Core/Shell Nanoparticles with Lecithin Lipid Cores for Protein Delivery

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Core/shell nanoparticles with lipid core, were prepared and characterized as a sustained delivery system for protein. The lipid core is composed of protein-loaded lecithin and the polymeric shell is composed of Pluronics (poly(ethylene oxide)−poly(propylene oxide)−poly(ethylene oxide) triblock copolymer, F-127). Based on the preparation method in the previous report by us, the freeze-drying of protein-loaded lecithin was performed in the F-127 aqueous solution containing trehalose used as a cryoprotectant to form stabilized core/shell nanoparticles. Cryo-TEM (transmittance electron microscopy) and a particle size analyzer were used to observe the formation of stabilized core/shell nanoparticles. For the application of core/shell nanoparticles as a protein drug carrier, lysozyme and vascular endothelial growth factor (VEGF) were loaded into the core/shell nanoparticles by electrostatic interaction, and the drug release pattern was observed by manipulating the polymeric shell.

Introduction

Among the various approaches investigated, extensive research has been conducted on the ability of colloidal systems, such as liposomes and polymeric nanoparticles, to provide an optimal delivery system of protein drugs.

Various types of liposomal formulations have been utilized as drug delivery vehicles for the sustained release of protein drugs, and some have been evaluated for clinical applications.[1−3] Most of the reported studies involve traditional liposomes (unilamellar[4,5] or multilamellar vesicular systems[6,7]) and multivesicular liposomes (DepoFoam).[8−11] However, the liposomal system exhibits mechanical instability in the physiological condition, which results in a total release of the internal aqueous content (burst effect).[4−7] Multivesicular liposomes have been introduced to overcome this difficulty.[8−11] Each multivesicular liposome is composed of discontinuous internal aqueous chambers that are bounded by a continuous, nonconcentric network of lipid membranes with a higher aqueous volume-to-lipid ratio. This unique feature provides for the sustained release of encapsulated protein drug since, unlike the unilamellar vesicular system, a single breach in the external membrane of a multivesicular liposome will not result in a total release of internal aqueous content containing a protein drug. However, the sustained release formulation of protein drugs using multivesicular liposomes still remains the greatest challenge to the stability due to the internal aqueous content.

Nano/microparticles composed of biodegradable polyesters are currently the most suitable and preferable systems till today in the sustained delivery of protein drugs in parenteral or nonparenteral routes.[12−14] Because of widely available toxicological and chemical data, biocompatibility/histocompatibility, predictable biodegradation kinetics, ease of fabrication, and variety in copolymer ratio and molecular weight, polyesters are approved for clinical applications.[15,16] Despite their many advantages, polyesters such as PLGA [poly(lactide-*co*-glycolide)] also have some inherent shortcomings. They are quite hydrophobic compared with most of the protein drugs to be encapsulated. A lack of protein compatibility leads to stability problems of protein drugs during storage or in vivo release conditions.[17] Since the hydration and degradation of PLGA are prerequisites for the release of protein drugs during the bioerosion phase, this results in an acidic microenvironment due to the formation of lactic acid and glycolic acid, which leads to the denaturation (hydrolytic degradation and aggregation) of protein drugs.[18]

In our previous report, the core/shell nanoparticles with a lecithin lipid core were prepared and characterized as a delivery system for liphophilic drugs.[19] A further study has been performed to evaluate the core/shell nanoparticles as a protein delivery system. VEGF (vascular endothelial growth factor) was used as a model drug. VEGF is a potent mitogen in embryonic and somatic angiogenesis with a unique specificity for vascular endothelial cells.[20,21] VEGF has been considered as a potential treatment for strokes due to its angiogenic and direct neuroprotective action.[22] One potential mechanism for the treatment of stroke is the induction of angiogenesis with increased oxygen availability. The extent of newly formed vessels is probably an important factor in determining the improvements in the blood flow, culminating in the recovery and repair of neurons and a reduction in ischemic damage. To achieve an optimum therapeutic effect, the sustained release of VEGF is required, and new types of drug delivery systems should be designed. Kim et al. reported on the pharmacokinetic characterization of VEGF release from the PLGA microparticles.[23] Later, the improved system for the sustained delivery of VEGF using heparin-functionalized PLGA nanoparticles was demonstrated by Chung

