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Stability Studies of Biodegradable Polymersomes Prepared by Emulsion Solvent Evaporation Method

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Abstract: Di-block copolymers composed of two biocompatible polymers, poly(ethylene glycol) and poly(D,L-lactide), were synthesized by ring-opening polymerization for preparing polymer vesicles (polymersomes). Emulsion solvent evaporation method was used to fabricate the polymersomes. Scanning electron microscope (SEM) images confirmed that polymersomes have a hollow structure inside. Confocal laser microscope and optical microscope were also used to verify the hollow structure of polymersomes. Polymersomes having various sizes from several hundred nanometers to a few micrometers were fabricated. The size of the polymersomes could be readily controlled by altering the relative hydrodynamic volume fraction ratio between hydrophilic and hydrophobic blocks in the copolymer structure, and by varying the fabrication methods. They showed greatly enhanced stability with increased molecular weight of PEG. They maintained their physical and chemical structural integrities after repeated cycles of centrifugation/re-dispersion, and even after treatment with surfactants.

Keywords: self-assembly, polymersome, polymer vesicle, amphiphilic, di-block copolymer, biodegradable.

Introduction

Amphiphilic di-block copolymers self-assemble into a wide array of supramolecular structures such as micelles, rods, and wires by non-covalent interactions.[1-4] In particular, amphiphilic and biodegradable di-block copolymers self-associate to form spherical micelles in an aqueous solution.[5-13] Recently, novel nano-sized hollow polymer vesicles called polymersomes were paid much attention because of their unique self-assembly nature similar to that of phospholipids that form liposomes.[14,15] The di-block copolymers self-assemble to form a single-layered or multi-layered structure that surrounds an aqueous cavity, resembling the structures of lipid vesicles. It was demonstrated that the formation of polymersome is thermodynamically favorable by employing amphiphilic di-block polymers that have relatively a shorter hydrophilic segment than a hydrophobic segment. Balancing of hydrodynamic volume ratio between hydrophilic and hydrophobic blocks in the structure is a key determinant of whether a di-block copolymer spontaneously forms either micelles or polymersomes.[1,16] The volume ratio required for the self-assembly of polymersomes is known to be in the range of 10-35%.

Biodegradable polymersomes composed of a di-block copolymer of poly(D,L-lactic acid) (PDLLA) and poly(ethylene glycol) (PEG) draw particular attention because they can be utilized as biocompatible reservoir-type drug carriers encapsulating hydrophilic bioactive agents such as peptides, proteins, and genes.[16,17] Previously, poly(D,L-lactic-*co*-glycolic acid) (PLGA)-PEG di-block copolymers were synthesized to produce spherical core/shell type micelles in aqueous solution, which were mostly used to load hydrophobic anticancer drugs such as paclitaxel in the core.[18] Those PLGA-PEG di-block copolymers have relatively larger hydrophilic/hydrophobic volume ratios, resulting in the formation of micelles and/or micellar aggregates in aqueous solution. There have been many studies concerning the formation, stability characteristics, and applications of biodegradable polymeric micelles. Self-assembled micelles and vesicles, however, have some inherent stability problems similar to liposomes, which limit their practical applications.[19-21] For example, they tend to dissociate into individual molecules below a critical concentration. The stability problems for polymersomes have not been examined thoroughly.

In this study, various PDLLA-PEG polymersomes with different sizes were fabricated by systematically varying the volume ratio between PDLLA and PEG segments. Oil-in-water emulsion solvent evaporation method was used to prepare polymersomes. Their stability characteristics as a function of hydrophilic/hydrophobic volume ratio were examined by changing thermal incubation conditions, repeating centrifugation/re-dispersion cycles, and treating with surfactant.

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Experimental

Materials. Methoxy-poly(ethylene glycol) (mPEG) with Mw of 2,000 and 5,000, fluorescene-isothiocyanate (FITC)-dextran (Mw, 21,200), and nile red dye, and sodium dodecyl sulfate were purchased from Sigma-Aldrich (Milwaukee, WI, USA). D,L-lactide was purchased from PURAC (The Netherlands) and re-crystallized with ethyl acetate. Stannous octoate, ethyl acetate, diethyl ether, chloroform, and other reagents were of analytical quality.

