

## Preparation and Reconstitution of Core-shell Type Nanoparticles of Poly( $\epsilon$ -caprolactone)/Poly(ethyleneglycol)/Poly( $\epsilon$ -caprolactone) Triblock Copolymers

Young-Il Jeong,<sup>†</sup> Jae-Gon Ryu, Young-Hoon Kim,<sup>‡</sup> and Sung-Ho Kim<sup>\*</sup>

College of Pharmacy, Chosun University, Gwangju 501-759, Korea

<sup>\*</sup>Université Paris-Sud XI-UMR CNRS 8612, Physico-Chimie-Pharmacotechnie-Biopharmacie,  
5, rue J.B. Clément, 92296 Châtenay-Malabry, France

<sup>‡</sup>Department of Chemistry, University of Massachusetts at Lowell, Lowell, Massachusetts 01854, U.S.A.

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One of the important characteristics of core-shell type nanoparticles is the long-term storage and reuse as an aqueous injection solution when required. For this reason, reconstruction of lyophilized core-shell type nanoparticles is considered to be essential. BAB type triblock copolymers differ from AB type diblock copolymers, which contain the A block as a hydrophilic part and the B block as a hydrophobic part, by not being easily redistributed into phosphate-buffered saline (PBS, pH 7.4, 0.1 M). Therefore, lyophilized core-shell type nanoparticles of CEC triblock copolymer were reconstituted using a sonication process with a bar-type sonicator in combination with a freezing-thawing process. Sonication for 30s only resuspended CEC nanoparticles in PBS; their particle size distribution showed a bimodal pattern, with a small fraction of aggregates, while the dialysis of aqueous nanoparticle solution showed a monomodal pattern with narrow size distribution. The bimodal size distribution pattern and the aggregates were reduced by further sonication for 120 s but these nanoparticles showed a wide size distribution. The initial burst of drug release was increased by reconstitution process. The reconstitution of CEC core-shell type nanoparticles by freezing-thawing resulted in trimodal distribution pattern and formed aggregates, although freezing-thawing process was easier than sonication. Drug release from CEC nanoparticles prepared by freezing-thawing was slower than from the original dialysis solution. Although core-shell type nanoparticles of CEC triblock copolymers were not easily redistributed into aqueous solution, reconstruction of CEC core-shell type nanoparticles was successively performed. Cytotoxicity testing of core-shell type nanoparticles of CEC-2 triblock copolymers containing clonazepam (CNZ) was performed using L929 cells. Cytotoxicity of CNZ was decreased by incorporation into nanoparticles.

**Keywords :** Triblock copolymer, Core-shell type nanoparticles, Cytotoxicity.

### Introduction

Since nanoparticles are acceptable for intravenous injection and enable site-specific drug delivery, nanoparticulate drug carriers have great potential in the therapy of several fatal diseases with reduced side-effects.<sup>1-3</sup> Many types of nanoparticles or colloidal carriers, such as nanospheres,<sup>1</sup> polymeric micelles,<sup>4</sup> liposomes,<sup>5,6</sup> and surface-modified nanoparticles<sup>7</sup> have been developed and suggested. Block copolymers exhibit surfactant behavior and then form core-shell type micelle structure due to their amphiphilic characters. Block copolymers consisting of a hydrophobic and hydrophilic blocks form polymer micelles<sup>4</sup> or core-shell type nanoparticles<sup>6</sup> in aqueous environment, in which a hydrophobic inner-core is surrounded by a hydrated outer-shell. Hydrophobic blocks form the inner-core of the structure and serve as a drug incorporation site<sup>9</sup> while the hydrophilic blocks form a hydrated outer-shell which cloak the hydrophobic core, and inhibits rapid uptake of carriers by the reticuloendothelial system (RES) and more active clearing

organs, such as liver, spleen, lung, and kidneys. The predominant advantages offered by core-shell type nanoparticles are reported to be; reduced toxic side effects of antitumor agents, the passive targeting to the specific sites, the solubilization of hydrophobic drugs, stable storage of drugs, long blood circulation times, favorable biodistribution, thermal stability, and lower interactions with RES.<sup>4</sup> Gref *et al.* reported that core-shell type nanoparticles of poly(lactide-co-ethylene glycol) diblock or multiblock copolymer and poly( $\epsilon$ -caprolactone-co-ethylene glycol) diblock copolymers circulated for long period of time in blood and provided sustained release of drugs.<sup>8</sup>

One of the major advantages of core-shell type nanoparticles or polymeric micelles is that they extend the storage period of the drug in a lyophilized form until use. When lyophilized nanoparticles are used for intravenous injection into the body, the core-shell type nanoparticles must be reconstituted. Therefore, it is necessary to confirm the proper reconstitution of lyophilized nanoparticles and this is an important factor for their actual use and administration by intravenous injection.

