Characterization of Pegylated Copolymeric Micelles and *In Vivo* Pharmacokinetics and Biodistribution Studies

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Abstract: The aim of this study was to evaluate the influence of pegylated copolymeric micelle carrier on the biodistribution of drug in rats. The copolymers were synthesized via a modified ring-opening copolymerization of lactone monomers (ε-caprolactone, δ-valerolactone, L-lactide) and poly(ethylene glycol) ($PEG_{10,000}$ and PEG_{4000}). The molecular weights and the polydispersities of synthesized copolymers were in the range of 15,000–31,000 g/mol and 1.7–2.7, respectively. All of the pegylated amphiphilic copolymers were micelles formed with low CMC values in the range of 10^{-7}–10^{-8}*M*. The drug-loaded micelles were prepared via a dialysis method. The average particle size of micelles was around 150–200 nm. The cytotoxicity in terms of cell viability after treated with PCL–PEG, PVL–PEG, and PLA–PEG micelles was insignificant. PCL–PEG and PVL–PEG micelles without branch side chain in structures had higher drug loading than PLA–PEG micelles. *In vitro* release profiles indicated the release of indomethacin from these micelles exhibited a sustained release behavior. The similar phenomenon was also observed *in vivo* in rats. The pegylated copolymeric micelles not only decreased drug uptake by the liver and kidney, but also prolonged drug retention in the blood.
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Keywords: poly(lactone); poly(ethylene glycol); micelles; pharmacokinetics; biodistribution

INTRODUCTION

The block copolymeric micelles possess several advantages, including good structural stability, slow dissociation rate, low critical micelle concentration (CMC), easy control of particle size, good solubilization of hydrophobic drugs, etc.[1] It has been applied in the field of drug targeting partly because of its unique characteristics in the body, including ability to reduce cytotoxicity of anticancer drugs, to accumulate anticancer drugs in tumor cells, to release drugs for an extended time, and to prevent rapid clearance by the reticular endothelial system (RES) because of their size and surface characteristics.[2] It has been reported that adriamycin-loaded micelles show dramatically higher antitumor activity *in vivo* than the free drug itself.[3,4] This is because macromolecules are too large to pass through the normal vessel walls, but they are relatively easy to extravasate into and accumulate within tumor tissue.[5–9] The functionalization of the outer surface of polymeric micelles by poly(ethylene glycol) (PEG), pluronics, and poloxamines reduces protein opsonization on the surface of nanoparticles and subsequent phagocytosis by the nonparenchymal cells of the liver. In other words, the avoidance of RES's uptake of nanoparticles and an increase in their circulation time in blood are achieved.[10] Otherwise, the pegylated copolymers possess amphiphilic property and form nanosized core–shell structure at the concentration above CMC. Furthermore, the cationic block copolymers consisting of a PEG block are able to spontaneously self-assemble with plasmid DNA to form a complex with a stable nature in the blood.[11]

The aim of this study was to evaluate the influence of copolymeric micelle carrier on the pharmacokinetics and biodistribution of drug in rats. The copolymers were synthesized via a modified ring-opening copolymerization of lactone monomers (ε-caprolactone, δ-valerolactone, L-lactide) and PEG ($PEG_{10,000}$ and PEG_{4000}). The possible reaction mechanism has been proposed by Cerrai et al. [12] and Kim et al.,[13] where the hydrogen atom of PEG end groups acted as an initiator to directly induce acyl-oxygen cleavage on lactone ring. The advantages of this method included the simple reaction condition and the nonuse of toxic catalysts and solvents. Consequently, no toxic substances resided in the resulting copolymers, and this was very important for a material to be used as a drug carrier or a medical device in the human body. The synthesized copolymers were characterized

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for their physical properties, cytotoxicity, and degradability. The drug-loaded micelles were then prepared via a dialysis method, and the drug release and the stability of micelle carriers were evaluated. Finally, the pharmacokinetics and biodistribution of drug delivered by micelle carriers were investigated *in vivo* in rats.

MATERIALS AND METHODS

Materials

PEG_{10000}, L-lactide (L-LA), δ-valerolactone (δ-VL) and ϵ-caprolactone (ϵ-CL) were purchased from Aldrich Chemicals (Wisconsin, USA). PEG_{4000} was from Wako Pure Chemicals (Osaka, Japan). Indomethacin and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide thiazolyl blue) were from Sigma Chemicals (Dorset, UK).

