Surfactant-Free Nanoparticles of Poly(DL-Lactide-*co*-Glycolide) Prepared with Poly(L-Lactide)/ Poly(Ethylene Glycol)

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ABSTRACT: Surfactant-free nanoparticles of poly(DL-lactide-*co*-glycolide) (PLGA) nanoparticles were prepared with or without poly(L-lactide)-poly(ethylene oxide) (LE) diblock copolymer (abbreviated as PLGA/LE and PLGA nanoparticles) by dialysis method. LE diblock copolymer was used to make PLGA nanoparticles to alternate conventional surfactant. The size of PLGA and PLGA/LE nanoparticles was 295.3 ± 171.3 and 307.6 ± 27.2 nm, respectively, suggesting LE diblock copolymer might be coated onto the surface of nanoparticles. Observation of scanning electron microscope (SEM) showed that PLGA/LE nanoparticles have spherical shapes ranging ~ 200–500 nm. In ^{1}H-NMR study, characteristic peaks of the methyl protons of PLGA disappeared in D_2O, whereas characteristic peaks of the methyl proton of both PEG and PLGA were shown in both $CDCl_3$ and D_2O, indicating that LE diblock copolymer coated on the surface of the PLGA nanoparticles. The higher the initial content of drug, the higher the drug contents and the lower the loading efficiency. PLGA/LE nanoparticles at higher drug contents resulted in slower adriamycin·HCl (ADR) release rate than that of lower drug contents. Also, slower release rate of ADR was achieved by entrapped into the PLGA/LE nanoparticles, whereas LE polymeric micelles showed rapid ADR release. © 2003 Wiley Periodicals, Inc. J Appl Polym Sci 89: 1116–1123, 2003

Key words: surfactant; diblock copolymer; gel permeation chromatography (GPC)

INTRODUCTION

Nanoparticles are widely used in the biomedical and biotechnological applications. In the drug delivery systems, nanoparticles have been primarily used for intravenous (iv) injection of drugs for drug targeting issues.[1–4] Drug targeting to specific sites of the body would be a great benefit in the therapy of several diseases, especially cancer treatment.[5–7] Therefore, the use of nanoparticles has attracted considerable interest to achieve these objectives.

Generally, the fate of nanoparticles after iv injection is greatly influenced by their interaction with the biological environment and their physicochemical properties. In particular, the effect of particle size and surface characteristics of nanoparticles has been shown to be of primary importance.[1,8] Administered particles several micrometers in diameter become filtered by the lung capillaries[9,10] and submicron particles are rapidly cleared by the reticuloendothelial system (RES).[11–13] Because RES is thought to be a major obstacle in the drug delivery to the site of drug action, rapid RES uptake can be avoided by alteration of the surface nature of nanoparticles.[9,11] The introduction of hydrophilic materials, such as poly(ethylene oxide) or poloxamer (PEO-polypropylene oxide) block copolymer on the nanoparticle surfaces can especially increase the blood circulation of carriers by reduction of the uptake of phagocytic cells.[13,14] Such applications of nanoparticles on the drug targeting to the specific body sites have advantages to avoid any surgery, which can always be the source of infection. Extensive and various research has been advanced to achieve the optimized carrier size and surface characteristics for extended blood circulation of carrier and effective delivery of drug to the target site.[3,4,7–9] Illum and coworkers[3,9,11,13,15] have been extensively investigating that altered biodistribution or targeted delivery to the specific site of the body of polystyrene nanoparticles are attained through surface modification by using hydrophilic block copolymer, poloxamer series (or pluronic series), which is absorbed onto the polysty-

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rene nanoparticle surface by hydrophobic interaction. A few years ago, it has been shown that the modification of biodegradable poly(lactide-*co*-glycolide) (PLGA) or polystyrene nanospheres by poly(lactide)-poly(ethylene glycol) (PEG) block copolymers to increase the surface hydrophilicity and decrease the surface charge of the nanospheres.[15] These coated nanospheres with block copolymers can alter the biodistribution in comparison to uncoated nanospheres. Langer and coworker[14,16] reported that core-shell-type nanospheres of PLGA-poly(ethylene oxide) (PEO) or poly(caprolactone)-PEO diblock copolymer can be made by a one-step procedure and the hydrophobic drug, lidocaine, was encapsulated in the nanospheres with high weight fraction, resulting in the sustained release of drugs. Also, it has been shown that these core-shell-type nanospheres have long blood circulation times, long enough to continuously deliver drugs.