Ring Opening Polymerization. Di-block copolymer, mPEG-PDLLA was synthesized by ring-opening polymerization method as described previously.[22] D,L-lactide was polymerized at the terminal hydroxyl group of mPEG. Briefly, mPEG was dried in a vacuum oven at 80 °C for 24 h. D,L-lactide and mPEG with varying stoichiometric molar ratios was put in a two-neck round flask. The flask was vacuumed for 1 h and purged with nitrogen gas. After melting the mixture at 140 °C, the ring opening polymerization reaction was initiated with addition of 0.05% (w/w) stannous octoate. The polymerization proceeded at 170 °C for 5 h. The di-block copolymer was precipitated in cold diethyl ether (-20 °C). The product was dried in vacuum, and then stored in a freezer until use. Molecular weights of mPEG-PDLLA di-block copolymers were determined by ^{1}H-NMR (Bruker AVANCE 400 spectrometer operating at 400 MHz) by using chloroform-*d* solution. The characteristics of the di-block copolymers are listed in Table I.

Formation of Polymersomes by Emulsion Solvent Evaporation Method. Oil-in-water emulsion solvent evaporation method was used to prepare polymersomes. One hundred microliter of chloroform containing 1 mg mPEG-PDLLA was added in 10 mL of deionized water with subsequent vortexing for 1 min. The chloroform in the resultant emulsion was completely evaporated with further magnetic stirring. After solvent evaporation, the solution was stored at 4 °C.

Morphology of Polymersomes. Optical microscopy (ECLIPSE TE300, Nikon, Japan) and confocal laser microscopy (Zeiss LSM 510 META, Carl Zeiss, Germany) were used to observe the morphology of polymersomes. In order to visualize the hollow structure of polymersome, Nile red and FITC-dextran were used as hydrophobic and hydrophilic dyes, respectively. Nile red stained a hydrophobic PDLLA phase, and FITC-dextran stained an aqueous phase. Polymersomes were prepared by adding 10 μg of Nile red in the polymer solution and adding 7 μg of FITC-dextran in the aqueous phase in the above preparation method. The dye-stained polymersomes were separated by centrifugation at 14,000 rpm for 3 min and re-dispersed in 2 mL of de-ionized water. Scanning electron microscopy (SEM, Philips 535M) was also used to observe the gross morphological shape of polymersomes.

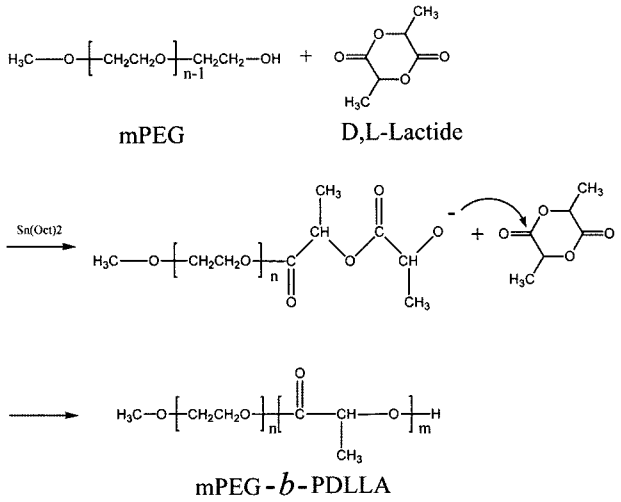

Figure 1. General scheme of ring-opening polymerization of mPEG-PDLLA di-block copolymer.

Effective Diameter of Polymersomes by Dynamic Light Scattering and Size Control of Polymersomes. Dynamic laser light scattering (DLS) measurement was carried out using a Brookhaven laser light scattering instrument (Zeta-Plus, Brookhaven Instruments Corporation, New York, U.S.A.), with a He-Ne laser operating at a wavelength of 677 nm at 25 °C. A scattering angle of 90° was used for all measurements. The concentration of the samples was 0.01% (w/v) in deionized water.

