Stability and Release Performance of a Series of Pegylated Copolymeric Micelles

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Received December 9, 2002; accepted December 24, 2002

Purpose. The aim of this work is to evaluate the capability of a series of biocompatible amphiphilic copolymers as a nano-sized drug carrier.

Methods. The influences of the type of lactone monomer, the feed molar ratios of lactone/PEG, and the molecular weight of PEG on the performance and release behavior of micelles are investigated.

Results. These pegylated amphiphilic copolymers efficiently form micelles with a low CMC value in the range of 10^{-6}–10^{-7} M. The average particle size of micelles is ~100 nm. The phenomenon of increasing particle size as increasing the chain length of poly(lactone) block is observed. The different hydrophobicity, based on chemical structure of poly(lactone), accounts for different interaction strength between indomethacin and hydrophobic inner core, which further influences the drug loading in copolymeric micelles and their release character. In addition, the PCL/PEG/PCL micellar solutions maintain their sizes at 4°C for 8 weeks without occurring significant aggregation or dissociation.

Conclusions. A series of biocompatible pegylated amphiphilic copolymers have been elucidated possessing micellization potential to form nano-sized micelles in an aqueous environment, which enable incorporate hydrophobic drug and regulate drug release.

KEY WORDS: poly(lactone); poly(ethylene glycol); micelles.

INTRODUCTION

Recently micellar carrier systems received much attention in drug delivery, partly because of their unique characteristics in the body, including ability to solubilize the hydrophobic molecules, possess thermodynamic stability, release of a drug for an extended time, and prevent rapid clearance by the RES due to their size and surface characteristics (1–3). Allen *et al.* (4) mentioned that the ideal micelle carrier system should possess several characteristics, which include the suitable size in the range of 10–200 nm, less than a millimolar range of critical micelle concentration, slow disassemble rate, retaining in the body for a long period, nontoxic degraded monomer, and ease of excretion by the kidney. The release of drug from micelles is generally governed by three mechanisms: the micelles enter the nucleus, the micelles enter the cells, and the micelles remain outside the cells where the drug is released (5). Because the micelles enter cells via an endocytosis rather than diffusion, and are faster in cancer cells than in normal cells, easy accumulation of anticancer drugs in tumor tissue results (6,7). The feasibility of polymeric micelles as a non-viral gene vector is an important perspective on carrier systems (8).

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The incorporation of drugs into micelles can be used to regulate the rate and extent of internalization of drugs into cells (9). Functionalization of the outer surface of the polymeric micelles by poly(ethylene glycol) modifies their physicochemical and biologic properties resulting in long-circulating characteristics and significant tumor accumulation. The binding of a potent anticancer agent, cisplatin, to poly(ethylene glycol)-poly(aspartic acid) block copolymer showed higher and more sustained serum levels in tumor tissue than cisplatin and much less renal damage without compromising anticancer cytotoxicity (10). A similar result was obtained in doxorubicin-conjugated poly(DL-lactic-co-glycolic acid) and poly(ethylene glycol) micelles and paclitaxel-loaded polymeric micelles (11,12). The fabrication of polymeric micelles sensitive to environmental changes, such as pH or temperature, is an intelligent carrier design. A sensitizer for the photodynamic therapy of cancer was incorporated in pH-sensitive *N*-isopropylacrylamide micelles, which showed higher potency when localized in tumor tissue (13). The nature of hydrophobic segments comprising the thermo-responsive inner core of polymeric micelle played an important role in controlling drug release and drug activity (14).

The aim of this work was to evaluate the capability of a series of characterized biocompatible amphiphilic copolymers as a drug carrier. The amphiphilic copolymers comprised hydrophobic poly(lactone) and hydrophilic poly(ethylene glycol) segments and were previously synthesized in the laboratory via a ring-opening copolymerization in the absence of toxic catalyst or initiator (15). The advantage of this method was avoiding toxic substances that resided in the resulting copolymers, and this was important for a material to be used as a drug carrier system or a medical device in the human body. Indomethacin was selected as a model drug to be incorporated inside the hydrophobic inner core of micelles to evaluate and regulate drug release. The influences of the type of lactone monomer (L-lactide, δ-valerolactone and ε-caprolactone), the feed molar ratio of lactone/PEG (40/1–200/1), and the molecular weight of PEG (PEG_{4000} and PEG_{10000}) on the performance and release behavior of drug-loaded micelles were investigated. The stability of micellar solution was further evaluated in an aqueous solution at 4°C.