The preparation method of nanoparticles is a critical problem for small nanoparticles.[17,18] The emulsion solvent evaporation method is widely employed for the preparation of nanoparticles or microspheres using PLGA[17–21] at present. In these methods, serious amounts of surfactants or emulsifiers are required to stabilize the dispersed oil droplets. In particular, poly(vinyl alcohol) (PVA) as a stabilizing emulsifier is most frequently used to make micro- or nanoparticles.[22,23] However, PVA has some problems in that PVA remains at the surface of the nanoparticles or microspheres and then it becomes difficult to remove it. It was known that PVA existing on the surface of PLGA micro- or nanoparticles change the biodegradability, biodistribution, and drug-release behavior.[24–27] After PVA detached from the microsphere surfaces in *in vivo*, it circulates for a long time without any favorable action[28] and is presently a suspected carcinogen.[29] Other surfactants such as Span series or Tween series, PEO, and poloxamer [PEO-poly(propylene oxide) block copolymer], etc., are also used to make and stabilize particles.[30,31] Furthermore, disadvantages of these methods are the difficulties and necessities of the removal of solvent and surfactant because of their toxicity and its solvent properties for polymer used: low particle yield, too many steps for the preparation, and use of too much surfactant for small nanoparticles.[30–32] Almost all of these surfactants are nonbiodegradable, nondigestible, and not always biocompatible. Also, these surfactants can affect the human body with an allergy-like reaction.

At this point, surfactant-free particulate system or surface-modified nanoparticles have been significantly investigated by several authors for a decade.[33–36] Surfactant-free nanocapsules of poly(DL-lactide) (PLA) based on nanoprecipitation technique were developed by Fessi et al.[34] and nanoprecipitation technique has been extensively employed by several groups to make nanoparticles. It was reported that PLGA or PLA microspheres can be prepared by using PLA oligomers[33] or PLA-PEG diblock copolymers,[37,38] which have an amphiphilic surfactant-like structure and behavior, as a means of surfactant instead of conventional surfactant. Because di- or triblock copolymers consisting of hydrophobic and hydrophilic block have a potential to self-aggregation in the aqueous environment, nanoparticles[14] or microspheres[37] using polylactide/PEO or poly(ϵ-caprolactone)/PEO block copolymers can be prepared without the use of surfactants or emulsifier. Moreover, because one of the problems with poly(ϵ-caprolactone) is the very slow degradation for several months, the presence of PEG hydrophilic segments may induce increased degradation rates of microspheres.

Recently, dialysis method was developed for the simple preparation of drug carriers such as liposomes and polymeric micelles.[39–41] Dialysis method is an acceptable simple and effective preparation method for small and narrow-sized distributed nanoparticles using block, graft copolymers, and other amphiphilic materials.[39–41]

For this study, we have prepared surfactant-free PLGA nanoparticles by using LE diblock copolymers instead of conventional surfactant and their physicochemical properties were analyzed *in vitro*. Adriamycin was entrapped into the surfactant-free PLGA nanoparticles coated with LE diblock copolymer for their potential as an anticancer drug.

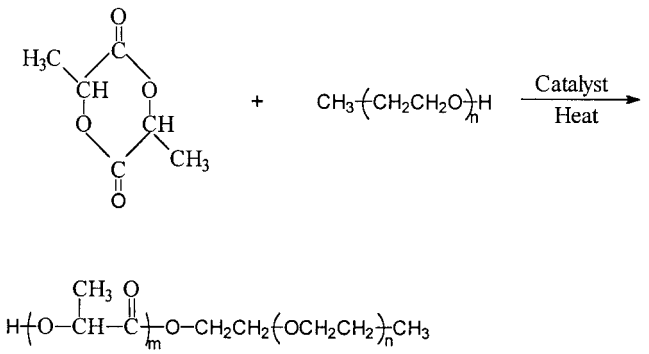
EXPERIMENTAL

Materials

PLGA 50/50, monomethoxy poly(ethylene glycol) (MePEG, M_w = 5000), and stannous 2-ethylhexanoate were purchased from Sigma Chemical Co. Ltd. (USA). (3S)-*cis*-3,6-Dimethyl-1,4-dioxane-2,5-dione (L-lactide) was purchased from Aldrich Chemical Co. (USA). Molecular weight of PLGA 50 : 50 was 40,100 Da from our GPC measurements, as described below. Adriamycin·HCl (ADR) was supplied from Dong-A Pharmaceutical Co. (Korea). Dimethylformamide (DMF), methylene chloride, diethyl ether, and acetone purchased from Aldrich Chemical Co. (Milwaukee, WI) as reagent grade were used without further purification.

