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Clonazepam Release from Core-shell Type Nanoparticles *In Vitro*

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AB-type amphiphilic copolymers (abbreviated as LE) composed of poly (L-leucine) (PLL) as the A component and poly (ethylene oxide) (PEO) as the B component were synthesized by the ring-opening polymerization of L-leucine N-carboxy-anhydride initiated by methoxy polyoxyethylene amine (Me-PEO-NH_2) and characterized. Core-shell type nanoparticles were prepared by the diafiltration method. Particle size distribution obtained by dynamic light scattering was dependent on PLL composition and the size for LE-1, LE-2 and LE-3 was 369.6± 267, 523.4±410 and 561.2±364 nm, respectively. Shapes of the nanoparticles observed by transmission electron microscope (TEM) were almostly spherical. The critical micelle concentration (CMC) of the nanoparticles determined by a fluorescence probe technique was dependent on the composition of hydrophobic PLL, and the CMC for LE-1, LE-2 and LE-3 was 2.0×10^{-6}, 1.7×10^{-6} and 1.5×10^{-6} (mol/l), respectively. Clonazepam release from core-shell type nanoparticles in vitro was dependent on PLL composition and drug loading content.

Key words : Core-shell type nanoparticle, Diafiltration method, Critical micelle concentration, Amphiphilic copolymers

INTRODUCTION

Nanoparticles are solid colloidal particles ranging in size from about 10 to 1000 nm. They are widely employed in various fields of life science such as separation technologies, histological studies, clinical diagnostic assays and drug delivery system (DDS) (1). Among DDSs, the nanoparticles were applied for site-specific drug carriers (2). Moghimi et al. prepared polystyrene nanoparticles coated with polyoxyethylene (POE)/polyoxypropylene (POP) block copolymers to control the rate of drainage from the subcutaneous injection site and to manipulate the lymphatic distribution (3). Maincent *et al.* reported that poly (hexyl cyanoacrylate) nanoparticles can be efficient for the treatment of cancers with dissemination of metastases in the abdominal cavity after intraperitoneal administration (4). However, these carriers have some drawbacks : rapid renal excretion, recognition by the reticuloendothelial system (RES) and too low stability in the physiological fluid. To overcome these problems, amphiphilic AB type block copolymer nanoparticles composed of hydrophilic and hydrophobic components, which have hydrophobic inner core and a hydrated hydrophilic outer shell in aqueous media were designed (5). These core-shell type nanoparticles hold separated functionalities. Hydrophobic components form inner core of the nanoparticle, which acts as a drug incorporation site and exhibits pharmacological activity. Hydrophobic drug may be easily incorporated in inner core of the polymeric carriers by hydrophobic interactions (5). Hydrophilic outer shell prevents interaction with various biocomponents such as protein and cell, which affects the pharmacokinetic properties (6) and biodistribution of the hydrophobic drug. The nanoparticles as vehicles to carry hydrophobic drugs have several advantages such as increased blood circulation time and reduced RES capture due to small size, solubilization of hydrophobic drug, high structural stability and high drug entrapment in the hydrophobic core (2,7). Recently, diafiltration method was developed for the preparation of nanoparticles based on polymeric micelles since it was simple and the nanoparticles were no-aggregation, small size, high yield, almostly spherical shape and had biodegradation (8).

In this study, we wish to report nanoparticles preparation of diblock copolymers composed of poly (L-leucine) (PLL) as the hydrophobic block and poly

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(ethylene oxide) (PEO) as the hydrophilic block by the diafiltration method in distilled water. PLL has been known as potentially biocompatible and biodegradable polypeptide (9). PEO is known as non-toxic, non-immunogenic water-soluble material and to reduce clearance of RES after intravenous injection (10-13). Anticonvulsant benzodiazepine, clonazepam (CZ), was selected as the hydrophobic model drug (water solubility: 14.7 μg/ml) because it had high interaction with protein *in vivo*. We have investigated physicochemical state of the nanoparticles and drug release from nanoparticles *in vitro*.