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et al.[24] However, the inherent shortcomings of PLGA nano/microparticles still remain. In this study, the core/shell nanoparticles with a lecithin core were designed and characterized as a sustained release system for VEGF based on our previous results. The release behavior and the stability of nanoparticles were evaluated for efficient protein delivery.

Materials and Methods

Materials. Pluronic F-127 (pluronics, poly(ethylene oxide)−poly(propylene oxide)−poly(ethylene oxide) triblock copolymer) was obtained as a gift from BASF corp., Korea and used as received. F-127 can be represented by the formula $(EO)_{100}(PO)_{65}(EO)_{100}$ on the basis of its nominal molecular weight of 12 600 and 75% PEO. L-α-Lecithin from soy beans (phosphatidylcholine content 40%) and lysozyme from chicken egg whites were purchased from Sigma (St. Louis, MO). Recombinant human vascular endothelial growth factor (VEGF) was obtained from PeproTech (Rocky Hill, NJ), and VEGF ELISA kit was the product of Neogen Corp. (Lexington, KY). The dialysis membrane (PVDF, MWCO = 500 000) was the product of Spectrum (Houston, TX).

Preparation of Core/Shell Nanoparticles. The lipid phase used as a core was composed of 40 wt % aqueous solution of lecithin from soy bean oil with the form of nanoparticles, which were prepared by sonication (Net power output = 750 W, frequency = 20 kHz) using a probe type ultrasonic wave homogenizer (Branson Sonifier model 185). The weighed amounts of the lipid phase, protein drug (lysozyme or VEGF), trehalose (cryoprotectant, 5 wt % of total weight of dried nanoparticles), and F-127 aqueous solution were mixed and subjected to freeze-drying to induce the formation of a polymeric shell on the surface of the protein drug-loaded lipid core.

Particle Size Distribution and Zeta Potential Measurements. Solutions of 30 mg of dried core/shell nanoparticles in 30 mL of phosphate buffered solution (PBS, pH 7.4) were prepared for the measurement of particle size distribution and zeta potential. The intensity autocorrelation was measured at a scattering angle (θ) of 90° with electrophoretic light scattering (ELS 8000, Otsuka Electronics) at 25 ± 0.1 °C. When the difference between the measured and the calculated baselines was less than 0.1%, the correlation function was accepted. A nonlinear regularized inverse Laplacian transformation technique was used to obtain the distribution of the decay constant. The mean diameter (d) was evaluated by the Stokes−Einstein equation. Experiments were repeated three times.

Cryogenic Transmittance Electron Microscope (Cryo-TEM) Measurements. The samples were prepared as a thin liquid film (less than 0.25 μm thick) supported on a cryo-grid. Thin aqueous films can be produced by applying approximately 7 μL of the aqueous solution containing core/shell nanoparticles to a holey grid, followed by the removal of excess fluid by blotting onto a filter paper for 7 s. (The aqueous solution containing core/shell nanoparticles was prepared by mixing 1 mg of nanoparticles and 30 mL of distilled−deionized water.) This left a thin layer of the grid hole. Immediately, the grid then was plunged in liquid ethane, before evaporation occurred from the thinly spread sample. The frozen grids were stored in liquid nitrogen and transferred in a GATAN model 630 cryotransfer (Gatan, Inc., Warrendale, PA) under liquid nitrogen at approximately −185 °C. Direct imaging was carried out at a temperature of approximately −170 °C and with a 120 kV acceleration voltage, using the images acquired with a Multiscan 600W CCD camera (Gatan, Inc., Warrendale, PA)