In this study, we synthesized the triblock copolymers composed of poly( $\epsilon$ -caprolactone) (PCL) and poly(ethylene

<sup>†</sup>To whom correspondence should be addressed. Phone: +82-62-220-3745; Fax: +82-62-222-5414; e-mail: shkim@mail.chosun.ac.kr

glycol) (PEG) (abbreviated as CEC). PCL is a biodegradable hydrophobic polymer and PEG is a non-immunogenic, non-toxic water-soluble polymers. We have prepared the core-shell type nanoparticles of CEC using CNZ as a hydrophobic drug using a dialysis method followed by lyophilization to investigate the reconstitution characteristics of core-shell nanoparticles of CEC triblock copolymer.

### Experimental Section

**Materials.** PEG (M.W. = 8,000) was purchased from Sigma Chem. Co. USA.;  $\epsilon$ -Caprolactone was purchased from Aldrich Chem. Co. Inc., USA; CNZ was supplied from Roche Co. Switzerland; 1,4-Dioxane, dichloromethane, methanol, and diethyl ether were used reagent grade without further purification.

**Synthesis of triblock copolymers of CEC.** CEC triblock copolymers were synthesized by a non-catalyzed ring opening polymerization of  $\epsilon$ -caprolactone in the presence of PEG.<sup>10</sup> PEG and  $\epsilon$ -caprolactone were mixed in a round-bottomed flask under vacuum condition. The mixture was cooled and degassed with vacuum pump. The round-bottom flask was sealed and placed in an oil bath at 185 °C. After the polymerization was completed, the resultant product was cooled at room temperature and dissolved in dichloromethane. The solution was precipitated by excess amount of cold ethanol and filtered to remove the unreacted PEG homopolymers and  $\epsilon$ -caprolactone monomers. The precipitates were then washed with diethyl ether three times and then dried in a vacuum oven for 3 days.

**<sup>1</sup>H Nuclear magnetic resonance spectrometer (NMR) measurement.** <sup>1</sup>H NMR spectra of the copolymers were measured in CDCl<sub>3</sub> to estimate the copolymer compositions and the molecular weight of the PCL blocks using a 300 MHz NMR spectrometer (FT-NMR, Bruker AC-300F, 300 MHz). As the number-average molecular weight of PEG was known, it was possible to estimate the number-average molecular weight of the PCL block, and the copolymer composition from the assigned polymer peak intensities in the NMR spectrum.

**Preparation of core-shell type nanoparticles.** The core-shell type nanoparticles of CEC triblock copolymers containing CNZ were prepared by dialysis method as reported previously.<sup>11</sup> 100 mg of CEC triblock copolymer and 50 mg of CNZ was dissolved in 20 mL of 1,4-dioxane and the solution was then stirred at room temperature to solubilize the polymer entirely. To form core-shell type nanoparticles, the solution was dialyzed with molecular weight cut-off (MWCO) 12,000 g/mol dialysis tube against 1.0 L  $\times$  3 of distilled water for 3 hrs, and the distilled water was then exchanged at intervals of 3-4 hrs over a period of 24 hrs. The solution was then analyzed or freeze-dried.

To measure the drug loading content, the freeze-dried samples of CEC nanoparticles were suspended in ethanol and vigorously stirred for 12 hrs and sonicated for 1 hr. The resulting solution was centrifuged with 12,000 g for 20 min

and supernatant was then sampled to determine the drug concentration using an UV spectrophotometer (Shimadzu UV-1201) at 310 nm. The drug contents were calculated using the following equation: (drug weight in the nanoparticles/weight of nanoparticles)  $\times$  100. Loading efficiency was calculated as: (Residual drug amount in the nanoparticles/initial feeding amount of drug)  $\times$  100.

**Transmission electron microscopy (TEM) measurements.** A drop of nanoparticles suspension containing 0.1 wt.-% phosphotungstic acids was placed on a carbon film coated on a copper grid. It was observed at 80 kV in a JEOL JEM-2000 FX II.

**Photon correlation spectroscopy (PCS) measurements.** PCS was performed using a Zetasizer 3000 (Malvern instruments, England) with a He-Ne laser beam at a wavelength of 633 nm at 25 °C (scattering angle of 90°). A nanoparticle solution prepared by the dialysis method was used for particle size measurement (concentration: 0.1 wt.-%) and measured without filtering.

**Reconstitution of CEC core-shell type nanoparticles by sonication.** To reconstitute the lyophilized nanoparticles, bar type sonicator was used. 20 mg of lyophilized nanoparticles were suspended in 10 mL PBS by sonication for 30s or 2 min (30s  $\times$  4) at 15 watts using a bar-type sonicator and/or filtered out using a 0.22  $\mu$ m syringe filter (Sterivex, 0.22  $\mu$ m filter unit, Millipore, USA). To avoid heating of nanoparticle solution by sonication, the procedure was performed in an ice bath. The drug release experiment was performed under the conditions described above.