EXPERIMENTAL

Material

Methoxy polyoxyethylene amine (Me-PEO-NH_2, M. W. ca. 12,500) was kindly provided by Japan Oil and Fat Co.. Dimethyl sulfoxide (DMSO), methylene dichloride and diethyl ether were purchased from Sigma Chemical Co. and used without further purification. Clonazepam (CZ) was obtained from Roche, Switzerland.

Synthesis of LE diblock copolymers

PLL/PEO (abbreviated as LE) diblock copolymers were prepared by ring-opening polymerization of LL-NCA initiated with Me-PEO-NH_2 as similar method reported previously (14,15). The reaction mixture was poured into a large excess of diethyl ether to precipitate the PLL/PEO diblock copolymers. The resulting copolymer was washed with diethyl ether and then dried *in vacuo*.

Measurement of ^{1}H NMR spectroscopy

^{1}H NMR spectroscopy of the LE diblock copolymers was measured to estimate the composition and molecular weight of the block copolymers, (JEOL FX 90Q NMR spectrometer) in deuterated trifluoroacetic acid (CF_3COOD). As the number-average molecular weight (12,500) of PEO is known, the number-average molecular weight of the PLL block of the block copolymer can be estimated from the copolymer composition via the peak intensities assigned to both polymer blocks.

Measurement of infrared (IR) spectroscopy

IR spectra of samples prepared by KBr method were measured with Bruker IFS-66 FTIR spectrometer between 4,000 and 400 cm^{-1}.

Preparation of nanoparticles and CZ-loaded nanoparticles

The 20 mg of PLL/PEO block copolymers was entirely dissolved in 4 ml of dimethyl sulfoxide (DMSO) and subsequently CZ was added. The solution was stirred at room temperature for 12 hrs. The resulting solution was dialyzed using molecular weight cut-off 12,000 g/mol dialysis membrane with distilled water for 24 hrs and freeze-dried (16).

Measurement of fluorescence spectroscopy

Fluorescence measurements were carried out using CZ as a probe to estimate critical micelle concentration (CMC) of LE nanoparticles in doubly distilled water. Emission spectra were measured with varying copolymer concentration by a fluorometer (Shimadzu RF-5,000) at room temperature. The CMC of the block copolymers was estimated from fluorescence emission spectra with excitation wavelength of 306 nm.

Measurement of transmission electron microscope (TEM)

A drop of nanoparticles suspension in ethyl alcohol was placed on a copper gride coated with carbon film for observation of nanoparticle shape using TEM (JEOL JEN-2,000 FX II). The specimen on the copper gride was not stained. The accelerating voltage of the TEM was 80 kV.

Dynamic light scattering (DLS) measurement

Dynamic light scattering was measured for particle size distribution using a S4,700 (Malvern Instruments, England) with an argon laser beam at a wavelength of 488 nm and value is expressed in weight-averaged scales as unimode. The scattering angle of 90° was used. The lyophilized sample was sonicated for 1 min in deionized water (concentration : 0.1 w/v%) and measured without filtering.

In vitro release test

The release test of CZ from LE nanoparticles was carried out as followed : CZ loaded nanoparticles and 1 ml phosphate buffered saline (PBS, pH=7.4) were put into dialysis membrane and then dialysis membrane was introduced into vial with 10 ml PBS in a shaking incubater at 37°C and 1 ml aliquot was taken and replaced with fresh PBS at specific time points. The concentration of the released CZ was determined by a UV spectrophotometer (Shimadzu, the model of UV-1,201) at 306 nm and expressed by the total release amount of CZ (w/w %).