In Vitro Lysozyme Release from Core/Shell Nanoparticles. Lysozyme-loaded nanoparticles (10 mg) were suspended in 0.2 mL of PBS, and this solution was subsequently put into a dialysis tube. The dialysis tube was placed into 15 mL of PBS containing 2.0 mM sodium azide and 0.1% (w/v) bovine serum albumin (BSA) and kept in a reciprocal shaking water bath (Jeio Tech., Korea) at 37 °C and 35 rpm. At each time point, the whole medium was taken and replaced with a fresh release medium. The amount of released lysozyme in the aqueous media was analyzed by reversed-phase HPLC using a Shodex RSpak RP 18−415 column and trifluroacetic acid/acetonitrile (0.1/99.9 v/v %) mobile phase at a flow rate of 1 mL/min. The elute was monitored by UV absorption at 220 nm.

For lysozyme activity assay, a suspension of 0.1 mg/mL *M. lysodeikticus* was prepared in PBS (37 °C). To 2.95 mL of *M. lysodeikticus* suspension was added 0.05 mL of lysozyme solution, and the solution was mixed immediately. The turbidity of the suspension was measured at 450 nm by a Shimadzu UV-1601 spectrophotometer. One unit activity corresponds to a decrease in turbidity of 0.001 per minute at 450 nm.

In Vitro VEGF Release from Core/Shell Nanoparticles. The same conditions used in the lysozyme release experiment were employed. The amount of released VEGF into the release medium was determined by ELISA analysis (Molecular Devices, USA).

Results and Discussion

In our previous report, we presented a new approach for the preparation of the core/shell nanoparticles consisting of a core of lecithin surrounded by F-127.[19] On the basis of this preparation method, the core/shell nanoparticles have been characterized as a protein delivery system.

Aqueous concentrated lecithin mixtures show typical lamellar liquid crystalline behavior and the individual lamellae tend to form a spherical supramolecular structure.[25] As reported in previous studies,[25,26] lecithin forms an anionic nanolipid, and it can form an ionic complex with positively charged molecules. For the preparation of core/shell nanoparticles, lecithin nanolipids were mixed with F-127 aqueous solution and freeze-dried. To find out the optimum composition for the preparation of core/shell nanoparticles, the changes of the size and surface charge of freeze-dried core/shell nanoparticles with VEGF-loaded lipid cores were observed with a variation of the lecithin/F-127 (w/w) ratio in PBS as shown in Figure 1. The loading amount of each nanoparticles was approximately 23.0 ± 3.4 ng/mg. In the case of VEGF-loaded lecithin nanolipids without F-127, the average diameter of the nanolipids was 96.8 ± 3.1 nm, and the surface charge from zeta potential measurements was −40.4 ± 2.1 mV. With the formation of core/shell nanoparticles, an anionic character of lecithin nanolipids used as core was diminished due to the presence of the polymeric shell. However, the negative value of the surface charge was amplified by increasing the lecithin content in the core/shell nanoparticles. With the increase of the lecithin/F-127 (w/w) ratio, the size of core/shell nanoparticles was increased up to 0.5 of the lecithin/F-127 (w/w) ratio and decreased after the maximum. Core/shell nanoparticles with a powdery form were formed with less than 0.5 of the lecithin/F-127 (w/w) ratio; however, the leakage of lecithin from the core/shell nanoparticles was observed with more than 0.5 of the lecithin/F-127 (w/w) ratio verified by the formation of core/shell nanoparticles in the powder/oil mixture form. This indicates that the stabilized core/shell nanoparticles were formed with less than 0.5 of the lecithin/F-127 (w/w) ratio and the decrease in the size of core/shell nanoparticles after 0.5 of the lecithin/F-127 (w/w) ratio may be due to presence of lecithin nanolipids released from the unstable core/shell nanoparticles.