Synthesis of poly(L-lactide)/PEG diblock copolymer

Poly(L-lactide) (PLLA)/PEG (abbreviated as LE) diblock copolymer was synthesized by ring-opening polymerization of L-lactide to the one-end hydroxyl group of MePEG as reported by Zhu et al.[42] as shown in Scheme 1. The preweighed amounts of L-lactide and MePEG were mixed in a round-bottomed flask and



Scheme 1 Synthesis of poly(L-lactide)-poly(ethylene glycol) diblock copolymer.

melted at 100°C in an oil bath. Stannous 2-ethylhexanoate (0.5 wt %) was added to the round-bottomed flask and evacuated with a vacuum pump. Then, the flask was placed in an oil bath at 180°C to start the polymerization. After 6 h, resultant product was dissolved in methylene chloride and precipitated into diethyl ether several times. The precipitants were harvested by filtration and the resultant product was dried in a vacuum oven at 40°C for 3 days.

The molecular weight was estimated by ^{1}H-NMR measurement by using $CDCl_3$. From the characteristic peaks of PLLA [5.1 and 1.5 ppm of methylene proton (CH and CH_3), respectively] and PEG (3.7 ppm of methylene proton), the copolymer composition and number-averaged molecular weight was estimated as 1220 of PLLA block and total molecular weight of LE diblock copolymer was calculated as about 6220.

Gel permeation chromatography (GPC) measurement

M_w of PLGA was measured from a Waters LC system coupled with a Waters 410 differential refractometer by using Waters Styragel™ HR1, HR2, and HR4 columns at a flow rate of 1 mL/min. THF was used as an eluant. Average M_w was evaluated by polystyrene as a standard.[43]

Preparation of PLGA/LE nanoparticles

Preparation of PLGA nanoparticles coated with LE diblock copolymer was carried out by dialysis method. Briefly, 20 mg PLGA and 5 mg LE diblock copolymer was dissolved in 4 mL DMF and solubilized entirely. Subsequently, 20 mg ADR in 1 mL DMF with 1.3 equiv triethylamine was added into the above solution. The solution was introduced into a dialysis tube (molecular cutoff, 12,000 g/mol) and dialyzed against 1.0 L of acetate buffer (pH 5.5, 0.1*M*) for 2 h and then 1 L × 4 of distilled water for 12 h. Then, the solution was analyzed or freeze-dried.

For evaluation of drug loading content, ADR-loaded PLGA/LE was dissolved into the DMF and measured by using UV spectrophotometer (Shimadzu UV-1201) at 479 nm. Drug loading contents and loading efficiency were calculated as follows: drug loading contents = [(the amount of remained drug in the nanoparticles)/(total amount of nanoparticles)] × 100; loading efficiency = [(amount of remained drug in the nanoparticles)/(initial amount of drug)] × 100.

Scanning electron microscope (SEM) observation

The morphology of the nanoparticles was observed by using a SEM (JEOL, JSM-5400, Japan). One drop of the nanoparticle suspension was placed on a graphite surface. After freeze-drying, the sample was coated with gold/palladium by using Ion Sputter (JEOL, JFC-1100). Coating was provided at 20 mA for 4 min. Observation was performed at 25 kV.

Measurement of fluorescence spectroscopy

To measure the critical micelle concentration of LE diblock copolymer using fluorescence spectroscopy, LE diblock copolymer solutions without drugs were prepared as follows: 20 mg LE block copolymer was dissolved in 5 mL DMF and dialyzed by using a molecular cutoff of 12,000 g/mol dialysis tube (Sigma) against 1 L × 3 of distilled water for 3 h and then 3–4 h for 2 days. Resultant solution was adjusted to the various concentrations of block copolymers.