Size Change of Polymersomes. One milliliter of freshly prepared polymersome solution was incubated in a temperature-controlled incubator at 4 and 37 °C. The size change of polymersomes was measured by DLS measurement as described above. Optical microscopy was also used to verify

Table I. mPEG-PDLLA Di-block Copolymers and Size of Polymersomes

Polymer Code	Di-block Copolymer Structure		Mw (mPEG)	Mw (PDLLA)	Volume Fraction[c]	Polymersome Diameter (nm)
EG-LA1	EG^a_{45}	$-{}_{DL}LA^b_{264}$	2,000	18,000	0.12	446.7 ± 2.5
EG-LA2	EG_{45}	$-{}_{DL}LA_{625}$	2,000	45,000	0.05	1298.9 ± 28.9
EG-LA3	EG_{114}	$-{}_{DL}LA_{556}$	5,000	20,000	0.14	404.3 ± 8.0
EG-LA4	EG_{114}	$-{}_{DL}LA_{1417}$	5,000	102,000	0.06	1060.4 ± 129.1

[a]EG: Ethylene glycol. [b]$_{DL}$LA: D,L-lactic acid. [c](Volume Fraction) = (hydrodynamic volume of PEG) / (total volume of copolymer).

the change in size of polymersomes during incubation.

Chemical Stability. One milliliter of freshly prepared polymersome solution was mixed with 1.5 mL of 2% sodium dodecyl sulfate aqueous solution. The size change was measured by DLS after 1 h. Relative transmittance of polymersome solution before and after sodium dodecyl sulfate (SDS) treatment was measured by using spectrophotometer (Shimadzu UV-1601, Japan) at 500 nm.

Mechanical Strength Against Centrifugation. Ten milliliter of freshly prepared polymersome solution was centrifuged at various speeds (3,000, 6,000, 9,000, 12,000, 14,000 rpm). The size of polymersomes before and after the centrifugation was measured by DLS. SEM was also used to visualize the change of polymersomes before and after centrifugation.

Results and Discussion

Four types of mPEG-PDLLA di-block copolymers with varying molecular weights were synthesized as shown in Table I. It is known that di-block copolymers self-assemble to form polymersomes when the di-block copolymer has a hydrophilic/hydrophobic volume fraction of 10-35%.[1-4] The volume fraction is the ratio of hydrodynamic volume of hydrophilic part to total volume of the copolymer structure. The PEG/PDLLA volume fraction in the structure was adjusted from 5 to 14%, while PEG Mw was fixed at 2,000 and 5,000. The di-block copolymers had amorphous PDLLA as a hydrophobic block. The amorphous PDLLA blocks are expected to more readily associate via hydrophobic interaction to form stable polymersomes in aqueous solution. When semi-crystalline poly(L-lactic acid) (PLLA) block was used instead of PDLLA block, mPEG-PLLA di-block copolymers irreversibly aggregated and were precipitated in aqueous solution due to crystallization.

Confocal laser microscopy and SEM were used to confirm the hollow sphere structure. Figure 2(A) indicates that polymersomes retain spherical morphology. Confocal laser microscopy image gave direct evidence for the hollow spherical morphology. Intense red fluorescence intensity of hydrophobic dye (Nile Red) in the outermost shell region in Figure 2(B) indicates that amphiphilic di-block copolymers were segregated at the shell part of the polymersomes, while the interior was filled up with an aqueous fluid. This suggests that poorly soluble drugs such as paclitaxel and amphotericin can be loaded in the outer shell layer of polymersomes. The size distribution of polymersomes ranged from a few hundred nanometer to a few micrometer. The interior hollow region was also visualized using FITC-dextran as a hydrophilic dye. Figure 2(C) shows that FITC-dextran was encapsulated in the interior aqueous space of