RESULTS AND DISCUSSION

LE diblock copolymers were prepared by ring-opening polymerization of LL-NCA initiated with Me-PEO-NH_2 in a methylene dichloride solution as scheme shown in Fig 1. It is assumed that the polymerization

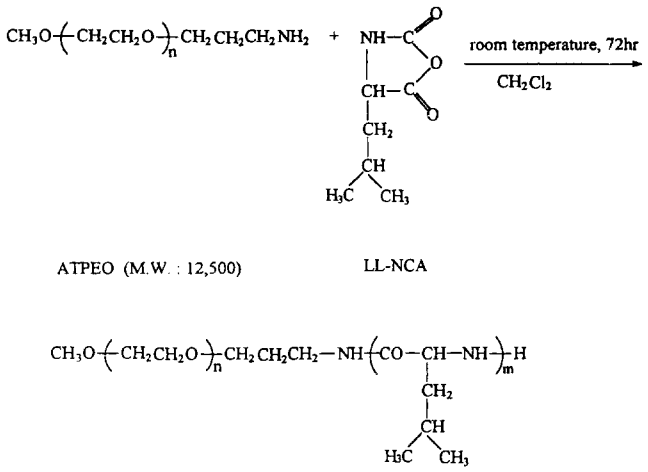

Fig. 1. Synthesis scheme of PLL/PEO diblock copolymer.

mechanism is the primary-amine mechanism in which the initiator amine undergoes a nucleophilic addition to the C-5 carboxy group of the NCA, as suggested by Goodman *et al.* (17).

Fig. 2 shows typical ^{1}H NMR spectrum of the LE-1 block copolymer. Table I are listed the amount of PEO and the molecular weight of copolymers obtained from ^{1}H NMR spectra. The block copolymer composition and the number-average molecular weight ($\overline{Mn}$) were estimated from peak intensities of methylene proton signal (3.9 ppm) of the PEO block and methyl proton signal (0.9 ppm) of PLL block in the spectrum.

Table I. Composition and molecular weight of LE diblock copolymers prepared

Sample	Content of monomeric unit in mol-%		Mn
	PLL	PEO	
LE-1	19.2	80.8	20,100
LE-2	25.7	74.3	23,600
LE-3	40.4	59.6	34,300

*M.W. of PEO: 12,500.
*Composition and molecular weight of the block copolymer was estimated by 1H NMR measurement.

Fig. 3 shows FTIR spectra of LE diblock copolymers and PLL homopolymer in the region of 1,800~500 cm^{-1}. The amide I, II and V bands of these LE block copolymers appear at 1,650 cm^{-1}, 1,550 cm^{-1}, 615 cm^{-1}, respectively, at the same wavenumbers as for the PLL homopolymer. It was found that the PLL block exists in the α-helical conformation, as in PLL homopolymer.

Fig. 4 shows the fluorescence emission spectra of CZ/LE-2 against various concentration of LE-2 in distilled water. It is regarded that by bringing CZ and LE diblock copolymer into an aqueous milieu from a good solvent for both species (in this case DMSO), CZ could be entrapped in core-shell type nanoparti-