**Reconstitution of CEC core-shell type nanoparticles by freezing-thawing.** The dialyzed solutions of CEC nanoparticles prepared by above method were frozen at -20 °C in the refrigerator, and after 3 days thawed to determine the particle size and drug release characteristics.

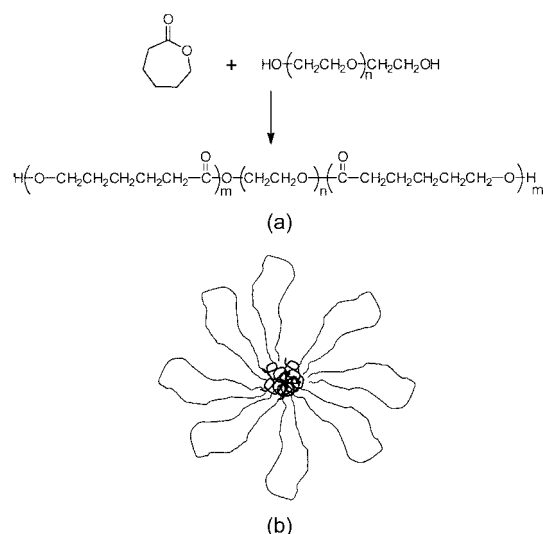
**In vitro cytotoxicity assay.** A mouse connective tissue fibroblast cell line, L 929 cells were used for *in vitro* biocompatibility testing cultivated at DMEM medium at 37 °C in CO<sub>2</sub> (5%) incubator (CO<sub>2</sub> incubator, Sanyo Co. LTD., Japan). Assays were always performed in the exponential growth phase of the Cells.

Cytotoxicity of CNZ, CNZ-loaded nanoparticles, and CEC empty nanoparticles against L929 cells was monitored by a MTT colorimetric assay. L929 cells maintained in DMEM medium (37 °C, 5% CO<sub>2</sub> incubator) were seeded in 96 wells to a  $5 \times 10^3$  cells/well. CNZ, CNZ-loaded nanoparticles, and CEC empty nanoparticles reconstituted by sonication into PBS and filtered by 0.22  $\mu$ m syringe filter (Sterivex, 0.22  $\mu$ m filter unit, Millipore, USA) described above section were added to 96 wells. After 2 day of incubation, 30  $\mu$ L of MTT solution (5 mg/mL) was added each well and cultured for 4 hrs at CO<sub>2</sub> incubator (37 °C). After 4 hrs, supernatant was removed and 100  $\mu$ L of DMSO was added and the plates were read with a ELISA reader (microplate reader (570 nm)) (THERMOMax microplate reader, Molecular Devices, USA). The values expressed in results were mean of 8 wells, *i.e.* n = 8.

## Results and Discussion

**Preparation of CEC core-shell type nanoparticles.** CEC triblock copolymers were prepared by ring-opening polymerization of  $\epsilon$ -caprolactone monomer in the presence of PEG without any other catalysts, as previously reported.<sup>10</sup> An active hydrogen atom at one end of the PEG chain acts as an initiator and induces a selective acyl-oxygen cleavage of  $\epsilon$ -caprolactone, as shown in Scheme 1(a). PCL homopolymer is a semicrystalline polymer and has hydrophobic characteristics, which contribute to its long degradation time *in vivo*. The introduction of  $\epsilon$ -caprolactone into PEG can be used to reduce the degradation time significantly, and this may result in better physico-chemical properties and processibility. CEC triblock copolymers with different molecular weight were prepared by changing the molar ratio of PEG homopolymer/ $\epsilon$ -caprolactone monomer; molecular weights and composition were determined by <sup>1</sup>H NMR spectroscopy. The ratio of PEG to  $\epsilon$ -caprolactone was obtained from the peak intensities of the methylene proton of the PEG chain and methylene proton in  $\epsilon$ -caprolactone units, which were assigned at 3.7 ppm and 4.13 ppm, respectively. The calculated number-averaged molecular weights of PCL was 7550 and total M.W. was 15,550 (M.W. of PEG was 8,000).

Figure 1 shows the particle size distribution (a) and TEM images (b) of the core-shell type nanoparticles of CEC triblock copolymers just after dialysis procedure. CEC core-shell type nanoparticles were  $51.7 \pm 13.7$  nm in size and had monomodal distribution. Also, morphology of CEC core-shell type nanoparticles revealed good spherical shapes at diameter of 50-70 nm, which agreed well with the particle size measurements. After dialysis procedure, core-shell type nanoparticles of CEC triblock copolymer showed good spherical appearance and small particle sizes with narrow size distribution, as was reported previously.<sup>11,12</sup> Saito and Ishizu reported that flower-shaped polymeric micelle based on poly(2-vinyl pyridine-*b*-styrene-*b*-2-vinyl pyridine) was formed in a selective solvent which is good solvent for A