MATERIALS AND METHODS

Materials

PEG_{10000} (average MW 10,000), L-lactide (L-LA), δ-valerolactone (δ-VL) and ε-caprolactone (ε-CL) were from Aldrich Chemical Company, Inc., (WI, USA). PEG_{4000} (average MW 3,000) was from Wako Pure Chemical Ind. LTD (Osaka, Japan). Indomethacin was from Sigma Chemical Co. (Dorset, UK).

Synthesis of Copolymers

The modified ring-opening copolymerization in the absence of catalyst or initiator was applied to synthesize six types of triblock copolymers comprising PLA/PEG_{4000}/PLA, PVL/PEG_{4000}/PVL, PCL/PEG_{4000}/PCL, PLA/PEG_{10000}/PLA, PVL/PEG_{10000}/PVL, and PCL/PEG_{10000}/PCL (16). Various molar ratios of L-LA, δ-VL or ε-CL monomer and

0724-8741/03/0400-0668/0 © 2003 Plenum Publishing Corporation

PEG were weighed and frozen in liquid N_2. The mixture was then vacuumized for 5 min, and immersed in an oil bath for copolymerization. The synthesized product was dissolved in dichloromethane, and extracted with *n*-heptane ×5. The dichloromethane layer was collected and the solvent was removed by rotary evaporation. The finally obtained copolymers were collected and further dried at 40–45°C. The composition of copolymers was determined by 200 MHz ^{1}H-NMR (Bruker DPX-200, USA). The molecular weight distribution in terms of polydispersity of synthesized copolymers was determined by GPC equipped with a refractive index detector (Shimadzu RID-10A, Japan).

Critical Micelle Concentration (CMC) Determination

The CMC of amphiphilic triblock copolymeric micelles was determined with a fluorescence spectrophotometer (F-4500, Hitachi, Tokyo, Japan) using pyrene as a fluorescence probe. The fluorescence excitation spectra of pyrene were measured at various concentrations of copolymers as mentioned by Wang *et al.* (17). The concentration of pyrene was kept at 6.0×10^{-7} M. The emission wavelength was set at 390 nm, and the intensities obtained from excitation wavelengths at 333.6 and 336.4 nm were recorded. The ratio of fluorescence intensity at 333.6 and 336.4 nm ($I_{333.6}/I_{336.4}$) was calculated and plotted against the logarithm of the copolymer concentrations. The CMC value was defined as the midpoint of the transition region before achieved micellar region.

Preparation of Drug Loaded Micelles

Indomethacin and pegylated copolymers comprising various compositions of copolymers were previously dissolved in acetone, after that de-ionized water (Milli-Q plus, Waters, Millipore, USA) was added slowly. The solution was then placed in a dialysis bag (Spectrum®, CA, USA), immersed in 1 L of de-ionized water, and dialyzed for 24 h. The micelle solution was sonicated and centrifuged. The supernatant was collected and sucrose was added as a lyoprotective agent during freeze-drying (18). The yield of micelles was calculated based on the following equation:

$$\text{Yield}\,(\%) = \frac{\text{the weight of micelles after freeze-drying}}{\text{the total weight of copolymer, drug, and lyoprotective agent added initially}} \times 100\%$$

The average particle size of micelles was measured with a particle sizer (Coulter® N4 Plus, Haieleah, Florida, USA) at $\theta = 62.6°$. The sample was negatively stained with 2%w/w phosphotungstic acid, and their morphologies were observed with a transmission electron microscope (Hitachi H-7100, Japan) under 75,000 voltage.