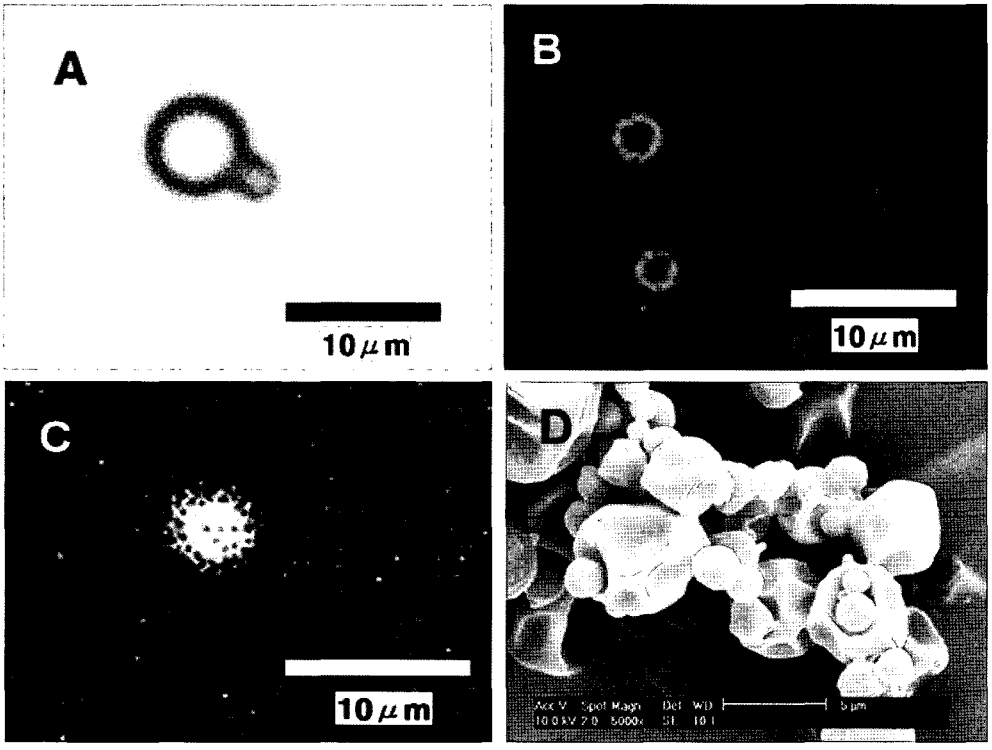

Figure 2. Microscopy images of polymersomes. (A) Optical microscopy image of polymersome made from EG-LA2. Scale bar is 10 μm, (B) confocal laser microscopy image of polymersome made from EG-LA2. Scale bar is 10 μm, (C) confocal laser microscopy image of encapsulated FITC-dextran in polymersomes made from EG-LA3. Scale bar is 10 μm, and (D) SEM image after lyophilization of polymersomes made from EG-LA1. Scale bar is 5 μm.

polymersomes. SEM image in Figure 2(D) also indicates that polymersomes had hollow spherical morphologies. Since they had to be lyophilized for SEM observation, the polymersomes appeared in the SEM picture were collapsed and shrunken as a result of dehydration. This also suggests that the polymersomes had an aqueous phase cavity surrounded by a self-assembled di-block copolymer shell layer.

Table I shows the size of polymersomes with different mPEG-PDLLA di-block copolymers. The size of polymersomes ranged from 400 nm to 2 μm on average depending on the volume fraction. The volume fraction, which is defined as the relative hydrodynamic volume ratio of hydrophilic PEG part to the total mPEG-PDLLA part in the di-block copolymer structure, is a major factor in governing the size of polymer vesicles. The size of polymersomes is dictated by the bending elasticity of a self-assembled bilayer structure, which was affected by the hydrophilic/hydrophobic balance of the amphiphilic di-block copolymer structure. Figure 3 shows that the size of polymersomes was inversely proportional to the volume fraction of amphiphilic di-block copolymers. The size of polymersomes increased with increasing molecular weight of PDLLA part when fixing Mw of PEG part at 2,000 or 5,000. In the case of PEG Mw 2,000, the size increased from 446.7±2.5 nm to 1,298±28.9 nm, as the molecular weight of PDLLA segment was increased from 18,000 to 45,000. This is likely due to the fact that the curvature of polymer vesicles is determined mainly by packing density of amphiphilic molecules that form the bilayer structure. In aqueous solution, individual mPEG-PDLLA molecules having a high volume fraction might have a wedge shape structure because of bulky hydrated PEG segment, while those with low volume fraction might have a more or less cylindrical shape structure. As the chain length of the PEG block becomes longer, it would be more hydrated with increasing hydrodynamic volume. When the di-block copolymers with high volume fraction were self-assembled to form a bilayer structure, steric repulsion between the adjacently positioned bulky PEG chains is likely to occur, resulting in reduced curvature with ultimately producing smaller polymersomes. Thus, the volume fraction plays a critical role in determining the packing density of the bilayer structure that controls the curvature of polymersomes.