Critical micelle concentration (CMC) of the LE diblock copolymers was estimated to prove the potential of micelle formation by the measurement of fluorescence spectroscopy (Shimadzu F-7000 spectrofluorometer, Shimadzu Co. Ltd., Tokyo, Japan) by using pyrene as a probe.[44,45] To get the sample solutions, a known amount of pyrene in acetone was added to each of a series of 20-mL vials and the acetone was evaporated. The amount was adjusted to give a pyrene concentration in the final solution of either $6.0 \times 10^{-7}M$. Ten milliliters of various concentrations of block copolymer solutions was added to each vial and then was heated for 3 h at 65°C to equilibrate the pyrene and the micelles and left to cool overnight at room temperature. Emission wavelength was 390 nm for excitation spectra. Excitation and emission bandwidths were 1.5 and 1.5 nm, respectively.

Photon correlation spectroscopy (PCS) measurements

PCS was measured with a Zetasizer 3000 (Malvern Instruments, U.K.) with He-Ne laser beam at a wavelength of 633 nm at 25°C (scattering angle, 90°). A nanoparticle solution prepared by dialysis method

was used for particle size measurement (concentration, 0.1 wt %) and measured without filtering.

In vitro release studies

The release experiment *in vitro* was carried out as follows: 10 mg ADR-loaded PLGA/LE nanoparticles and 5 mL phosphate-buffered saline (PBS, 0.1*M*, pH 7.4) were put into a dialysis tube and then the dialysis tube was introduced into a vial with 100 mL PBS. For control, the equivalent amount of free ADR dissolved in 5 mL PBS was put into a dialysis tube and then the dialysis tube was introduced into the vial with 100 mL PBS. At specific time intervals, whole medium was taken and replaced with fresh PBS. The concentration of the released ADR was determined by a UV spectrophotometer (Shimadzu UV-1201) at 479 nm.

RESULTS AND DISCUSSION

Characterization of PLGA nanoparticles coated with LE diblock copolymer

LE diblock copolymer was synthesized for use as a surfactant and surface modification of nanoparticles. It can be expected that LE diblock copolymer should be coated onto the PLGA nanoparticles by hydrophobic interactions. Due to the surfactant behavior, LE diblock copolymer can form polymeric micelle through a self-assembling process and also can be used as a hydrophobic drug carrier.[43] Fluorescence probe technique was used to measure their CMC.[44–46] Wilhelm et al.[44] reported a micelle formation of polystyrene (PS) and PEO di- or triblock copolymers in water by using a fluorescence technique with pyrene as a hydrophobic probe and determined CMC from fluorescence and excitation spectra, as pyrene partitions between aqueous and micellar environments.

The formation of polymeric micelle of LE diblock copolymer prepared by dialysis technique was confirmed by a fluorescence probe technique using pyrene as a hydrophobic probe. Fluorescence excitation spectra of LE block copolymer at various concentrations in the presence of pyrene ($6.0 \times 10^{-7}M$) is shown in Figure 1(a). Pyrene will be preferentially partitioned into hydrophobic cores with a change of the photophysical properties of the molecules. In the excitation spectrum, a red shift was observed with increasing concentration of LE block copolymer. A red shift of pyrene in the excitation spectrum was observed in the study of micelle formation of PS-PEO block copolymers.[44] The (0, 0) bands in the pyrene excitation spectra were examined and compared with the intensity ratio $I_{336.2}/I_{334.2}$. This ratio takes the value characteristic of pyrene, in water at low concentrations, and the value of pyrene, entirely in the hydrophobic domain. A plot of $I_{336.2}/I_{334.2}$ versus log c is