Scheme 1

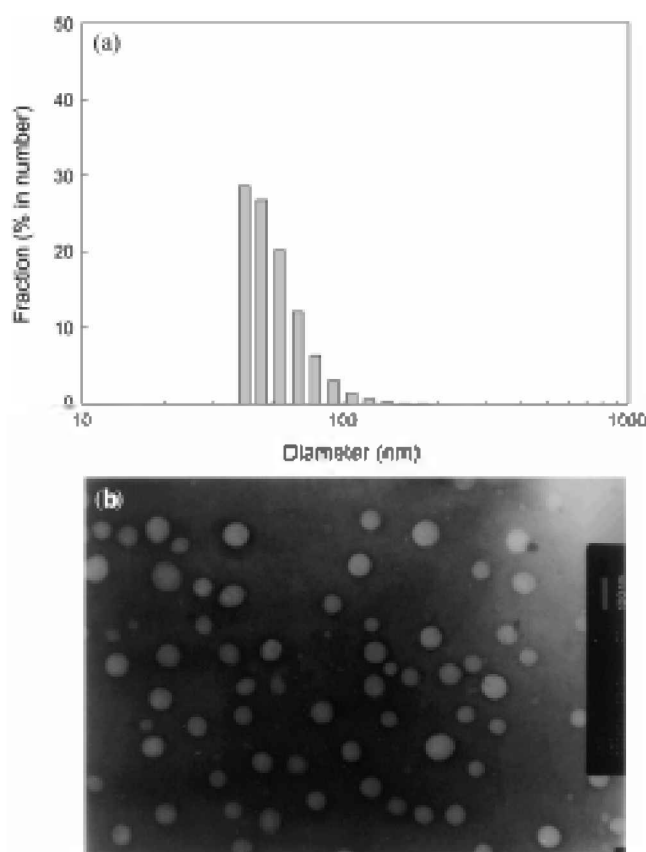
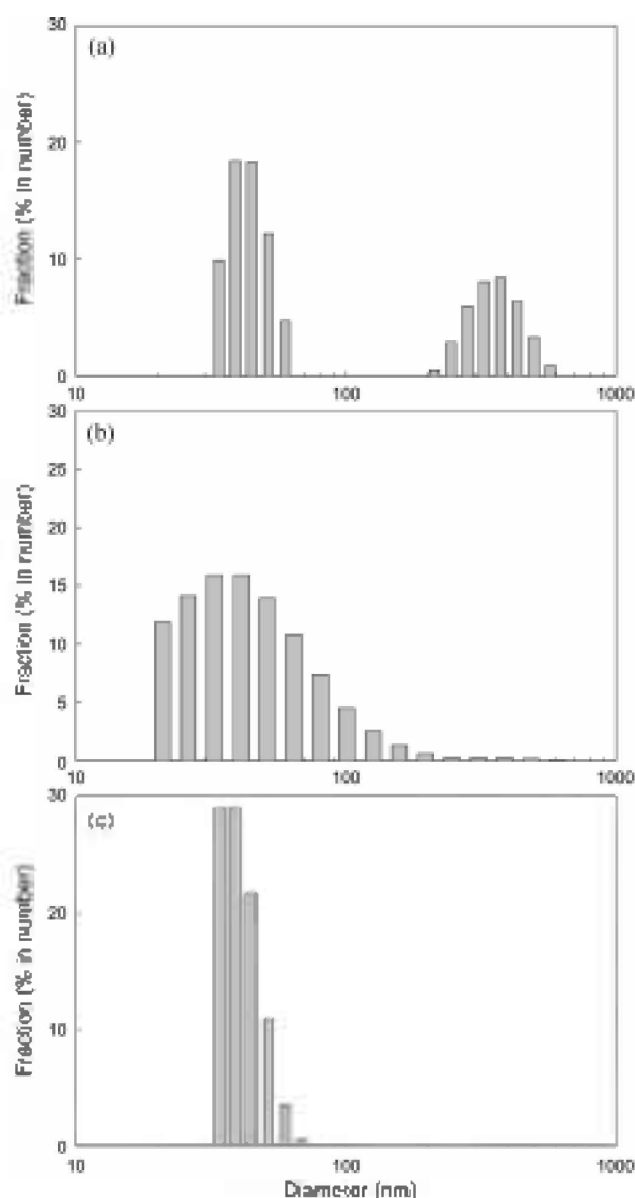


Figure 1. The particle size distribution (a) and TEM images (b) of core-shell type nanoparticles of CEC triblock copolymers after dialysis.

and poor solvent for B sequences.<sup>13</sup> These flower type micelles have a loop structured outershell. On the other hand, many studies have been performed on aqueous solutions of triblock copolymers of PEO-poly(propylene oxide)(PPO)-PEO, which are associated into spherical micelle along with rodlike and possibly layered micelles.<sup>14</sup> Aqueous suspensions of the reversed block copolymer architecture, PPO-PEO-PPO, show rather different phase behavior, but this system is also dominated by spherical micelles in a wide region of its phase diagram.<sup>15,16</sup> Mortensen reported that aqueous solutions of PPO-PEO-PPO associate into a homogeneous phase composed of interconnected network of micelles, in which micellular cores of hydrophobic poly(propylene oxide) are interconnected by hydrophilic poly(ethylene oxide).<sup>15,16</sup> Since CEC triblock are BAB type triblock copolymer, we expected that CEC core-shell type nanoparticles had similar molecular architectures to the flower type spherical polymeric micelles in water (Scheme 1(b)).