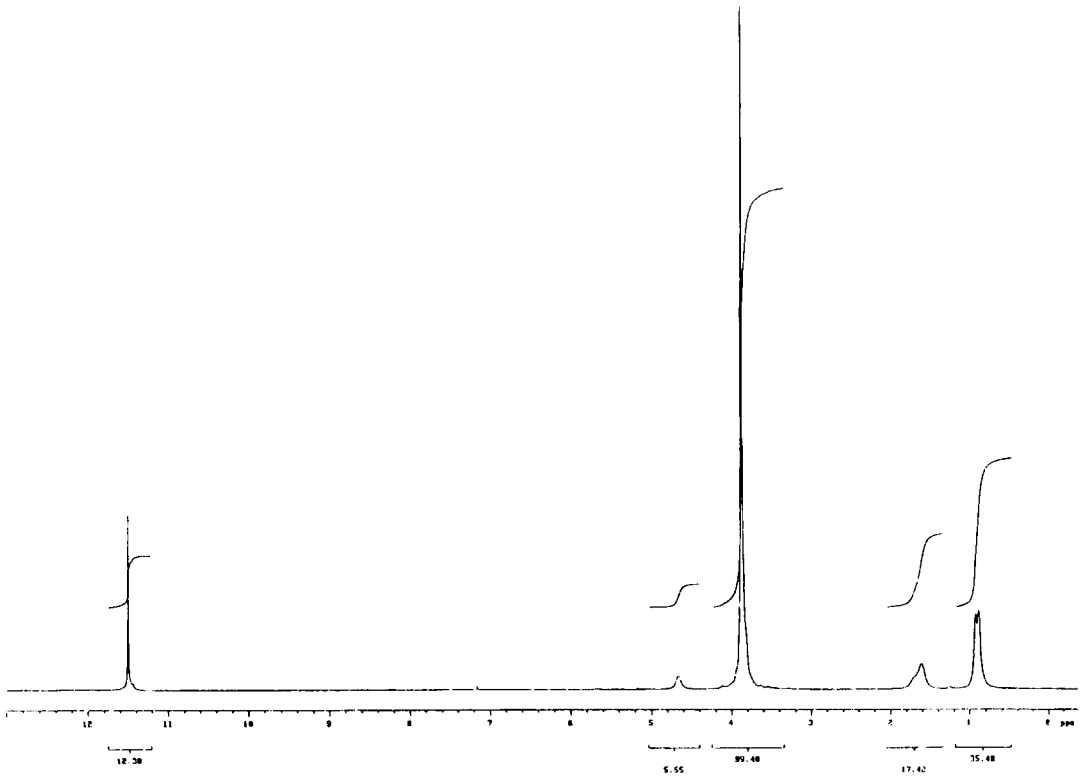

Fig. 2. ^{1}H NMR spectrum of LE-1 block copolymer in trifluoroacetic acid-d1.

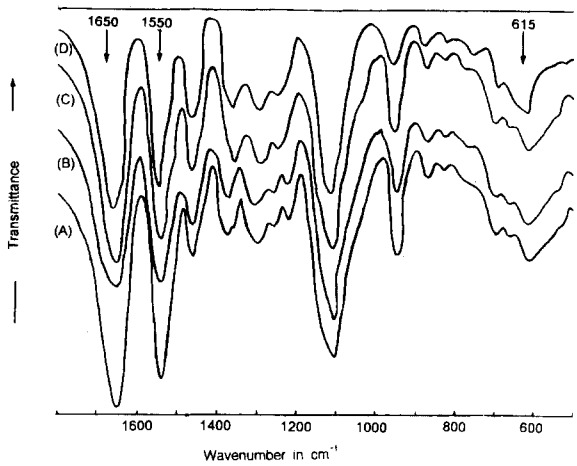

Fig. 3. IR spectra of LE diblock copolymers and PLL homopolymer; (A) PLL homopolymer, (B) LE-1, (C) LE-2, (D) LE-3.

cles. And this was done using a simple dialysis procedure. As this DMSO is removed, LE nanoparticles were formed and have a prospect of incorporating CZ. Furthermore, CZ was preferentially partitioned into hydrophobic microdomains with a concurrent change in the molecule's photophysical properties (18). The total fluorescence intensity was increased and another peak at ca. 370 nm appeared with increasing the concentration of LE-2. This may reflect a change in the vibrational structure of CZ monomer emission upon micellization of LE-2. The preferential partitioning of CZ into hydrophobic domains was used to determine micropolarities (hydrophobicity of the micellar cores) of molecular assemblies, indicating that CMC of the LE-2 block copolymer. It was already reported that the use of the fluorescence probe pyrene, the emission characteristics of which was changed with the onset of association of the block copolymer (19,20). The CMC values for the polymeric micelles according to the composition