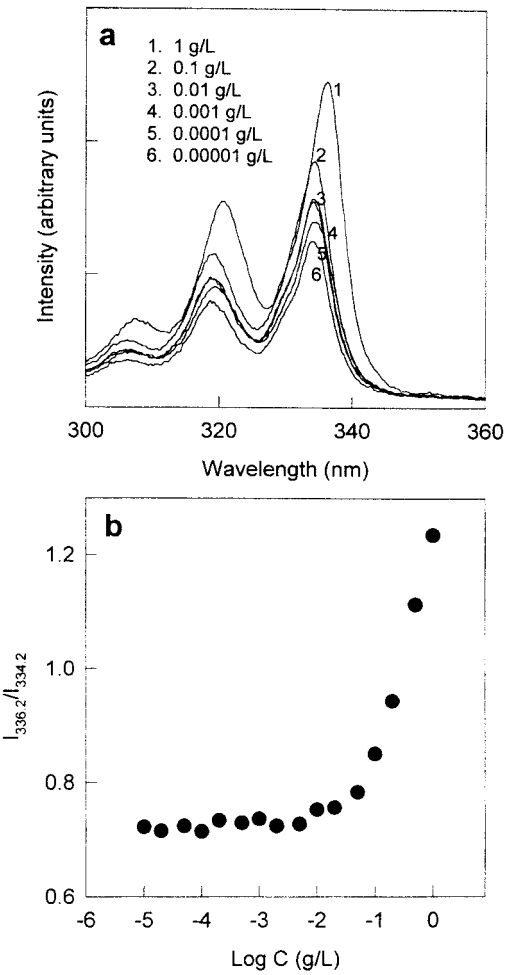


Figure 1 Fluorescence excitation spectra of pyrene/LE against concentration of LE in distilled water (emission wavelength: 390.0 nm) (a) and plots of the intensity ratio of $I_{336.2}/I_{334.2}$ from pyrene excitation spectra versus log C for block copolymer against concentration of LE in distilled water (b).

shown in Figure 1(b). A flat region in the low concentration extreme and sigmoidal region in the crossover region was noted. This result indicated that signal change in the region of 0.065 g/L can be used to evaluate the CMC values of LE block copolymer.

PLGA nanoparticles noncoated and coated with LE diblock copolymer (abbreviated as PLGA and PLGA/LE nanoparticles, respectively) were prepared by dialysis technique. Figure 2 shows the comparison of particle size distribution of PLGA nanoparticles without (PLGA) Figure 2(a) and with (PLGA/LE) Figure 2(b) LE diblock copolymers. Particle size of PLGA nanoparticles without and with LE was 295.3 ± 171.3 and 307.6 ± 27.2 nm, respectively. These results indicated that mean size of PLGA nanoparticles was slightly increased and LE coating layer can be evaluated as about 6 nm. After addition of LE diblock copolymer, size distribution of PLGA nanoparticles showed narrower distribution than that of noncoated LG nanoparticles. It was thought that LE

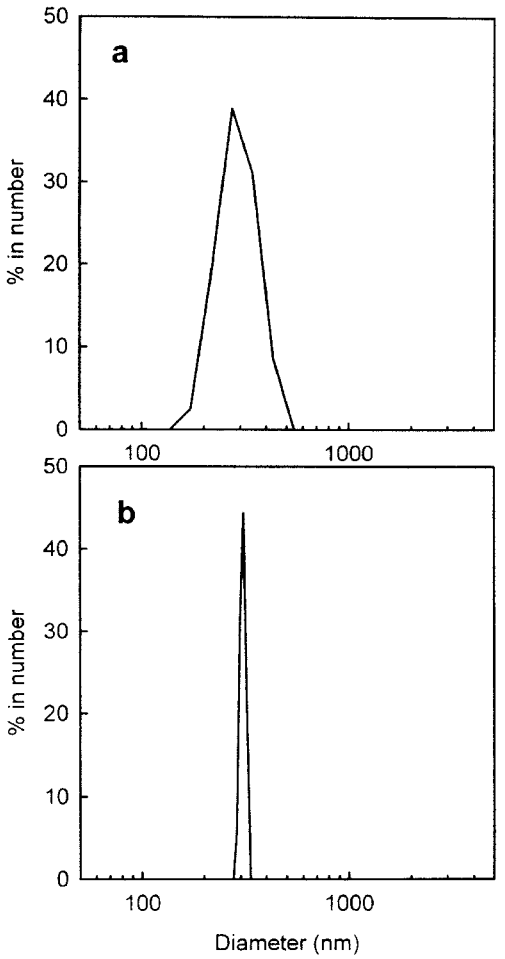


Figure 2 Particle size distribution of PLGA nanoparticles without (a) and with (b) LE.