Loading Efficiency

Drug-loaded micelles were dissolved, and the concentration of indomethacin was determined with a validated UV spectrophotometer at 318 nm (Jasco model 7800, Tokyo, Japan). The percentage of drug loaded in micelles was calculated as follows:

$$\text{Loading}\,(\%) = \frac{\text{the amount of drug in micelles}}{\text{the amount of drug added initially}} \times 100\%$$

In Vitro Release Study

Drug-loaded micelles were placed in a dialysis device containing pH 7.2 phosphate buffer solution. The dialysis device was then sealed with the dialysis membrane (Spectrum®, CA, USA, cut off MW 6,000 – 8,000), and immersed in the same medium. The release of indomethacin from micelles was conducted at 37 ± 0.5°C, and the stirring speed was set at 50 rpm. Samples (1 mL) were withdrawn at specific time points for 14 days, and the release medium was replaced by the same volume of fresh medium. The sample solution was determined with a validated UV spectrophotometer at 318 nm. The cumulative amount of drug released at each sampling point was corrected with the volume of the release medium.

Stability Study

The blank micelles of PCL/PEG/PCL were placed in the de-ionized water and stored at 4°C for 8 weeks. The particle

Table I. The Composition, Molecular Weight and Polydispersity of Copolymers

Molar ratio	M_n (NMR)	M_w/M_n (GPC)	Molar ratio	M_n (NMR)	M_w/M_n (GPC)
LA/PEG_{4000}/LA			LA/PEG_{10000}/LA		
69/1/69	22800	1.8	74/1/74	31300	2.7
38/1/38	13900	1.6	72/1/72	30700	3.3
24/1/24	10000	1.5	37/1/37	20600	2.5
7/1/7	4900	1.1	28/1/28	18000	2.5
VL/PEG_{4000}/VL			VL/PEG_{10000}/VL		
62/1/62	15400	1.8	36/1/36	17200	1.7
51/1/51	13200	1.9	33/1/33	16600	1.9
41/1/41	11200	2.0	8/1/8	11600	1.5
11/1/11	5200	1.2			
CL/PEG_{4000}/CL			CL/PEG_{10000}/CL		
99/1/99	25600	1.8	92/1/92	31000	2.2
70/1/70	18900	1.9	63/1/63	24300	2.0
27/1/27	9100	1.4	33/1/33	17500	1.6
18/1/18	7000	1.7	15/1/15	13400	1.8

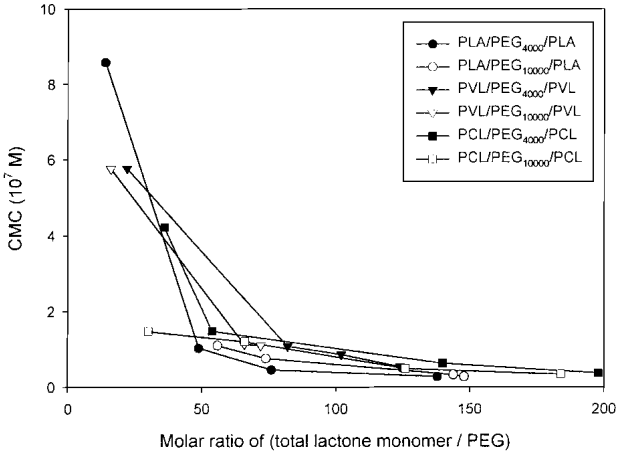

Fig. 1. The CMC of six types of micelles with various compositions.

size of micelles was measured at the beginning and at each 7-day interval after storage. The ratio of particle size following storage to initial size was calculated.