As described schematically in Figure 2, the complex formation between lecithin and protein drugs can be induced by choosing a proper protein with a high isoelectric point (>8) and protein-loaded core/shell nanoparticles can be obtained based on the preparation method of core/shell nanoparticles in our previous study. For efficient loading of model protein drugs, the complex formation was induced between lecithin and model

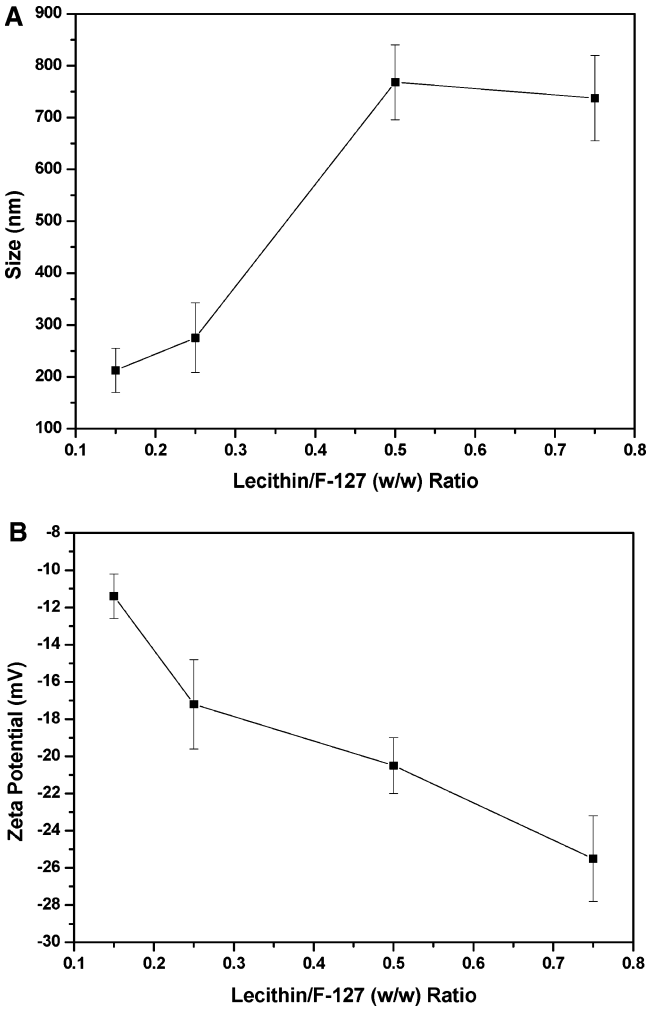

Figure 1. Changes of size (A) and zeta potential (B) of VEGF-loaded core/shell nanoparticles as a function of lecithin/F-127 (w/w) ratio. The total number of experiments is three.

protein drugs, such as lysozyme (the isoelectric point, pH 11.1) and VEGF (the isoelectric point, pH 8.4) in the F-127 aqueous solution containing trehalose, and was freeze-dried to form the polymeric shell.

In the freeze-drying process, trehalose used as a cryoprotectant plays two roles. The first is to preserve the core/shell structure of nanoparticles, which is very important to accomplish a sustained release pattern of protein drugs. The other is to preserve the activity of loaded protein drugs. It has been reported that globular proteins invariantly show higher melting (denaturation) temperatures in the presence of sugars (in dilute solution), and concentrated sugar systems confer textural consistency that prevents microbial attack in foods. It is not clear, however, whether these two different types of stabilization have a common physicochemical origin, and in particular, whether water−sugar interactions are the only basis of both phenomena. Among all of the sugars, trehalose has received the greatest attention, because of both its wide role in nature and its potential use as a highly efficient natural preservative.[28−29]

To observe the core/shell structure, Cryo-TEM pictures were taken with of VEGF-loaded core/shell nanoparticles with 0.25 of the lecithin/F-127 (w/w) ratio. As shown in Figure 3, the formation of core/shell nanoparticles was clearly demonstrated.