diblock copolymer acts as a stabilizing agent and then the size distribution of the nanoparticles has narrowed. Further evidence of LE coated onto the surface of PLGA nanoparticles was obtained with ^{1}H-NMR in $CDCl_3$ and D_2O, as shown in Figure 3. Because both of the PLGA and LE block copolymers can be dissolved in $CDCl_3$ [Fig. 3(a, b)], PLGA nanoparticle formation with LE coating (PLGA/LE) is not expected because both polymers exist in a liquid state in $CDCl_3$. In $CDCl_3$, characteristic peak of the protons of the PLGA was shown in 1.6, 4.8, and 5.2 ppm [Fig. 3(a)]. As shown in Figure 3(b), both the characteristic proton peaks of PLGA copolymer and LE block copolymer were shown in 1.6, 4.8, and 5.2 ppm (characteristic peaks of PLGA and PLA block) and 3.7 ppm (characteristic peaks of PEG). However, these characteristic peaks of the methyl protons in the PLGA disappeared in D_2O [Fig. 3(c)], whereas characteristic peaks of the methyl proton of PEG appearing in 3.6 ppm was shown in both $CDCl_3$ and D_2O, indicating that LE diblock copolymer was coated on the surface of the PLGA nanoparticles.

In vitro drug release study

The effect of the initial drug feeding amount on the particle size and drug loading contents of PLGA/LE nanoparticles were summarized in Table I. The increased initial contents of ADR resulted in increased particle size and drug contents but loading efficiency was decreased. Also, increased drug contents resulted in not only increased particle size but also broad size distribution. Above all, drug content of LE diblock copolymer itself was significantly lower than PLGA/LE nanoparticles, indicating that the initial content of drug significantly affected the drug contents, particle size, and physicochemical properties of PLGA/LE nanoparticles.

To study the drug release behavior, the ADR-entrapped PLGA/LE nanoparticles were reconstituted

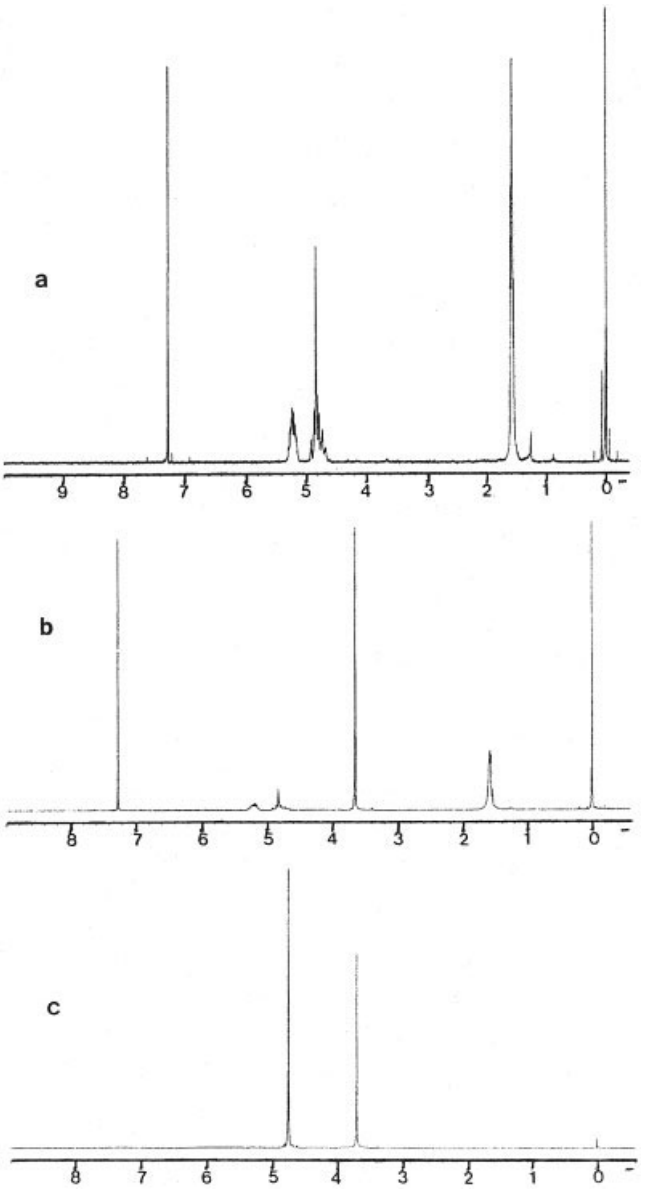


Figure 3 ^{1}H-NMR spectra of PLGA nanoparticles in $CDCl_3$ (a), and PLGA/LE nanoparticles in $CDCl_3$ (b) and D_2O (c).