**Reconstitution of lyophilized nanoparticles by sonication.** For a long-term storage, the nanoparticle solution is generally freeze-dried and stored as a powder form under refrigeration. The lyophilized nanoparticle powder must be easily reconstituted into aqueous saline solution for injection. To study the reconstitution of CEC core-shell type nanoparticles, lyophilized CEC core-shell type nanoparticles containing CNZ as a hydrophobic model drug was redi-



**Figure 2.** Reconstitution of lyophilized core-shell type nanoparticles of CEC triblock copolymers by sonication for 30 s (a), 120 s (30 s X 4) (b) using a bar type sonicator, and after filtration of sample (a) using 0.22  $\mu\text{m}$  syringe filter (c).

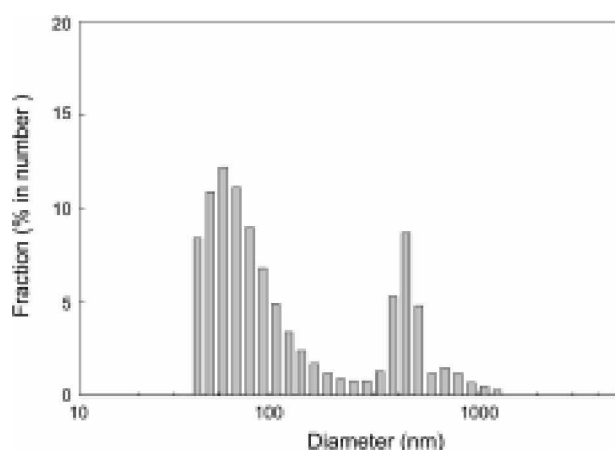
distributed into PBS (0.1 M, pH 7.4). Contrary to what was expected, the core-shell nanoparticles of the CEC triblock copolymers were not easily reconstituted into an aqueous medium either aggregation or precipitation into the aqueous medium. The reason for these results remained unclear. It is suggested that one of the reason is the limited motion of the PEG domain in the BAB type triblock copolymer, which composes of hydrophobic B block and the hydrophilic A block. To obtain a well-reconstituted CEC nanoparticle solution, the sonication using a bar type sonicator was used for 30 s; particle sizes distributions are shown in Figure 2(a). At least 30 s of sonication was required to obtain a clear solution. As a result of sonication, CEC core-shell type nanoparticles were redistributed into the aqueous medium

**Table 1.** Reconstitution of core-shell type nanoparticles of CEC triblock copolymer

	Particle size (nm)	Drug contents (wt.-%)
After dialysis	$51.7 \pm 13.7$	10.7
Reconstitutes <sup>a</sup>		
(30 s sonication)	$43.9 \pm 15.9$ (63.6%)	10.5
	$360.9 \pm 217.0$ (36.4%)	
(120 s sonication)	$50.9 \pm 51.6$	10.4
After filtration <sup>b</sup>	$41.2 \pm 10.4$	8.1
Freezing and thawing	$72.8 \pm 42.9$ (72.3%)	10.7
	$426.4 \pm 154.0$ (22.3%)	
	$791.6 \pm 240.0$ (5.4%)	

<sup>a</sup>Lyophilized nanoparticles (20 mg) was reconstituted into 10 mL of PBS by sonication at 15 Watts using bar type sonicator. 120s sonication was composed of 4 times of 30s. <sup>b</sup>Filtration of reconstitutes (30s sonication) was performed by 0.22  $\mu\text{m}$  syringe filter.

