA–MePEG nanoparticles. Int. J. Pharm. 254, 69–72.

Nishikawa, M., Miyazaki, C., Yamashita, F., Takakura, Y., Hashida, M., 1995. Galactosylated proteins are recognized by the liver according to the surface density of galactose moieties. Am. J. Physiol. 268, 849–856.

Noguchi, A., Takahashi, T., Yamaguchi, T., Kitamura, K., Takakura, Y., Hashida, M., Sezaki, H., 1991. Tumor localization and in vivo antitumor activity of the immunoconjugate composed of anti-human colon cancer monoclonal antibody and mitomycin C-dextran conjugate. Jpn. J. Cancer Res. 82, 219–226.

Ogawa, Y., Yamamoto, M., Takada, S., Okada, H., Shimamoto, T., 1988a. Controlled-release of euprolide acetate from polylactic acid or copoly(lactic/glycolic) acid microcapsules: influence of molecular weight and copolymer ratio of polymer. Chem. Pharm. Bull. 36, 1502–1507.

Ogawa, Y., Okada, H., Yamamoto, M., Shimamoto, T., 1988b. In vivo release profiles of leuprolide acetate from microcapsules prepared with polylactic acids or copoly(lactic/glycolic) acids and in vivo degradation of these polymers. Chem. Pharm. Bull. 36, 2576–2581.

Onishi, H., Machida, Y., Machida, Y., 2003. Antitumor properties of irinotecan-containing nanoparticles prepared using poly(DL-lactic acid) and poly(ethylene glycol)–block–poly(propylene glycol)–block–poly(ethylene glycol). Biol. Pharm. Bull. 26, 116–119.

Otsuka, H., Nagasaki, Y., Kataoka, K., 2000. Surface characterization of functionalized polylactide through the coating with heterobifunctional poly(ethylene glycol)/polylactide block copolymers. Biomacromolecules 1, 39–48.

Ozalp, Y., Ozdemir, N., Hasirci, V., 2002. Vancomycin release from poly(DL-lactide) and poly(lactide-co-glycolide) disks. J. Microencapsul. 19, 83–94.

Panagi, Z., Beletsi, A., Evangelatos, G., Livaniou, E., Ithakissios, D.S., Avgoustakis, K., 2001. Effect of dose on the biodistribution and pharmacokinetics of PLGA and PLGA–mPEG nanoparticles. Int. J. Pharm. 221, 143–152.

Pimm, M.V., Perkins, A.C., Duncan, R., Ulbrich, K., 1993. Targeting of *N*-(2-hydroxypropyl)methacrylamide copolymer–doxorubicin conjugate to the hepatocyte galactose-receptor in mice: visualisation and quantification by gamma scintigraphy as a basis for clinical targeting studies. J. Drug Target. 1, 125–131.

Xinteng, Z., Weisan, P., Ruhua, Z., Feng, Z., 2002. Preparation and evaluation of poly (DL-lactic acid) (PLA) or DL-lactide/glycolide copolymer (PLGA) microspheres with estradiol. Pharmazie 57, 695–697.

Yamamoto, Y., Nagasaki, Y., Kato, Y., Sugiyama, Y., Kataoka, K., 2001. Long-circulating poly(ethylene glycol)–poly(DL-lactide) block copolymer micelles with modulated surface charge. J. Contr. Release 77, 27–38.