Figure 4 shows the release pattern of lysozyme from the core/shell nanoparticles as a function of the lecithin/F-127 (w/w) ratio. The loading amount of each nanoparticles is 0.50 ± 0.12

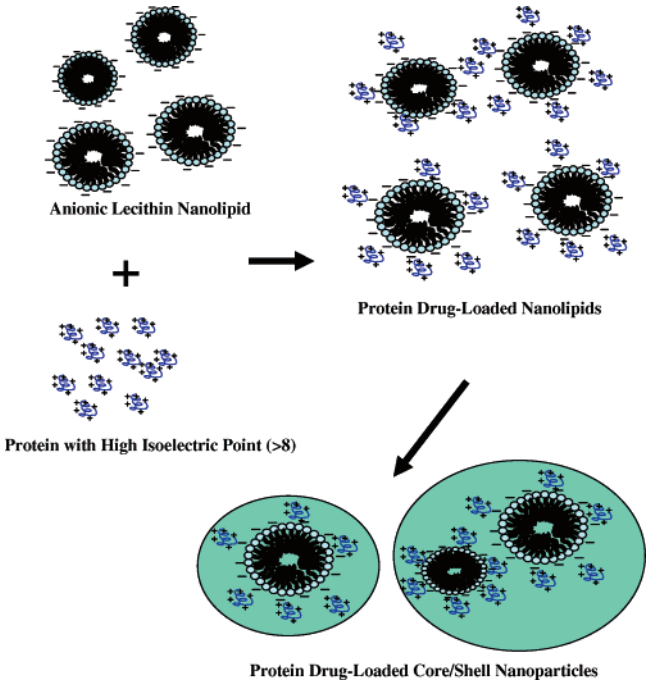

Figure 2. Schematic description for the formation of core/shell nanoparticles with protein drug-loaded lipid cores.

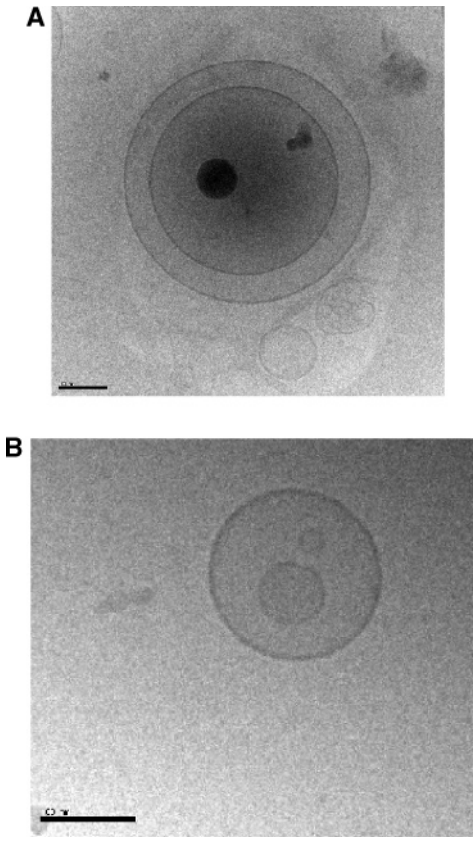

Figure 3. Cryo-TEM pictures of VEGF-loaded core/shell nanoparticles. (A) Nanoparticle with one lipid core and (B) Nanoparticle with two lipid cores. Dots in (A) are the ice contaminants and bar length is 100 nm.

wt %. Because lecithin used as a core forms an anionic nanolipid, the protein with cationic character in the physiological condition is suitable to induce the electrostatic interactions for efficient loading and lysozyme is selected as a model protein. Lysozyme is a structurally stable protein and is expected to