( $43.9 \pm 15.9$  nm (63.6% in fraction) but their particle size revealed a bimodal distribution and a high fraction of aggregated nanoparticles ( $360.9 \pm 217.0$  nm (36.4% in fraction) nm as shown in Figure 2(a). Drug contents were not significantly changed after reconstitution. These results were summarized in Table 1. To remove aggregates from the solution further sonication was performed for 120 s, which had to be performed four times of 30 s to avoid heating of the solution, and the resultant nanoparticles solution showed a monomodal size distribution with a mean size of  $50.9 = 51.6$  nm (Figure 2(b)). However, this solution had a very wide size distribution. This phenomenon can be explained as secondary aggregation as follows: (1) individual micelles are further associated by the hydrophobic-hydrophobic interactions between exposed cores, and (2) the multilayer structure with alternating concentric layers of solvated and undissolved blocks.<sup>9,11</sup> Further sonication did not resulted in small particles or narrower size distribution. It is believed that sonication for a long time may be induce drug leakage from the nanoparticles and change the nanoparticle morphology, which in turn might affect the physico-chemical properties of the nanoparticles. Therefore, as short period of sonication as possible is required to reconstitute core-shell type nanoparticles of CEC triblock copolymer. The required nanoparticle solution with small particle size and narrow size distribution was obtained by filtering the nanoparticle suspension using 0.22  $\mu\text{m}$  syringe filter (Figure 2(c)). After filtration, the drug contents decreased from 10.5 wt.-% to 8.1 wt.-%. These results indicated that the aggregated fraction had both a larger particle size (Figure 2(a)) and a higher drug loading than the small one. Fessi *et al.* reported that a highly soluble aqueous surfactant, such as poloxamer is needed for physical stability of the nanocapsule suspension, although it was possible to make nanocapsules in the absence of such a surfactant.<sup>17</sup> Also, they reported that the presence of at least one emulsifiers was needed for wall coating formation and suspension stabilization and that maximum suspension stability was achieved with a combination of two kinds of emulsifiers since, when prepared with only one emulsifier, nanocapsules

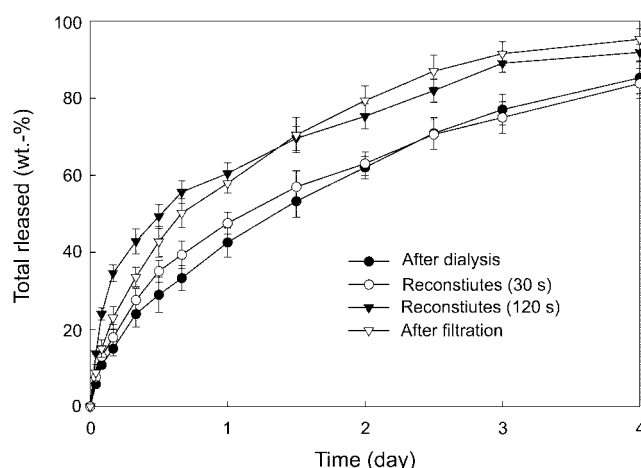


**Figure 3.** Reconstitution of core-shell type nanoparticles of CEC triblock copolymers by freezing and thawing.

would sediment and form a cake which was difficult to redisperse. In this report, we have tried to reconstitute after freeze-drying but physical stability was not maintained after reconstitution, *i.e.* CEC core-shell type nanoparticles were largely aggregated by reconstitution after freeze-drying. Of course, we can store nanoparticles in a frozen state without drying but drugs or biological agents in a frozen-nanoparticles may be easily lose their biological activities in the long term storage.

**Reconstitution of lyophilized nanoparticles by freezing-thawing.** Another possibility of storing and reconstituting core-shell type nanoparticles of CEC triblock copolymer involves freezing the aqueous nanoparticle solution and storing it in a refrigerator until use. When needed the frozen nanoparticles suspension can be thawed in a warm water bath (37 °C) to obtain the nanoparticle solution. Using the protocol, a nanoparticle solution of CEC triblock copolymer was frozen and, after 3 day thawed and the particle size was measured, as shown in Figure 3. After thawing of frozen nanoparticle suspension, its particle size distribution was significantly changed. Their mean sizes were slightly increased but particle size distribution had changed from monomodal to bi- or tri-modal. The reason for the aggregation remains unclear. We considered several possibilities, as follows: (1) secondary aggregation during freezing and thawing procedure of nanoparticle suspension, (2) temperature responsive changes of CEC triblock copolymers, *i.e.* solubility properties of CEC triblock copolymer was decreased in a cold water during freezing process, (3) thermal property change of the CEC triblock copolymer at low temperature, and (4) the formation of a multilayer structure with alternating concentric layers of solvated and undissolved blocks.

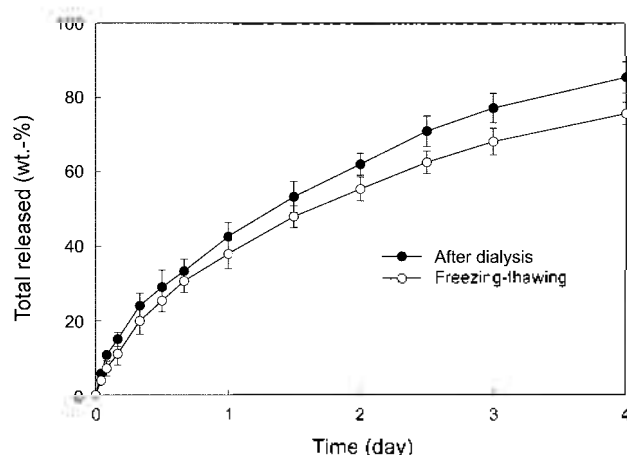
**Drug release from reconstituted core-shell type nanoparticles.** To investigate drug release characteristics of reconstituted core-shell type nanoparticles of CEC triblock copolymer, drug release experiments were performed on each of the sample in Figure 1, 2, and 3. Figure 4 shows the effect of reconstitution on the drug release from core-shell