RESULTS AND DISCUSSION

The Properties of Drug-Loaded Micelles

Various compositions of six types of amphiphilic copolymers, comprised hydrophobic poly(lactone) (PLA, PVL or PCL) and hydrophilic poly(ethylene glycol) (PEG_{4000} or PEG_{10000}), were previously synthesized via a ring-opening copolymerization in the absence of toxic catalyst or initiator. The conversion of different types of lactone monomers into PLA/PEG_{4000}/PLA, PVL/PEG_{4000}/PVL, PCL/PEG_{4000}/PCL, PLA/PEG_{10000}/PLA, PVL/PEG_{10000}/PVL, and PCL/PEG_{10000}/PCL copolymers were 69.0, 56.0, 102.5, 63.5, 50.0, and 92.0% respectively. The conversion efficiency of CL monomer was higher than that of VL and LA monomers. This result indicated that the ring-opening of CL monomer was efficient under a current copolymerization condition that resulted in the final composition of synthesized copolymers close to their initial feed ratios. Table I lists the number-average molecular weights (M_n) and polydispersity (M_w/M_n) of six types of copolymers corresponding to their compositions. The number average molecular weights (M_n) of these copolymers ranged within thirty thousand daltons depending on their compositions. The polydispersity of copolymers was affected by the molecular weight of PEG and the type of monomers. The molecular weight distribution of copolymers comprising PEG_{4000} was less broad than PEG_{10000}. The polydispersity of PCL/PEG_{4000}/PCL was consistent in the range of 1.4–1.9 including wide molar ratio of CL/PEG_{4000}/CL ranging from 18/1/18–99/1/99. However, PLA/PEG_{4000}/PLA copolymers had the smallest polydispersity (1.1–1.8) rather than PVA/PEG_{4000}/PVL and PCL/PEG_{4000}/PCL copolymers.

These copolymers proved their biocompatibility through an *in vitro* cytotoxic test (15). The capability of these amphiphilic copolymers as a nano-sized drug carrier was further evaluated. Figure 1 shows the CMC values of copolymers comprising different types of poly(lactone) segment and different molecular weights of PEG segment. All of the pegylated amphiphilic copolymers were spontaneously formed micelles with a low CMC value in the range of 10^{-6}–10^{-7} M in an aqueous environment. The micellization efficiency of these copolymers was similar irrespective of the type of hydrophobic poly(lactone) segment and the molecular weight of hydrophilic PEG segment. However, a dependence of hydrophobic chain length was observed. The possible structure of micelles formed by pegylated triblock copolymer has been proposed by Maiti and Chatterji (19). They proposed the PEG chain folded back and inserted the hydrophobic terminals into the micellar core according to the micellization and adsorption energy. Langer *et al.* has used ^{1}H-NMR technique to elucidate the structure of diblock PLGA-PEG nanoparticles in an aqueous environment. They confirmed the formation of core-corona structure under this condition, where the chain mobility of PEG was similar to that dissolved in solution (20). The PEG domain acted as the hydrophilic outer shell extended in the aqueous environment, and poly(lactone) domain played as the inner core of the micelles. Riley *et al.* found (21) the molecular weight of PLA block in the range of 3-15 kDa formed highly colloidally stable micellar, like nanoparticles in an aqueous medium, with a complete surface coverage of PEG. They also mentioned that increase in the chain length of PLA up to 30-110 kDa would decrease PEG surface coverage and present PLA on the particle surface. The au-

Table II. The Yield and Average Particle Size of Copolymeric Micelles

Molar ratio	Yield (%)	Size (nm)	Molar ratio	Yield (%)	Size (nm)
LA/PEG_{4000}/LA			LA/PEG_{10000}/LA		
69/1/69	94.1 ± 3.9	97.7 ± 1.5	74/1/74	96.8 ± 1.0	143.1 ± 12.9
38/1/38	96.3 ± 0.5	90.7 ± 2.8	72/1/72	96.5 ± 1.0	106.8 ± 8.6
24/1/24	92.3 ± 0.6	64.5 ± 1.1	37/1/37	96.0 ± 2.1	101.9 ± 7.2
7/1/7	99.0 ± 0.9	53.7 ± 2.9	28/1/28	97.0 ± 3.6	92.0 ± 8.4
VL/PEG_{4000}/VL			VL/PEG_{10000}/VL		
62/1/62	96.12 ± 2.3	121.0 ± 10.3	36/1/36	96.9 ± 1.9	150.0 ± 4.9
51/1/51	97.3 ± 1.2	84.8 ± 1.8	33/1/33	96.4 ± 1.9	98.7 ± 3.3
41/1/41	96.7 ± 1.8	72.4 ± 3.0	8/1/8	96.9 ± 0.3	97.9 ± 12.0
11/11/11	97.7 ± 5.8	67.4 ± 2.6			
CL/PEG_{4000}/CL			CL/PEG_{10000}/CL		
99/1/99	91.9	84.4	92/1/92	93.8	132.2
70/1/70	87.0	75.5	63/1/63	95.0	96.7
27/1/27	97.3	72.6	33/1/33	97.0	99.7
18/1/18	95.0	60.8	15/1/15	96.0	93.9