TABLE I
Characterization of ADR-Entrapped PLGA/LE Nanoparticles

Sample	Polymer (mg)		Drug (mg)	Drug loading contents (wt %)	Loading efficiency (wt %)	Particle size (nm) (% in area)		
	PLGA	LE				Intensity average	Volume average	Number average
LE	—	20	20	7.1	7.65	63.5 ± 11.0 (82.8) 370.6 ± 90.5 (17.2)	63.8 ± 22.1 (93.5) 403.0 ± 151.0 (6.5)	63.6 ± 22.2
PLGA	20	—	—	—	—	295.8 ± 120.2	312.2 ± 181.3	295.3 ± 171.3
PLGA/LE	20	5	—	—	—	307.5 ± 14.8	307.8 ± 27.0	307.6 ± 27.2
PLGA/LE-1	20	5	20	15.7	18.63	324.9 ± 38.3	328.8 ± 76.2	326.8 ± 73.7
PLGA/LE-2	20	5	40	21.1	13.38	331.5 ± 78.3	359.6 ± 193.0	348.4 ± 176.6

in PBS (pH 7.4, 0.1*M*) and their results are shown in Figure 4. Particle size of PLGA/LE nanoparticles before and after lyophilization was 348.4 ± 176.6 and 367.6 ± 147.9 nm, respectively. These results indicate that PLGA/LE nanoparticles are able to be stored in freeze-dried form and simply reconstituted into the aqueous medium. Also, their particle size was not significantly affected by the lyophilization process. The morphology of reconstituted PLGA/LE-2 nanoparticles was observed by SEM, as shown in Figure 5.

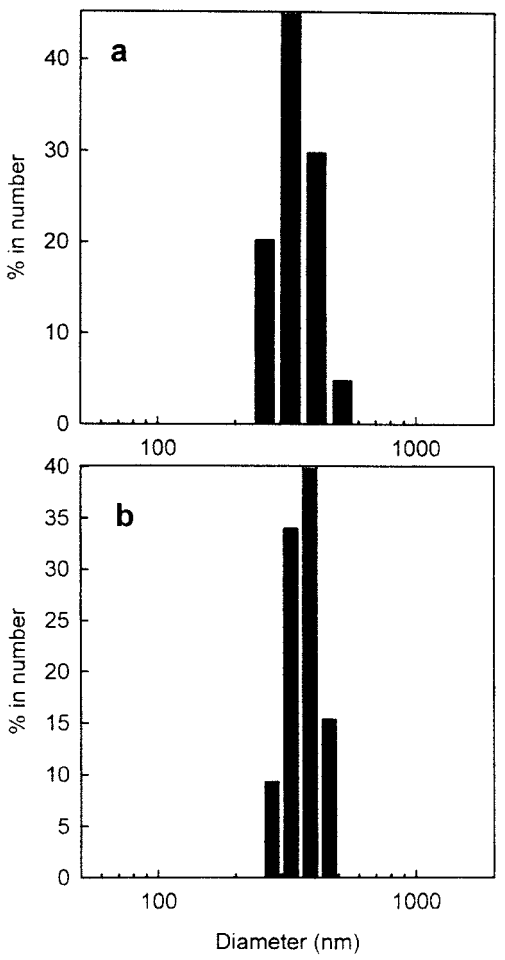


Figure 4 Reconstitution of PLGA/LE nanoparticles before (a) and after (b) lyophilization.

PLGA/LE-2 nanoparticles have maintained a spherical shape and their size ranged from 200 to 500 nm.

Figure 6 shows the release kinetics of ADR from PLGA/LE nanoparticles as a function of drug contents. In PLGA/LE nanoparticles, ADR is continuously released over 3 days and the release pattern revealed almost pseudo-zero-order kinetics. However, ADR release from LE block copolymer micelle finished in almost 2 days. The higher the drug contents, the slower the drug release kinetics. These phenomena were reported by several authors.[14,39,40] Gref et al.[14] reported that crystallization of hydrophobic drug occurred inside the nanoparticles and, especially, at the higher drug loading contents, a phase separation occurs, leading to the crystallization of part of the drug in nanoparticles. Then, hydrophobic drugs loaded into nanoparticles release more slowly at higher drug contents, differing from hydrophilic water-soluble drugs. Also, our group observed that ADR release rate from nanoparticles at higher drug contents (PLGA/LE-2) were shown to be slower than that of lower drug contents. On the other hand, at the low drug contents, ADR might be relatively present as a molecular dispersion inside the nanoparticles.[40] The crystallized drug should dissolve and diffuse more slowly into the