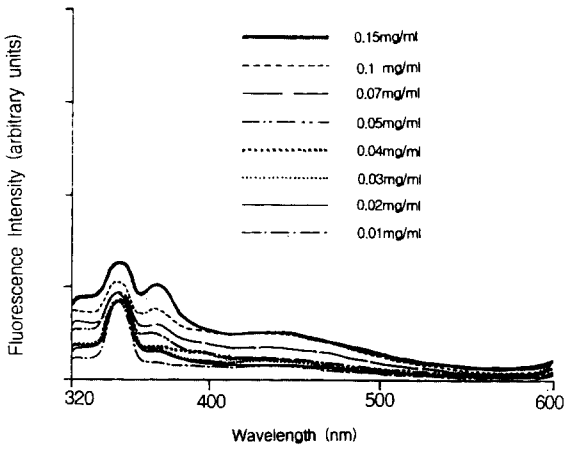

Fig. 4. Fluorescence emission spectra of CZ/LE-2 against concentration of LE-2 in distilled water.

Table II. CMC values for the LE diblock copolymers against PLL content estimated by fluorescence spectroscopy

Sample	PLL content (mol-%)	CMC (mol/L)
LE-1	19.2	2.0×10^{-6}
LE-2	25.7	1.7×10^{-6}
LE-3	40.4	1.5×10^{-6}

Table III. Particle size distribution of block copolymer nanoparticles against PLL content

Sample	PLL content (mol-%)	Particle sizea (nm)
LE-1	19.2	369.6±267
LE-2	25.7	523.4±410
LE-3	40.4	561.2±364

[a]The size of nanoparticles was expressed by weight average.

of the LE diblock copolymers are shown in Table II. As shown in Table II, the CMC for the polymers decreased with increasing PLL composition which is the hydrophobic part in the block copolymer. It is thought that the block copolymer micelles were easily formed with an increase of hydrophobic compositions.

Table III shows particle size distribution of LE nanoparticles measured by DLS. The size of the nanoparticles was dependent on the PLL composition in the block copolymer, as expected (21). It was found that the weight average particle size of the nanoparticles for LE-1, LE-2 and LE-3 was 369.6±267 nm, 523.4±410 nm and 561.2±364 nm, respectively. This results indicated that the more the PLL composition, the bigger the particle size.

Fig. 5 is the transmission electron micrograph (TEM) of the LE-2 diblock copolymer nanoparticles. Their shapes were almostly spherical shape and the sizes were ranged about 50~100 nm in diameter. The size of the nanoparticles observed by TEM was smaller compared to the result obtained from DLS. This phenomena accounted for secondary aggregation during measurement of DLS (22).

Fig. 6 is plot of total CZ released from the LE nanoparticles as a function of PLL content. It was found that the more PLL content, the slower the drug released. These results may be due to hydrophobic interaction between hydrophobic domain of polymer and hydrophobic drug. Also, the more hydrophobic domain of polymer should lead to the stronger hydrophobic interaction. It is thought that PLL as hydrophobic drug-loading segments form the core of the nanoparticle and the PEO as the hydrophilic segments of the block copolymer surround this core as a hydrated outer shell.

Fig. 7 shows total amount of CZ released from LE-2 nanoparticles against drug loading content. From this result, the more the drug content, the slower the drug release. At lower drug loading content, CZ is present as a dispersed state in the core segment whereas a crystallization of drug in the PLL core occurs at high-