Physical stability of polymersomes is a critical factor when they are applied as reservoir-type drug delivery carriers. Self-assembled lipid vesicles, liposomes, have been extensively utilized as drug delivery carriers, but their structures are easily disintegrated upon exposure to various formulation conditions such as incubation at higher temperature, freezing and/or drying, physical stresses, and contact with surface active agents. Polymerized liposomes have been introduced to overcome such stability problems.[21] Likewise, it was interesting to determine how stable polymersomes were in response to various conditions. Figure 4(a) and (b) show the size change of polymersomes (EG-LA1 and EG-LA3) at two different temperatures at 4 and 37 °C, respectively. EG-LA1 and EG-LA3 have close volume fraction values (0.12 for EG-LA1 and 0.14 for EG-LA3) with similar size distributions. However, EG-LA3 polymersomes showed greater

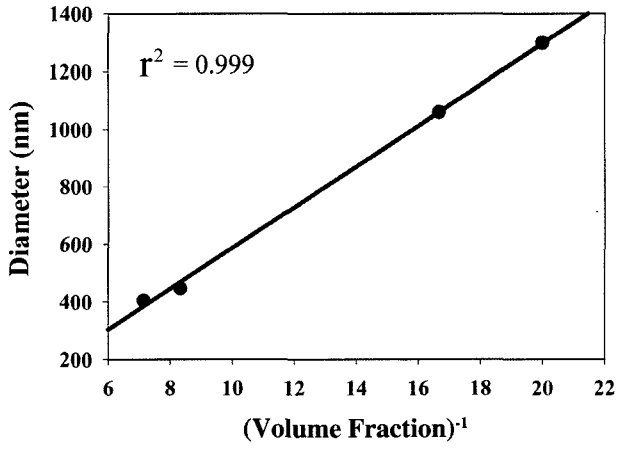

Figure 3. Correlation of polymersome size and volume fraction.

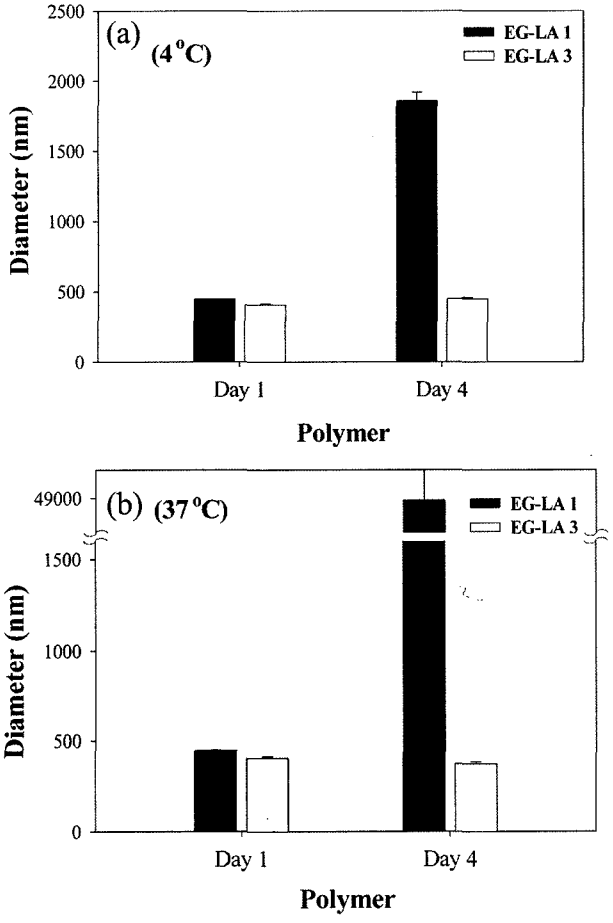

Figure 4. Size change of polymersome during incubation at (a) 4 °C and (b) 37 °C.

stability than EG-LA1 polymersomes after 4 day incubation at both of the temperatures. EG-LA1 polymersomes were likely to fuse together after collision, resulting in the formation of larger aggregates. It appears that the PEG molecular weight in the di-block copolymer structure is pivotally important in maintaining the stability of polymersomes. At a constant volume fraction value, the longer chain length of PEG (Mw 5,000) was superior to the shorter one (Mw 2,000) for stabilizing the polymersome structure. This was probably caused by the steric repulsive effect of PEG chains surrounded around the polymersomes. It is well known that chain length and surface density of PEG chains immobilized on the polymer surfaces are the two most critical factors for non-fouling surfaces that repel proteins and cells.[23] In the case of PEG-coated colloid particles, steric stabilization effect of PEG chains also prevents them from aggregation each other. From this point of view, it is evident that the polymersomes with higher PEG molecular weight exhibit better stability against coalescence.