Synthesis and Characterization of Copolymers

The modified ring-opening copolymerization in the absence of an external initiator was used to synthesize six types of triblock copolymers, including PCL–$PEG_{10,000}$ (C10), PCL–PEG_{4000} (C4), PVL–$PEG_{10,000}$ (V10), PVL–PEG_{4000} (V4), PLA–$PEG_{10,000}$ (L10), and PLA–PEG_{4000} (L4), as indicated in Table I.[14] The composition and number-average molecular weight (M_n) of copolymers were determined by 200 MHz ^1H NMR (Bruker DPX-200; Bruker, USA). The molecular weight distribution in terms of polydispersity of the synthesized copolymers was determined by gel permeation chromatography (7.8 mm × 30 cm; Waters, USA) equipped with a refractive index detector (Shimadzu RID-10A; Shimadzu, Japan). The crystallinity of the synthesized copolymers was identified using X-ray diffractometer (Rigaku, USA), and the diffraction angle was set in the range of 10–30° with a scan rate 1°/min. The CMC of amphiphilic copolymers was determined with a fluorescence spectrophotometer (F-4500; Hitachi, Tokyo, Japan), using pyrene as a fluorescence probe.

Degradation of Copolymers

The pegylated copolymers, 70 mg, were weighed in a glass tube (6 × 50 mm), melted at 70°C, and solidified in a freezer. The molded copolymer was placed in a glass screw-capped test tube containing 10 mL of pH 7.4 phosphate buffer solution and maintained at 37°C. Samples were removed at the specific time points, filtered through a 0.45-μm membrane, and washed with distilled water. The solid samples were collected and dried *in vacuo* at room temperature. After that the remaining copolymers were weighed to determine their dry weights. The percentage of remaining copolymers was calculated by dividing the dry weight by the initial weight.

In Vitro Cytotoxicity of Copolymers

Normal human fibroblast cells (IMR-90) was maintained in the minimum essential medium containing 10% fetal bovine serum in a 5% CO_2 atmosphere at 37°C. Culture medium was plated in 96-well plates overnight. Cells were then incubated in the various concentrations of polymeric solutions at three 10-fold dilutions ranging from 0.001 to 0.1 mg/mL for 48 h. For each polymer concentration, experiments were carried out in triplicate. The degree of cells survived was evaluated by using MTT assay and determined with a spectrophotometer (Spectra MAX PLUS, USA) at 550 nm. The ratio of cell survival with and without copolymer treatment was calculated.

Preparation of Drug-Loaded Micelles

Indomethacin and pegylated copolymers were dissolved in acetone, and deionized water was then added slowly. After that the solution was placed in a dialysis bag, immersed in 1 L of deionized water, and dialyzed for 24 h. Finally, the micelle solution was sonicated and centrifuged. The average particle size of the micelles was measured with a particle sizer (Coulter® N4 Plus, Haieleah, Florida, USA) at $\theta = 62.6°$. The amount of indomethacin incorporated in micelles was determined with a UV spectrophotometer at 318 nm (Jasco model 7800, Tokyo, Japan). The percentage of the drug loaded in micelles was calculated by dividing the amount of drug in micelles to the amount of drug added initially. The stability of micelles in terms of their size change was investigated at 4°C for 12 weeks. The particle size of micelles was measured at the beginning and at 1, 2, 3, 4, 5, 6, 7, 8, 10, and 12 weeks after storage. The ratio of the particle size after storage to the initial particle size was calculated.

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 dialysis membrane (Spectrum®, CA, USA, cut off MW 6000–8000), and was immersed in the same medium. The release of indomethacin from micelles was allowed to occur 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 analyzed at 318 nm using a UV spectrophotometer, and the cumulative amount of drug released at each sampling point was corrected with the volume of the release medium.

Pharmacokinetic Study and Tissue Distribution in Rats

The male Wistar rats (250–300 g) were used in this study. They were obtained from National Taiwan University Experimental Animal Center. All procedures were examined by the Ethics Committee on Animal Experiment at National Taiwan University, and the animal experiment was in accordance with "Guide for the Care and Use of Laboratory Animals" published by the National Institute of Health. The rats were given subcutaneous injections with 10 mg/kg of drug-loaded micelle solution and free drug solution, respectively. Blood