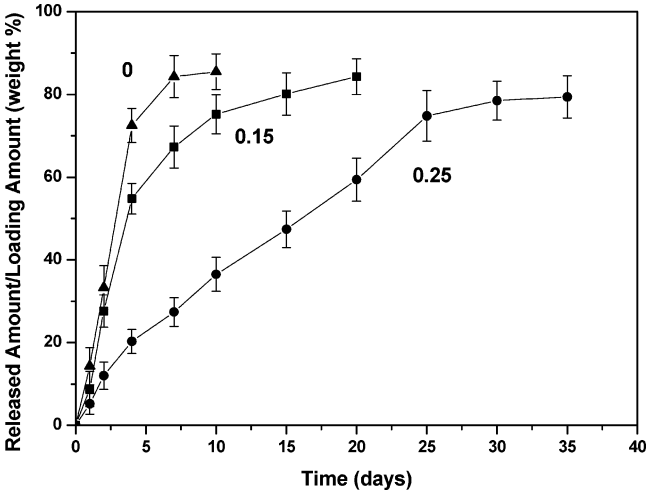

Figure 4. Release pattern of lysozyme from the core/shell nanoparticles as a function of the lecithin/F-127 (w/w) ratio. The total number of experiments is three.

Table 1. Enzyme Activity of Lysozyme in the 10-Day Released Sample[a]

formulation	specific enzyme activity (EU/mg) × 10^2 (mean± S. D., n=3)
control	39.4 ± 2.9
lysozyme released from the core/shell nanoparticle formed with 0.25 of the lecithin/F-127 (w/w) ratio	34.1 ± 1.5

[a] Control: Lysozyme (0.65% w/v in release media) kept at 37 °C in oscillating water bath for 1 week.

undergo little conformational alteration with the electrostatic interactions.[30] Results with lysozyme will give us an insight into the influence of electrostatic interactions between lecithin nanolipids and VEGF which has a similar ionic character. In the case of 0 of the lecithin/F-127 (w/w) ratio, a significant burst was observed, releasing about 85% of the initial loading amount during a one-week period. With the formation of core/shell nanoparticles, a sustained release pattern was observed, releasing about 80% of the initial loading amount during a 30-day period. Table 1 provides data on the specific enzyme activity of lysozyme in the release media after the 10-day release experiment. In comparison to the activity of native lysozyme, 86.6% of the activity was preserved, indicating that the activity of lysozyme was preserved during the freeze-drying process.

The release of VEGF from the nanoparticles was observed as a function of the lecithin/F-127 (w/w) ratio as shown in Figure 5. The release pattern of VEGF is similar to that of lysozyme. By increasing the lecithin/F-127 (w/w) ratio, the lecithin content in the core increases with anionic character as presented in the Figure 1. This leads to the increase in the electrostatic interactions between lecithin cores and VEGF resulting in the decrease of the release rate of VEGF with a sustained release pattern. In the case of 0 of the lecithin/F-127 (w/w) ratio, the freeze-dried mixture of VEGF and F-127, which was resuspended in release medium for the release experiment, was used to observe the interaction between VEGF and F-127. A significant burst was observed, releasing about 85% of the initial loading amount during a 9-day period. These results indicate that the sustained release pattern was accomplished with the formation of the core/shell nanoparticles and the electrostatic

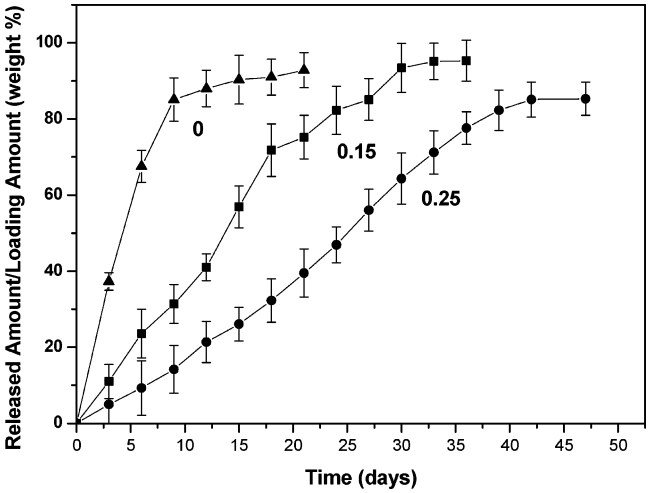

Figure 5. Release pattern of VEGF from the core/shell nanoparticles as a function of the lecithin/F-127 (w/w) ratio. The total number of experiments is three.