To further prove that EG-LA3 polymersomes had an excellent colloidal stability against coalescence, they were subject to centrifugation with different speeds. The precipitated polymersomes were re-dispersed in aqueous solution. Figure 5(a) shows that re-dispersed polymersomes exhibited nearly the same solution turbidity even after increasing the centrifugation speed. In addition, diameter of polymersome remained unaltered after centrifugation (centrifuge speed 14,000 rpm). This indicates that polymersomes with suitable chemical composition and molecular weight could maintain their structural integrity when they were concentrated.

Sodium dodecyl sulfate (SDS), an anionic detergent, is commonly used to break up the self-assembling structures of micelles, liposomes, and cell membranes. Thus it will be of interest to see the effect of SDS on the stability of polymersomes. In this study, 1% (w/w) SDS solution was added to the polymersome solutions. As shown in Figure 6, the size of EG-LA3 polymersomes was slightly reduced after the treatment of 1% SDS solution, but they were still present as nano-particulate polymersomes. In addition, there was no significant change in relative transmittance values of polymersome solution before and after the SDS treatment; which means that polymersomes were stable even in the presence of strong detergent. The polymersome solution did not show any visual evidence of turbidity change such as precipitation and clearance in the presence of SDS. This might be attributed to the fact that surface active SDS molecules could not be accessible into the hydrophobic interior of PDLLA layer imbedded in the self-assembled bilayer structure of di-block copolymers.