TABLE I. The Composition, Polydispersity, and CMC of Amphiphilic Copolymers

Copolymer	Symbol	Molar ratio of lactone/PEG	Weight % of lactone	M_w/M_n	CMC (10^8 mole/L)
PCL-$PEG_{10,000}$	C10	184/1	68	2.2	3.39
PCL-PEG_{4000}	C4	198/1	88	1.8	3.67
PVL$PEG_{10,000}$	V10	72/1	42	1.7	11.00
PVL-PEG_{4000}	V4	124/1	81	1.8	5.40
PLA-$PEG_{10,000}$	L10	296/1	68	2.7	2.83
PLA-PEG_{4000}	L4	276/1	87	1.8	2.75

samples were collected at specific time points up to 24 h postdose. The plasma concentrations of indomethacin were analyzed with a high-performance liquid chromatography (HPLC) equipped with a reverse-phase column (LiChroCART RP-18, 250–254 mm, 7 μm) and a UV spectrophotometer (Shimadzu SPD-6AV) at a wavelength of 280 nm. The mobile phase was constituted by methanol and 1% acetic acid (73:27 v/v, pH 3.0) and its flow rate was 1.0 mL/min. The intra- and interday precision and accuracy of the HPLC analytical method were validated before sample analysis. The pharmacokinetic parameters were calculated by using WinNonlin software (Version 4.0.1; Pharsight, USA), and the differences between two groups were statistically compared using *t*-test ($\alpha = 0.05$). In the tissue distribution study, the rats were sacrificed at 0.5, 1, 4, and 8 h after administration of micelles and free drug solution, respectively. The heart, liver, lung, and kidney were excised, washed with saline, blotted dry, and weighed. The tissue samples were then homogenized and extracted. The drug concentration in each tissue was assayed using HPLC. The total amount of drug accumulated per unit weight of tissue within 8 h was calculated, and the difference in each tissue between two groups with or without micelle carriers was statistically compared using *t*-test ($\alpha = 0.05$).

RESULTS AND DISCUSSION

Characterization of Amphiphilic Copolymers

Table I lists the composition, polydispersity, and CMC of six copolymers. Their molecular weights and the polydispersities were in the range of 15,000–31,000 g/mol and 1.7–2.7, respectively. Their X-ray diffraction spectra are shown in Figure 1. PEG, PVL, and PCL had special diffraction peaks 2θ at 19°, 22°, and 21°, respectively. High molecular weight $PEG_{10,000}$ was crystallized in all copolymers, whereas the crystallization of PEG_{4000} did not occur. The X-ray spectra of PLA–PEG were different from PCL–PEG and PVL–PEG copolymers. The crystallinity of PLA segment in PLA–PEG copolymers was quite low and most PLA behaved as an amorphous phase. The efficient and rapid crystallization of PEG hindered PLA crystallization in PLA–PEG copolymers. The biocompatibility of synthesized amphiphilic copolymers was evaluated *in vitro* cytotoxicity test using normal human fibroblast cells. Figure 2 shows the ratio of survival cells after treated with 10^{-3}–10^{-1} mg/mL of six types of copolymers. All of these copolymers showed similar *in vitro* cytotoxicity, and more than 90% of cells were viable after treated with copolymers irrespective of the chain length of hydrophilic PEG block and the type of poly(lactone) block. In other words, all of these copolymers might be recognized as biocompatible in the studied concentration range. All of the pegylated amphiphilic copolymers were able to form micelles with a low CMC in the range of 10^{-7}–$10^{-8}M$, and their micellization efficiency was similar independent of copolymers.

Degradation of Copolymers

Figure 3 shows the weight of pegylated copolymers remaining in pH 7.4 phosphate buffer for 360 days. The prominent weight loss was observed during the first 7 days, and there were 1.5, 6.9, 7.2, and 22.1% of PCL–PEG_{4000} (C4), PCL–$PEG_{10,000}$ (C10), PVL–PEG_{4000} (V4), and PVL–$PEG_{10,000}$ (V10) lost, respectively. The possible reason accounted for weight loss in the first phase could be due to the solubilization of uncopolymerized PEG polymer in aqueous medium. Fur-