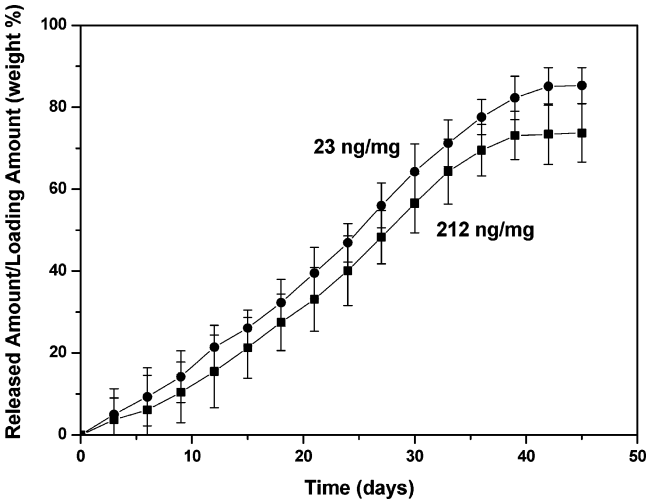

Figure 6. Release pattern of VEGF from the core/shell nanoparticles as a function of loading amount. VEGF-loaded core/shell nanoparticles were formed with 0.25 of the lecithin/F-127 (w/w) ratio and the total number of experiments is three.

interactions between lecithin cores, and the loaded proteins play an important role in determining the release pattern.

Figure 6 shows the release pattern of VEGF with the variation of loading amount. A similar release rate was observed with the variation of loading amount and the released amount can be regulated by loading amount.

To observe the stability of nanoparticles in the aqueous media, the size distribution was observed with the core/shell nanoparticles and nanolipid after 2-week equilibrium in the aqueous medium. If the particles are unstable in the aqueous medium, they tend to aggregate to form the agglomerate.[31] This leads to the unexpected change in the release of loaded drug from the particles. As shown in Figure 7, the core/shell nanoparticles did not show an aggregation indicating that the core/shell nanoparticles were stable in the aqueous medium. In the case of VEGF-loaded (or adsorbed) lecithin nanolipid without a polymeric shell, a slight aggregation was observed at the early stage of formation (see Figure 8A); furthermore, a significant aggregation was observed after 2-week equilibrium in the aqueous medium (see Figure 8B). VEGF-loaded nanolipid without a polymeric shell formed the agglomerate during the 2-week equilibrium in the aqueous medium, which might result

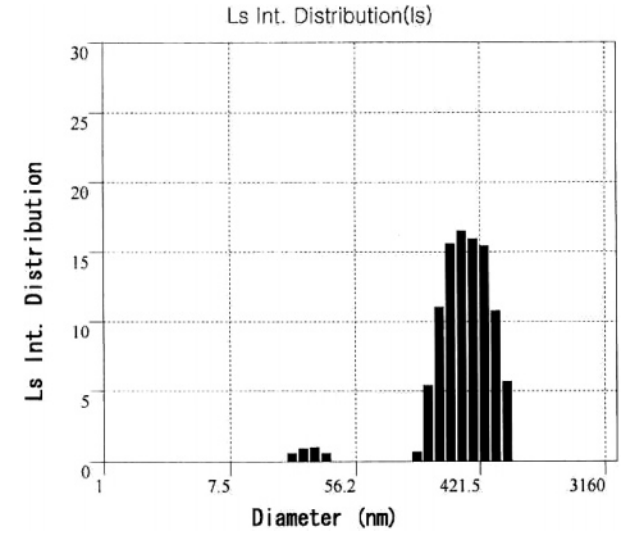

Figure 7. Size distribution of core/shell nanoparticles after 2-week equilibrium in the aqueous medium.