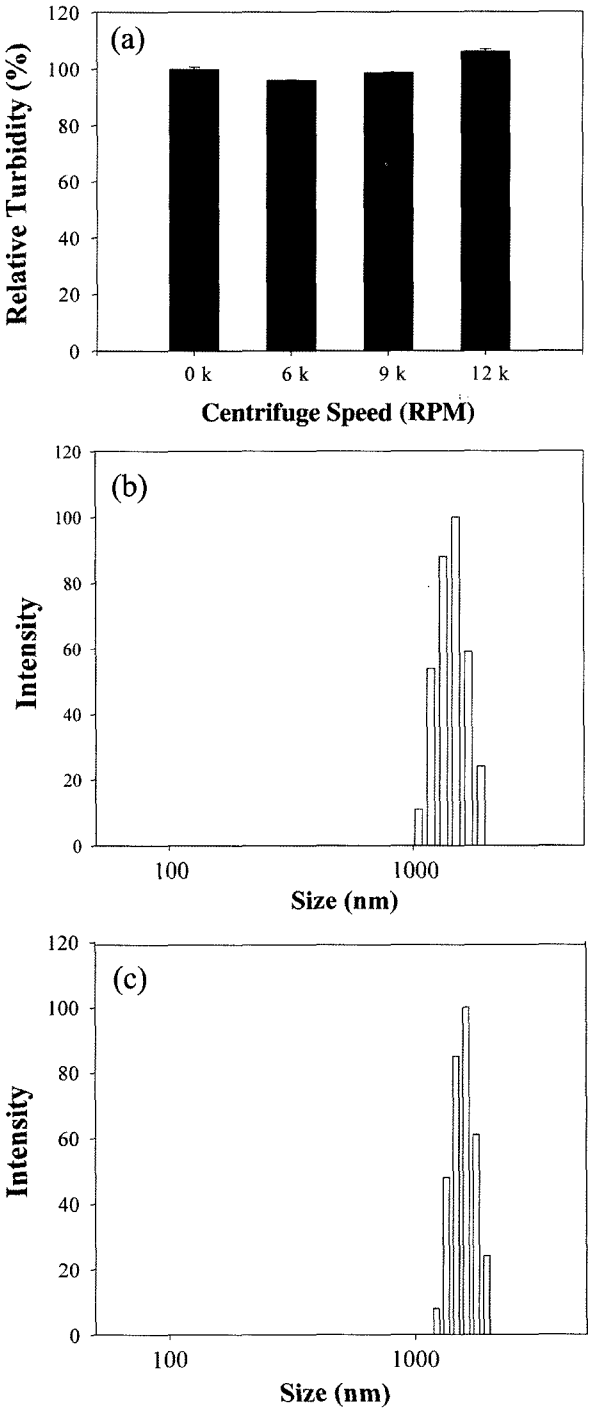

Figure 5. Mechanical strength of polymersome (EG-LA3) against centrifugation. (a) Change on relative turbidity after centrifugation, (b) size distribution of polymersome before centrifugation (14,000 rpm), and (c) size distribution of polymersome after centrifugation (14,000 rpm).

Conclusions

Biodegradable polymersomes consisting of mPEG-PDLLA di-block copolymers were fabricated using an emulsion solvent evaporation method. Various polymersomes with controllable sizes could be readily produced by adjusting the volume fraction between a hydrophilic PEG

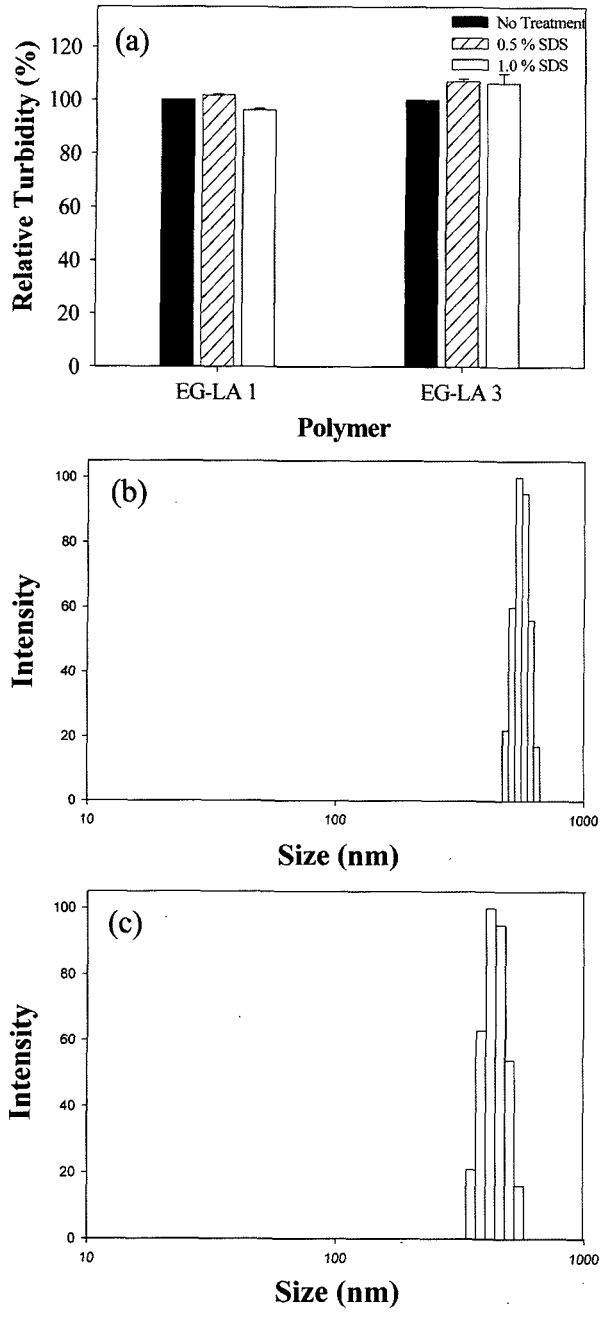

Figure 6. Chemical stability of polymersomes. (a) Relative turbidity of polymersomes, (b) size distribution of EG-LA3 before treatment of 1% SDS solution, and (c) size distribution of EG-LA3 after treatment of 1% SDS solution.

block and a hydrophobic PDLLA block in the copolymer structure. Robust and stable polymersomes could be obtained when their composition and molecular weight were optimally adjusted. The PEG molecular weight was particularly important for the stabilization. The biodegradable polymersomes having much higher stability than liposomes could be potentially applied for delivering bioactive macromolecular drugs such as peptides, proteins, and genes in a sustained or targeted manner.

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