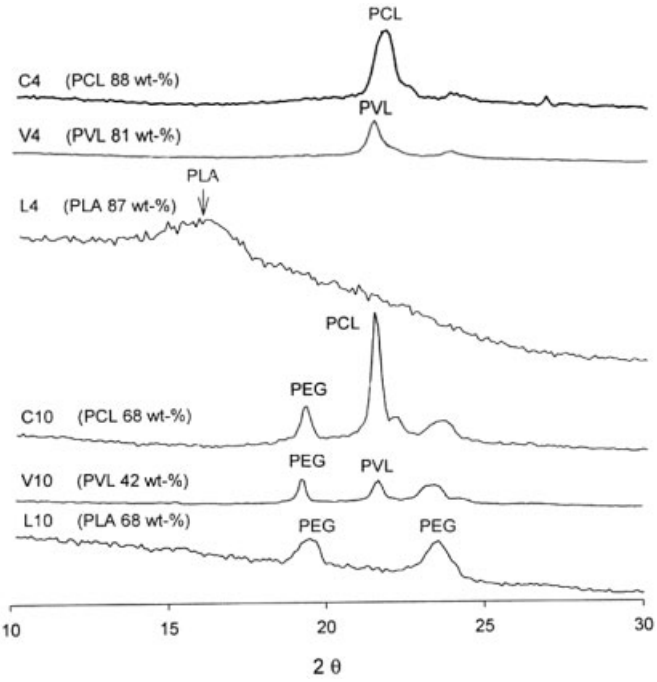

Figure 1. X-ray diffraction spectra of six types of copolymers: (a) C4, (b) V4, (c) L4, (d) C10, (e) V10, and (f) L10.

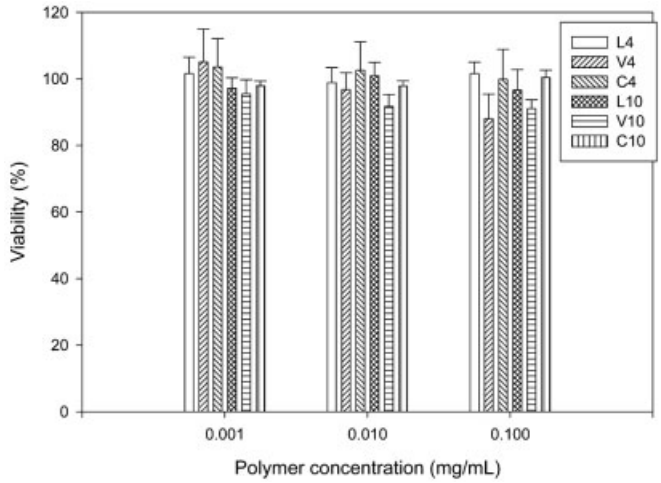

Figure 2. Cell viability in various concentrations of copolymeric micelles.

thermore, the decrease of polymer mass in the second phase was slow, and the total amounts of copolymers lost at the end of 360 days were 6.9, 15.1, 15.4, and 39.9% for PCL–PEG_{4000}, PCL–$PEG_{10,000}$, PVL–PEG_{4000}, and PVL–$PEG_{10,000}$, respectively. The gel permeation chromatograms showed a change of molecular weight distribution to small size direction in the second phase (data not shown). In other words, the decrease of molecular weight occurred continuously; however, the size of degraded copolymers was not small enough to dissolve in the medium to account for further weight loss in the second phase. The similar two-phase degradation phenomenon has been reported by Li et al. in alkaline medium (pH 10.6).[15]

Core–Shell Structure of Micelles

Theoretically, amphiphilic triblock copolymers enabled to form self-assemble micelles as soon as the concentration increased above the CMC, where the hydrophilic PEG chain

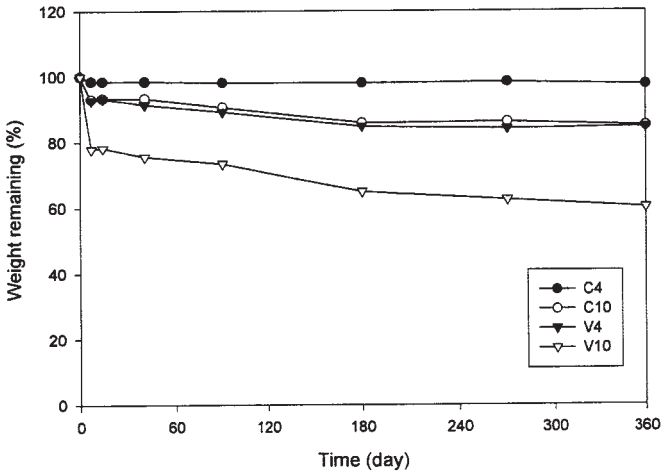

Figure 3. Weight of copolymers remaining in pH 7.4 phosphate buffer solutions at 37°C.