**Figure 4.** The effect of reconstitution on the drug release from core-shell type nanoparticles of CEC triblock copolymer.

type nanoparticles of CEC triblock copolymer. As shown in Figure 4, drug release from CEC core-shell type nanoparticles just after dialysis revealed an initial burst effect over 12 hrs and then pseudo-zero order release kinetics until 4 day. Reconstitution of core-shell type nanoparticles of CEC triblock copolymer by 30 s of sonication resulted in an increased initial burst release (after dialysis), *i.e.* about 50% of the drug was released during the first day and subsequently drug release showed similar pseudo-zero order kinetics. This phenomena is accelerated by additional sonication processing, *i.e.*, after an additional 120 s of sonication, the initial drug release burst accounted for about 60% of drug, indicating that drug might have leaked from the nanoparticles during the sonication process. To remove the aggregated nanoparticles, reconstituted nanoparticles by 30 s of sonication, were filtered using 0.22  $\mu\text{m}$  syringe filter, described as above. The filtered nanoparticles were obtained as a clear solution and their particle size was similar to the original nanoparticles, just after dialysis. However, this filtration process decreased their drug loading contents. These results might have been due to the higher drug loading of the aggregated fraction of the reconstituted nanoparticles, as shown in Table 1. Several possibilities were considered as follows: (1) exposure and aggregation of the inner-core of the core-shell type nanoparticles during reconstitution process, (2) during the reconstitution process the drug was further associated or entrapped in the aggregated particles by hydrophobic interaction between hydrophobic drug and exposed core of the core-shell type nanoparticles, and that the total drug loading was then decreased by removing the aggregated fraction of reconstituted nanoparticles.

Figure 5 shows the effect of freezing-thawing on the drug release from the core-shell type nanoparticles of CEC triblock copolymer. As shown in Figure 5, drug release from core-shell type nanoparticles of CEC became slower than nanoparticles, which were made by dialysis process. These results were probably due to the aggregation of particles during freezing-thawing process, because drug release was slower from the larger size of nanoparticles. As described



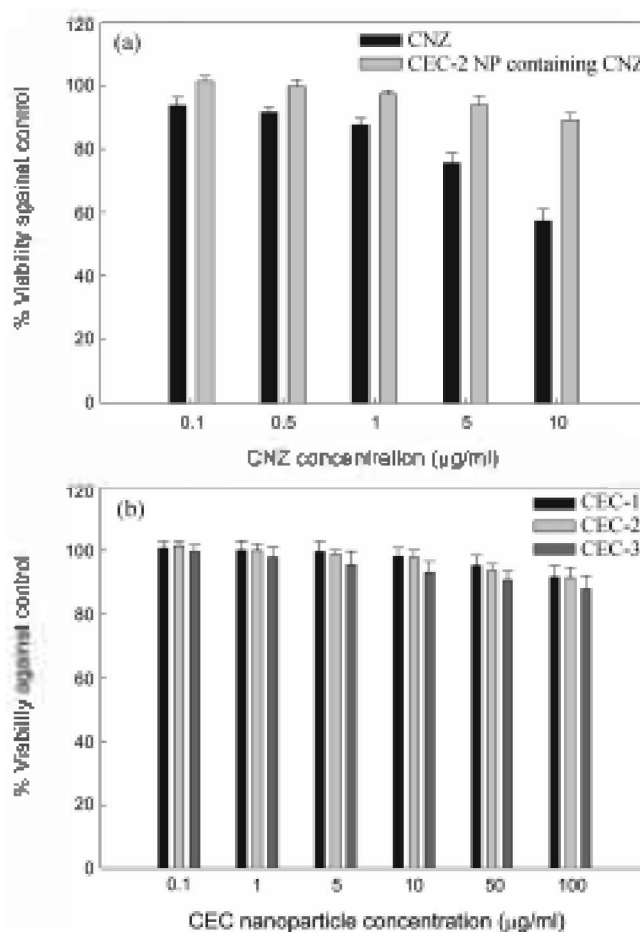
**Figure 5.** The effect of freezing-thawing on the drug release from core-shell type nanoparticles of CEC triblock copolymer.

above, larger particle size of nanoparticles can result in slower drug release rate due to the different diffusivity into the aqueous medium.

As shown in Figures 1-4 and Table 1, core-shell type nanoparticles of BAB type triblock copolymers have limitation in terms of their ease of reconstitution. The reconstitution of nanoparticles by sonication caused aggregation and the drug release rate was changed. These reconstitution processes are an important factor for actual application of nanoparticles by intravenous injection.