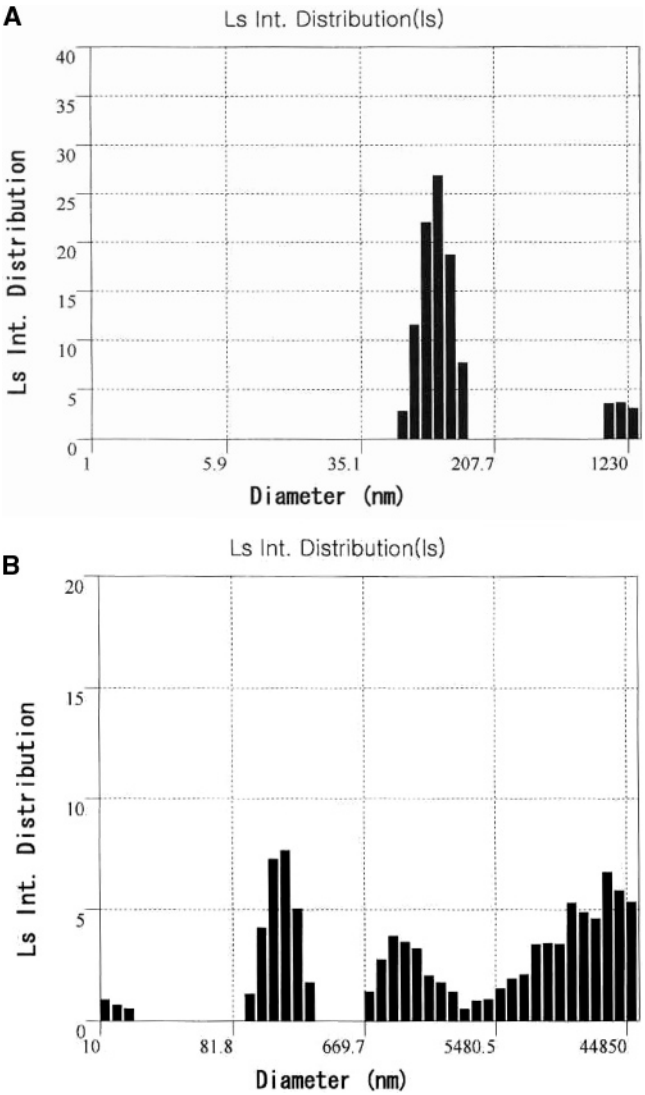

Figure 8. Size distributions of a VEGF-loaded nanolipid before (A) and after (B) 2-week equilibrium in the aqueous medium.

in the unexpected release pattern and the destabilization of loaded protein. This indicates that the improved stability of nanoparticles in the aqueous medium can be achieved with the formation of core/shell nanoparticles.

The core/shell nanoparticles in this study are intended to improve blood flow in the ischemic area of the body through the formation of new blood vessels, a process known as angiogenesis, along with the delivery of the angiogenic protein, VEGF. In this approach, the core/shell nanoparticles are directly injected into the ischemic area by an injection catheter, enabling the sustained and controlled release of VEGF in the area of the ischemic area with low blood flow. The feasibility of this approach will be reported later.

Conclusions

The core/shell nanoparticles with protein drug-loaded lipid cores were prepared in the powdery state with the maintenance of protein activity. With the formation of nanoparticles with core/shell structure, the stability of lecithin nanolipids was significantly increased, and the sustained release patterns were achieved with model protein drugs such as lysozyme and VEGF. This indicates that the trehalose, used as a cryoprotectant, can preserve the core/shell structure of nanoparticles as well as the activity of loaded protein drug during the freeze-drying, and the core/shell nanoparticles in this study can be utilized as a protein delivery system.

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