thors' copolymers have molecular weights close to their low molecular weight range; therefore, it is possible that PEG covered the particle surface.

Table II lists the yield and the average particle size of drug-loaded micelles formed by various compositions of copolymers that were prepared via a dialysis method. All of the recovery efficiencies were higher than 90% irrespective of the type of lactone, the chain length of poly(lactone), and the molecular weight of PEG. The average particle size of micelles was 100 nm. The phenomenon of increasing particle size as increasing the chain length of poly(lactone) block was observed. The morphology of micelles was observed with a

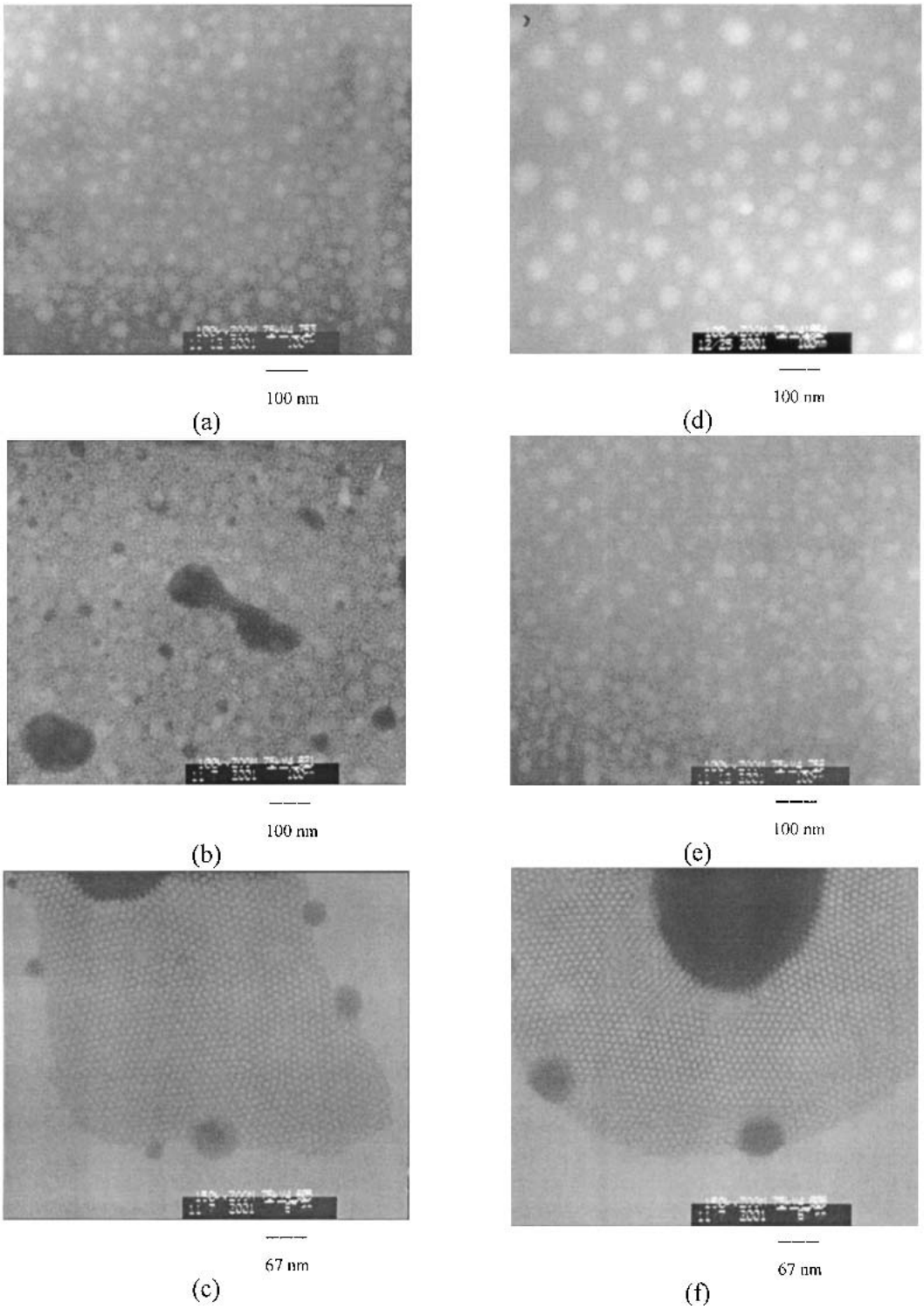

Fig. 2. TEM photographs of micelles formed by six different types of pegylated amphiphilic copolymers. (a) PLA/PEG_{4000}/PLA; (b) PVL/PEG_{4000}/PVL; (c) PCL/PEG_{4000}/PCL; (d) PLA/PEG_{10000}/PLA; (e) PVL/PEG_{10000}/PVL; (f) PCL/PEG_{10000}/PCL.

transmission electron microscope, and the photographs are shown in Fig. 2. All micelles showed a spherical shape with nano-size. Where two hydrophobic poly(lactone) terminals were inserted into the micellar core, the longer hydrophobic chain length enlarged the size of micelles. These results were consistent with the micellar structure proposed by Chatterji *et al.* (19).

Figure 3 shows the loading efficiency of indomethacin in micelles with various compositions. The loading efficiency of drug in micelles was increased as the hydrophobic poly(lactone) chain length increased. The loading efficiency of drug in PVL/PEG/PVL and PCL/PEG/PCL triblock copolymeric micelles was similar however, it was low in PLA/PEG/PLA micelles particularly for micelles comprised of high molecular weight PEG_{10000}. There were two, five, and six carbons in the backbone of each repeat unit of PLA, PVL, and PCL, respectively, the different hydrophobicity, based on their chemical structures, accounted for different interaction strength between indomethacin and poly(lactone) inner core (22).