**In vitro cytotoxicity test and biocompatibility of CEC triblock copolymer.** Several procedures have been described involving cell culture techniques for preliminary biocompatibility evaluation of materials intended for medical application.<sup>18</sup> To harmonize in vitro cytotoxicity test methods, an ISO norm was created by the International Standard Organization.<sup>19</sup> This document classifies the *in vitro* cytotoxicity test methods into three categories based on the preparation of the test material as follows: (1) test of extracts prepared from the polymer; (2) indirect contact methods, where cells and the test material are separated by a protective layer, for example an agar layer; (3) direct contact methods where the cells are put directly in contact with the test material. We used these *in vitro* biocompatibility-testing methods suggested by the ISO norm by seeding the cells directly into aqueous solution of core-shell type nanoparticles of CEC triblock copolymer. Mouse connective tissue fibroblasts L929 (ATCC cell line CCL 1, NCTC clone 929) are a standard model for biocompatibility testing, since they are easy to cultivate and because of their favorable doubling time of about 24 h. L929 cells are widely used for cytotoxicity studies and are recommended by many standard institutions.<sup>19-21</sup> The viability of the cells was quantitated by a colorimetric assay for cellular growth, the MTT assay,<sup>22</sup> based on the cleavage of a yellow tetrazolium salt (MTT) (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to purple formazan crystals by mitochondrial active cells.

We compared the cytotoxicity induced by free drug, CEC nanoparticles containing drug (equivalent amount as a drug),



**Figure 6.** Cytotoxicity of core-shell type nanoparticles of CEC triblock copolymers containing CNZ against L929 cells. CNZ and CEC-2 core-shell type nanoparticles (a), CEC empty nanoparticles (b). The values of viability was expressed as % against control values.

and empty nanoparticles with L929 cells. The viability was expressed in percent compared to control that is not treated with CEC nanoparticles or drug. Viability of samples were assayed after 2 day incubation. Figure 6 shows the cytotoxicity of CNZ, core-shell type nanoparticles of CEC-2 triblock copolymer containing CNZ, and empty nanoparticles of CEC block copolymer. CEC-2 core-shell type nanoparticles containing CNZ used for cytotoxicity test was prepared by reconstituted and filtered into PBS and diluted with DMEM medium for cell culture as similar to the method described in above section in Figure 2. Cytotoxicity of CNZ and CNZ-loaded CEC core-shell type nanoparticles was 87.5% and 97.2% at 1 µg/mL, 75.7% and 93.9% at 5 µg/mL, and 57.3% and 89.1% at 10 µg/mL. As shown in Figure 6(a), the cytotoxicity of CNZ was significantly decreased by incorporation of CNZ into core-shell type nanoparticles of CEC triblock copolymers. As shown in Figure 6(b), the cytotoxicity of empty nanoparticles was not significantly affected to the viability of L929 cells. At very high concentrations of empty nanoparticles, 100 µg/mL, cell biability was maintained above 90%. The cytotoxicity between CEC-1, CEC-2, and CEC-3 triblock were not significantly different.

Resultantly, it was thought that core-shell type nanoparticles of CEC triblock copolymer was not affected to the viability of L929 cells.

It was suggested that the reduced cytotoxicity of CNZ was due to several factors as follows:

1. Direct exposure of CNZ to cell was avoided by incorporation of drug into the nanoparticles.

2. Outer-shell, PEG, of the core-shell type nanoparticles of CEC-2 could reduce the interaction between nanoparticles and cells by stealth surfaces.

3. Sustained drug release characteristics might be helpful to maintain the cell viability.

Biocompatibility is defined as the ability of a material to perform an appropriate host response in a specific application,<sup>23</sup> is one of the key factors for the development of new polymeric delivery systems. An ideal material should be well tolerated, its use is not longer required, the material should be degraded and absorbed.

### Conclusion

Reconstitution of lyophilized core-shell type nanoparticles of CEC triblock copolymer was performed by sonication and filtration. Reconstitution by 30 s sonication resuspended CEC nanoparticles in PBS but their particle size distribution showed bimodal pattern with small fraction of aggregates, while dialysis of aqueous nanoparticle solution showed a monomodal pattern with a narrow size distribution. Bimodal size distribution pattern and aggregation were reduced by further sonication for 120 s but nanoparticles showed a wide size distribution. The initial burst of drug release was increased by reconstitution process. Reconstitution of CEC core-shell type nanoparticles by freeze-thawing resulted in a trimodal distribution pattern and formed aggregates although freezing-thawing in a trimodal distribution pattern and formed aggregates, although the freezing-thawing process were more simple than sonication process. Drug release from CEC nanoparticles prepared by freezing-thawing process was slower than from the original dialysis solution. Although core-shell type nanoparticles of CEC triblock copolymers were not easily redistributed into aqueous solution, reconstruction of CEC core-shell type nanoparticles was successfully performed. Cytotoxicity test of core-shell type nano-

particles of CEC triblock copolymers containing CNZ was performed using L929 cells. Cytotoxicity of CNZ was decreased by incorporation into the nanoparticles.

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