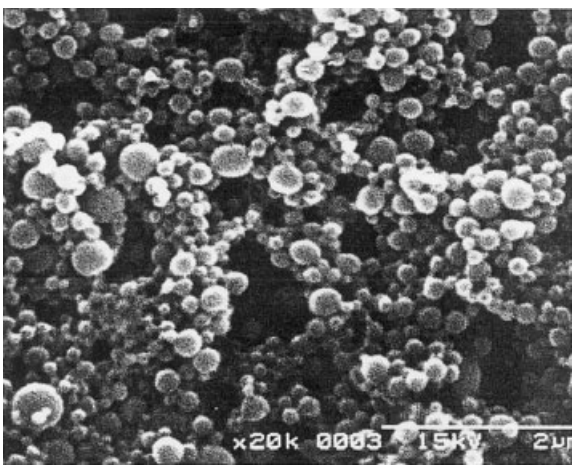


Figure 5 Morphological observation of PLGA/LE-2 nanoparticles using a scanning electron microscope.

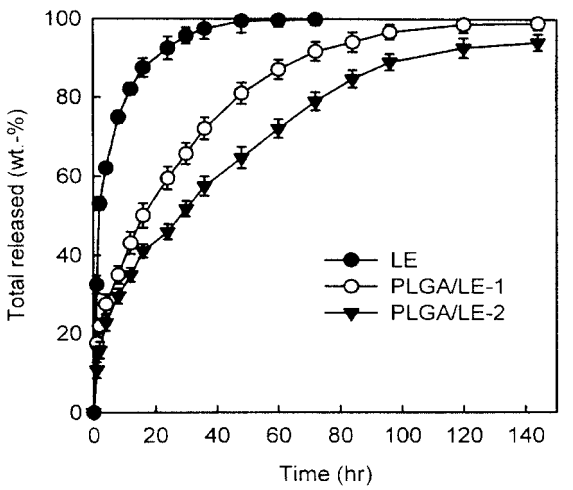


Figure 6 Release of ADR from LE polymeric micelle and PLGA/LE nanoparticles in PBS (0.1*M*, pH 7.4) at 37°C.

outer aqueous phase than that of molecular dispersion state. These characteristics of drug release behavior were supported by calorimetric analysis (data not shown) as reported previously.[39] Also, because of differences in the diffusivity of drug molecules to the outer aqueous phase, drug-release kinetics are affected not only by the drug contents but also by the nanoparticle size. Generally, the drug release rate is slower at the large nanoparticles than that of small nanoparticles reported elsewhere.[5] Resultantly, control of the drug-release kinetics can be achieved by optimizing the chemical nature of the used polymers, drug contents, used initial solvents, and the size of the nanoparticles.

CONCLUSION

In this article, PLGA nanoparticles coated with poly(L-lactide)-poly(ethylene oxide) diblock copolymer (abbreviated as PLGA/LE nanoparticles) were prepared by dialysis method as a novel carrier of the anticancer drug, ADR. PLGA/LE nanoparticles were prepared by dialysis method and their particle size distribution was measured by PCS for analysis of effect of LE coating. PLGA/LE nanoparticles containing ADR were prepared to analyze their loading capacity and *in vitro* release characteristics. The size of PLGA and PLGA/LE nanoparticles was 295.3 ± 171.3 and 307.6 ± 27.2 nm, respectively, indicating that LE diblock copolymer was coated onto the surface of nanoparticles. From the observation of SEM, PLGA/LE nanoparticles showed spherical shapes ranging from about 200 to 500 nm. In ^{1}H-NMR study, characteristic peaks of the methyl protons of PLGA disappeared in D_2O, whereas characteristic peaks of the methyl proton of both PEG and PLGA was shown in both $CDCl_3$ and D_2O, indicating that LE diblock copolymer coated on the surface of the PLGA nanoparticles. The higher the initial content of drug, the higher the drug contents and the lower the loading efficiency. PLGA/LE nanoparticles at the higher drug contents resulted in slower ADR release rate than that of the lower drug contents. Also, the slower release rate of ADR was achieved by entrapping the PLGA/LE nanoparticles, whereas LE polymeric micelles showed rapid ADR release.

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