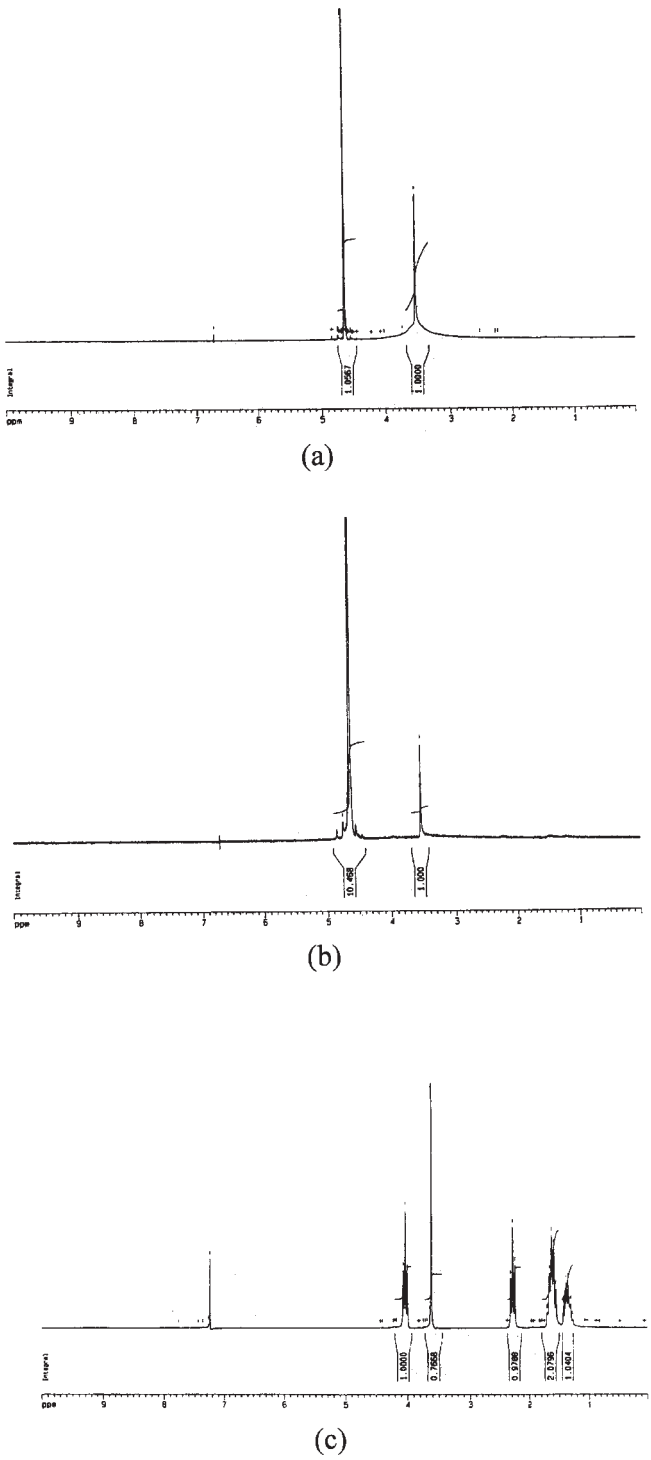

Figure 4. ^{1}H NMR spectra of PCL–PEG: (a) C10 in D_2O, (b) C4 in D_2O, and (c) C4 in $CDCl_3$.

was folded back and inserted the hydrophobic chains inside the core of micelles.[16,17] The ability to form core-shell structures by our amphiphilic copolymers was further evaluated by using ^{1}H-NMR technique. Figure 4 shows the ^{1}H-NMR spectra of PCL–$PEG_{10,000}$ and PCL–PEG_{4000} micelles in D_2O and in $CDCl_3$, respectively. Since PCL–PEG copolymer could be dissolved in $CDCl_3$, the peaks assigned to methylene protons