In Vitro Release Study

Figure 4 shows *in vitro* release of drug from PLA/PEG_{10000}/PLA micelles of different compositions of PLA and PEG in pH 7.2 buffer solutions. No significant burst release was observed in all of the micelles, and there was ~20% of indomethacin released during the first day. The release of drug during the first four days was similarly independent of the composition of copolymers, however, a dependence on PLA chain length was observed where slower release was obtained from micelles comprising higher molar ratio of lactone monomer. The significant influence of molecular weight of PEG hydrophilic outer shell of micelles on drug release was particularly observed in PLA/PEG/PLA micelles, where low molecular weight PEG_{4000} not only increased drug loaded in micelles, but also slowed down the drug release rate when compared to PEG_{10000}.

The effects of the type of hydrophobic poly(lactone) and drug loading efficiency on the percentage of drug released were further discussed. A slow release character was observed in PVL/PEG/PVL and PCL/PEG/PCL micelles when their molar ratios of lactone/PEG/lactone were higher than 40/1/40, where less than 40% of drug was released at the end of 14 days. The release of drug from PLA/PEG/PLA micelles was faster than from PVL/PEG/PVL and PCL/PEG/PCL micelles. The weak interaction between indomethacin and poly(lactide) inner core of micelles partially contribute to the fastest release of drug. Low drug loading in PLA/PEG/PLA micelles was another reason. The similar explanation has been proposed in norfloxacin-loaded poly(lactide-co-glycolide) nanoparticles (22).