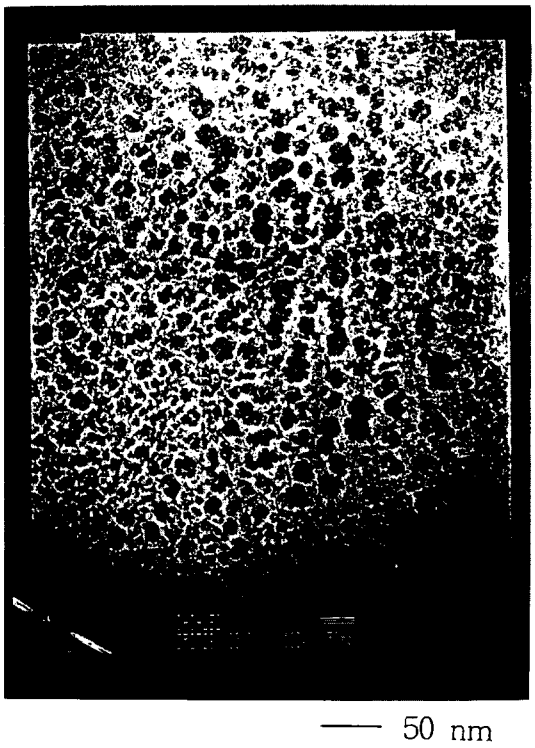

Fig. 5. Transmission electron micrograph of CZ/LE-2 nanoparticles.

er drug loading content. As reported elsewhere, the crystallized drug should be more slowly dissolved and diffused into the outer aqueous phase (23).

In summary, an amphiphilic AB type diblock copolymers composed of PLL as the A component and PEO as the B component were synthesized and characterized. Core-shell type nanoparticles were prepared by the diafiltration method. The particle size obtained by DLS was dependent on PLL composition. Shapes of the nanoparticles observed by TEM were almostly spherical. The CMC of the nanoparticles determined by a fluorescence probe technique was dependent on the composition of hydrophobic PLL. CZ release from core-shell type nanoparticles *in vitro* was dependent on PLL composition and drug loading contents.

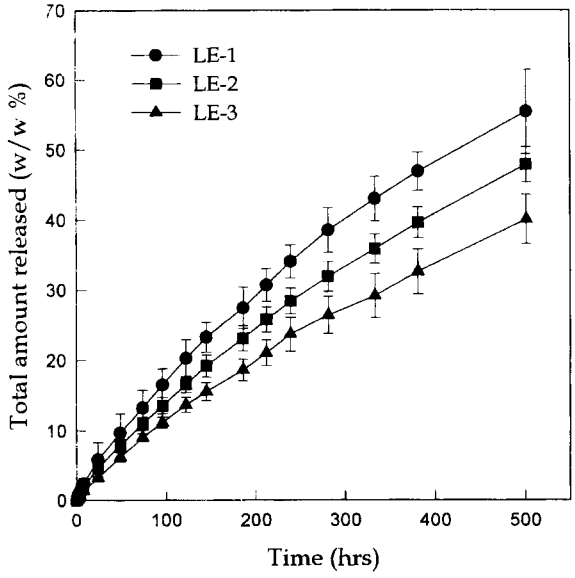

Fig. 6. A plot of the total amount of CZ released from nanoparticles as a function of PLL content.

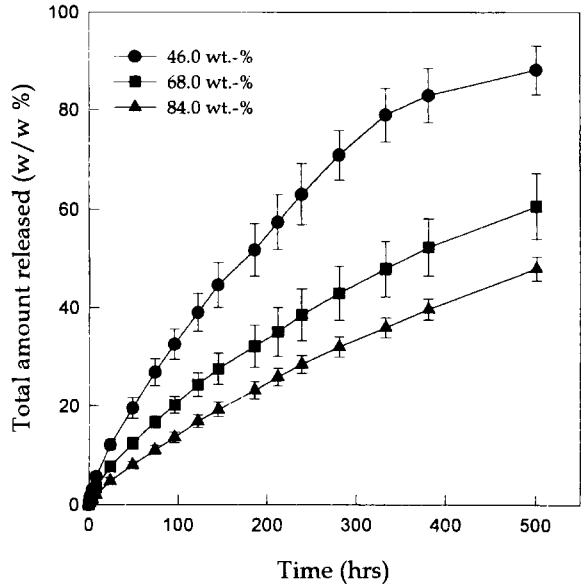

Fig. 7. A plot of the total amount of CZ released from LE-2 nanoparticles against drug loading content.

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