TABLE II. The Drug Loading and Particle Size of Micelles

Copolymer	Symbol	Drug loading (%)	Particle size (nm)
PCL-PEG$_{10,000}$	C10	77.5 ± 5.2	190.8 ± 8.4
PCL-PEG$_{4000}$	C4	72.7 ± 2.7	174.2 ± 15.3
PVL-PEG$_{10,000}$	V10	80.5 ± 2.2	159.0 ± 4.9
PVL-PEG$_{4000}$	V4	81.2 ± 1.7	185.0 ± 11.3
PLA-PEG$_{10,000}$	L10	7.3 ± 0.7	206.3 ± 4.3
PLA-PEG$_{4000}$	L4	19.6 ± 0.9	201.1 ± 22.3

of PCL and PEG were all clearly appeared in the spectra when $CDCl_3$ was used as the solvent [Figure 4(c)]. However, when the micelle was in water environment, there was only methylene protons corresponding to PEG resolved in the spectra, and the peaks corresponding to lactone repeat units were almost not visible. This result revealed that the poly(lactone) block was dispersed (instead of being dissolved) in the aqueous environment because of its hydrophobic character, and behaved as the solid core of the micelles. On the other hand, it was found that the shape of the methylene peak of PEG residues in D_2O was broader for PCL–PEG$_{10,000}$ micelles than for PCL–PEG$_{4000}$ micelles. Since the chain length of PEG$_{10,000}$ anchored to solid core was longer than that of PEG$_{4000}$ and also had less chain mobility than PEG$_{4000}$, it resulted in a broader bottom as shown in the NMR spectrum.[18]

Drug-Loaded Micelles

Table II lists the average particle size and drug-loading in micelles formed by six types of copolymers. The average particle size of micelles was around 150–200 nm. The morphology of micelles was observed with a transmission electron microscope, and all of micelles showed spherical shape with several hundred nanometers in size. The loading efficiency of drug in PCL–PEG and PVL–PEG micelles was similar in the range of (72.7 ± 2.7)% to (81.2 ± 1.7)%, however, it was quite low in PLA–PEG micelles. This result could be partially due to a less hydrophobic interaction between indomethacin and the poly(lactide) inner core.[19] It has been reported that the alkyl-branch side chain could hinder micellization efficiency due to steric hindrance.[20] Since a methyl side group was present in each monomer of PLA, it further hindered drug encapsulation during micellization and resulted in quite a low amount of drug loaded in PLA–PEG micelles. Figure 5 shows the change in particle size of copolymeric micelles stored in water at 4°C for 12 weeks. All of the micellar solutions maintained their sizes in the range of 1.0 ± 0.3 at the end of the study irrespective of the molecular weight of PEG and the type of lactone.[21] This result indicated that a stable micellar system was maintained, and there was no significant aggregation or dissociation occurred during storage.

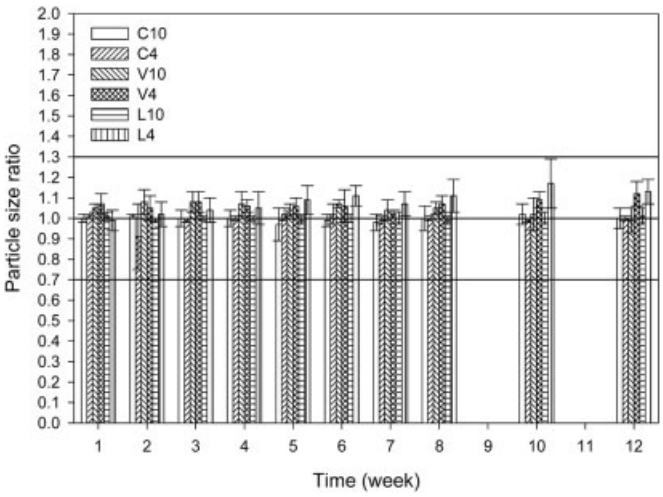

Figure 5. The particle size change of six types of micelles. The bars from left to right represented copolymers C10, C4, V10, V4, L10, and L4.

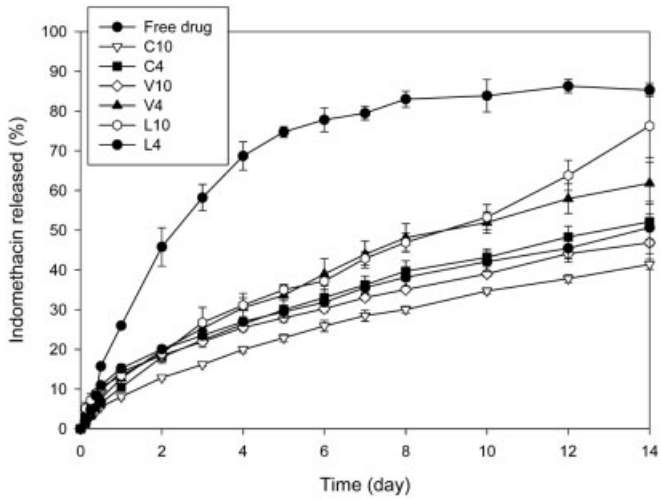

Figure 6. Release of indomethacin from micelles in pH 7.2 phosphate buffer solutions at 37°C.