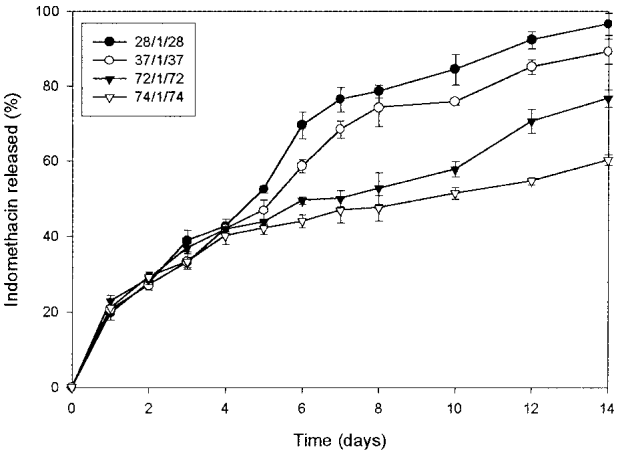

Fig. 4. The percentages of indomethacin released from PLA/PEG_{10000}/PLA micelles comprising various molar ratio of LA/PEG/LA in pH 7.2 phosphate buffer solutions.

Stability Study

Figure 5 shows the change of particle size of PCL/PEG/PCL micelles following storage in de-ionized water at 4°C for 8 weeks. If the ratio of particle size of micelles after storage to its initial size were in the range of 1.0 ± 0.3, indicating a stable micellar system maintained while outside this range, then it is possible that a significant aggregation or dissociation occurred (17). All of the micellar solutions maintained their sizes at the end of study irrespective of the molecular weight of PEG.

In conclusion, a series of biocompatible pegylated amphiphilic copolymers have been elucidated possessing micellization potential to form nano-sized micelles that incorporate hydrophobic drug and regulate drug release.

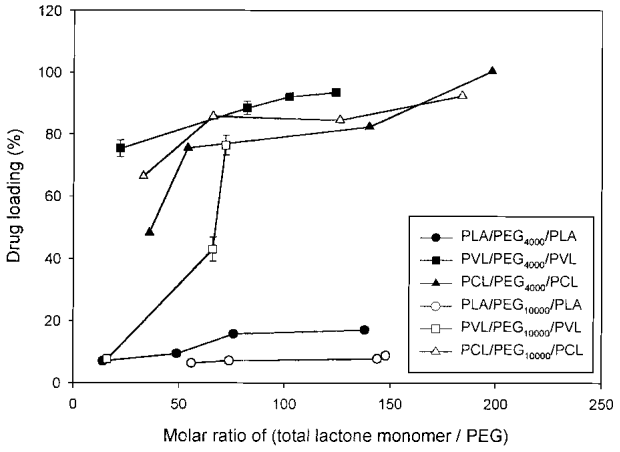

Fig. 3. The drug loading efficiency in six types of micelles with various compositions.

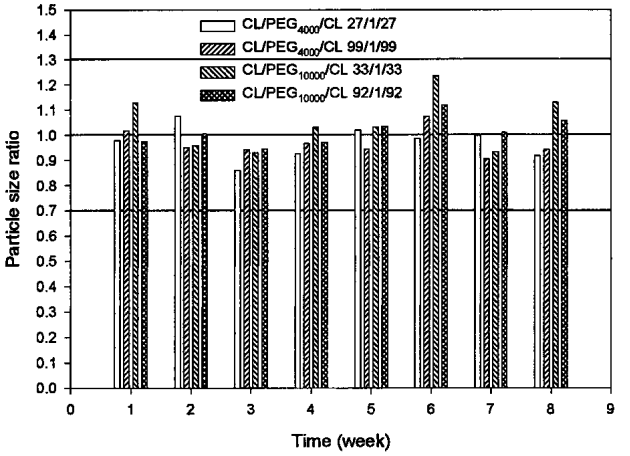

Fig. 5. The change of particle size of PCL/PEG/PCL micelles in de-ionized water at 4°C.

ACKNOWLEDGMENTS

The authors thank Professor J. W. Chern for his consultation in polymer synthesis. This work was supported by National Science Council in Taiwan (NSC 90-2320-B-002-105).

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