In Vitro Release Study

Figure 6 shows the *in vitro* release of drug from six types of copolymeric micelles in pH 7.2 buffer solutions. There was a sustained release observed among these micelles. The percentage of drug released at 7th day and the release rate during the first 7 days were calculated and shown in Figure 7. The result showed that these values were reduced 2–3-fold due to micelle carriers. Several factors might contribute to this outcome including the molecular weight, hydrophilic/hydrophobic composition, crystallinity, and polydispersity of copolymers, as well as molecular weight of PEG and the amount of drug loaded. Theoretically, the amorphous form, rather than the crystallized form, of polymer was preferred to allow drug release. The amorphous character of PLA was therefore more feasible for drug release than semicrystalline PCL and PVL was. However, low drug-loading in PLA–PEG micelles diminished the above phenomenon and resulted in a release rate

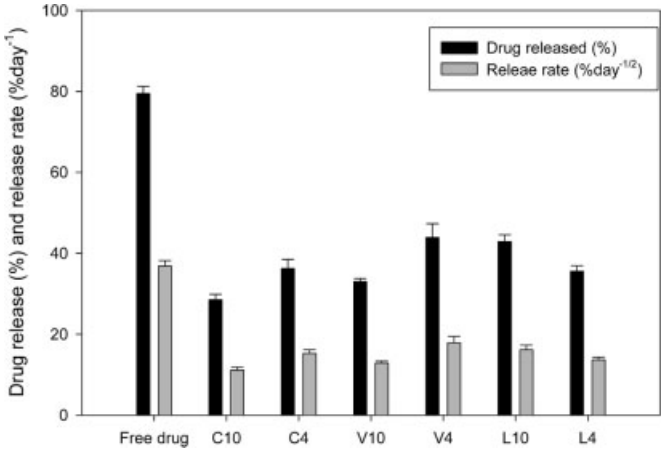

Figure 7. The percentage of indomethacin released at 7th day and the release rate during the first 7 days from micelles.

and extent from PLA–PEG micelles similar to those from PCL–PEG and PVL–PEG micelles.

Pharmacokinetics and Biodistribution in Rats

Before PCL–PEG$_{10,000}$ micelle was selected and applied in the *in vivo* study, the six types of synthetic copolymers were evaluated for their *in vitro* cytotoxicity, micellization efficiency, drug encapsulation efficiency, *in vitro* micelle stability, and *in vitro* release performance. First of all, the drug encapsulations in PLA–PEG$_{10,000}$ and PLA–PEG$_{4000}$ micelles were found much lower than the other micelles; therefore, they were excluded from the *in vivo* study. Furthermore, safety and biocompatibility are very important to the biomedical carrier; however, the PVL–PEG copolymers showed a higher *in vitro* cytotoxicity than PCL–PEG and PLA–PEG. It seems that PCL–PEG micelles, especially PCL–PEG$_{10,000}$ micelles with higher drug loading, lower *in vitro* cytotoxicity, and better *in vitro* sustained release performance, could be selected as the candidate for further *in vivo* study. Figure 8

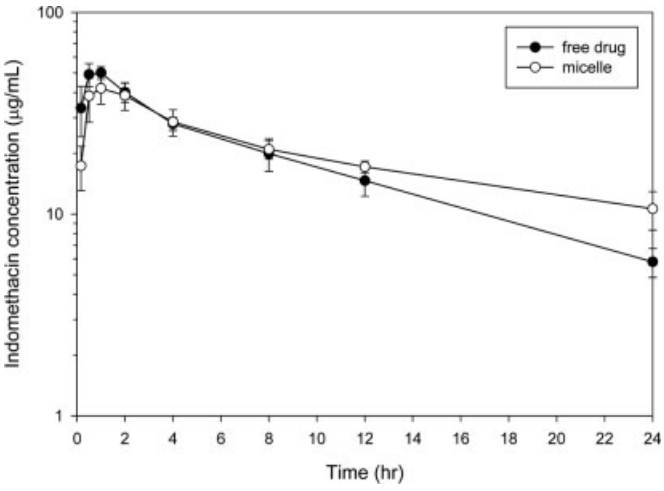

Figure 8. The plasma concentrations of indomethacin after subcutaneous administration of free drug solution and micelles, respectively.

TABLE III. The Pharmacokinetic Parameters of Indomethacin after Subcutaneous Administration of Drug Solution and Micelles

Parameter	Free drug	Micelle
λ (h^{-1})*	0.076 ± 0.007	0.047 ± 0.012
T_{max} (h)	0.80 ± 0.27	0.80 ± 0.27
C_{max} (μg/mL)	51.44 ± 5.05	42.93 ± 7.72
AUC_∞ (μg h/mL)*	518.90 ± 63.41	721.32 ± 117.28
V_D (mL/kg)*	220.53 ± 64.89	317.79 ± 44.91
CL (mL/h/kg)	14.65 ± 2.04	14.72 ± 2.33
$AUMC_\infty$ (μg h^2/mL)*	6232.04 ± 1001.07	15826.11 ± 5951.31
MRT (h)*	11.99 ± 0.83	21.36 ± 4.81

* Indicate significant difference ($p < 0.05$).

shows the concentration of drug in blood after subcutaneous administration of indomethacin solution and PCL–PEG$_{10,000}$ micelles, respectively. The concentration of drug delivered by micelles was lower than drug solution during the first hour, but an opposite concentration level was observed at 12–24 h. The maximum concentration was shown at 1.0 h, and the corresponding values were 51.44 ± 5.05 and 42.93 ± 7.72 μg/mL for drug solution and micelles, respectively. The plasma concentration of indomethacin showed a bi-exponential disposition, and the related pharmacokinetic parameters are listed in Table III. The drug delivered by micelles showed a slower elimination rate, as well as higher MRT, AUC, AUMC, and apparent volume of distribution (V_D) than the free drug solution ($p < 0.05$). This was due to the sustained release of drug and prolonged circulation time *in vivo* by micelles. The amounts of drug distributed in unit mass of heart, liver, lung, and kidney of rats at 0.5, 1, 4, and 8 h were then measured, and the total amount of drug accumulated in each organ within 8 h (AUC_{tissue}) was calculated and shown in Figure 9. There were statistically significant differences in liver and kidney after administration of micelles and drug solution, respectively ($p < 0.05$); however, no significant difference was observed in heart and lung. Decrease of drug uptake by RES-enriched liver and excretion organ-kidney because of micelle carriers was in accordance with increase in mean residence time (MRT) of drug from 11.99 ± 0.83 to 21.36 ± 4.81 h.

CONCLUSION

In vitro characteristics of polymeric micelles and *in vivo* biodistribution of drug delivered by micelle carriers were demonstrated. The pegylated poly(lactone)s, synthesized via a modified ring-opening copolymerization, had molecular weights in the range of 15,000–31,000 g/mol with polydispersity 1.7–2.7. These pegylated copolymers possessed amphiphilic property, and formed micelles with low CMC values in the range of 10^{-7}–$10^{-8}M$. PCL–PEG and PVL–PEG micelles without branch side chain in structures showed higher drug loading than PLA–PEG micelles. *In vitro* release

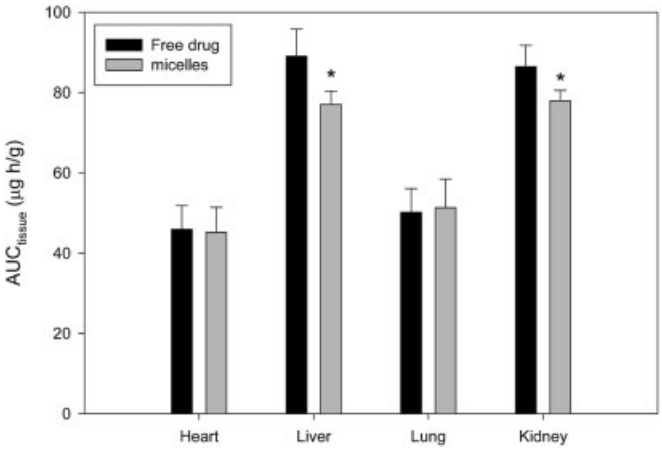

Figure 9. The total accumulation of indomethacin in each tissue within 8 h after subcutaneous administration of free drug solution and micelles, respectively.

profiles indicated that the release of indomethacin from these micelles exhibited a sustained release behavior. A similar phenomenon was also observed *in vivo* of rats. The pegylated copolymeric micelles not only decreased drug uptake by liver and kidney, but also prolonged drug retention in the blood.

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