Thermally Reversible Pluronic/Heparin Nanocapsules Exhibiting 1000-Fold Volume Transition

Seung Ho Choi,† Ji-Hwan Lee,‡ Sung-Min Choi,‡ and Tae Gwan Park*,†

Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon 305-701, South Korea, and Department of Nuclear and Quantum Engineering, Korea Advanced Institute of Science and Technology, Daejeon 305-701, South Korea

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Novel Pluronic/heparin composite nanocapsules that exhibit a thermally responsible swelling and deswelling behavior were synthesized. Pluronic F-127 preactivated with *p*-nitrophenyl chloroformate at its two terminal hydroxyl groups was dissolved in a methylene chloride phase. The organic phase was dispersed in an aqueous phase containing heparin. At an organic/aqueous interface, Pluronic-cross-linked heparin nanocapsules were produced. They exhibited a 1000-fold volume transition (ca. 336 nm at 25 °C; ca. 32 nm at 37 °C), and a reversible swelling and deswelling behavior when the temperature was cycled between 20 and 37 °C. The reversible volume transition of Pluronic nanocapsules was caused by micellization and demicellization of cross-linked Pluronic polymer chains within the nanocapsule structure in response to temperature. The morphological characters were investigated with transmission electron microscopy and small angle neutron scattering. Pluronic/heparin nanocapsules had an aqueous fluid-filled hollow interior with a surrounding shell layer below the critical temperature, but they became a collapsed core/shell structure similar to that of Pluronic micelles above it.

Introduction

Thermo-responsive synthetic hydrogels, exhibiting a lower critical solution temperature (LCST) behavior in an aqueous solution over a narrow temperature range, received much attention recently.[1−10] They swell and expand below the LCST but deswell and shrink above that temperature. The major cause for the LCST phenomenon is entropy driven hydrophobic interaction of polymer chains that have a delicate balance of hydrophilic and hydrophobic moieties in the structure.[3,4] Among them, poly(*N*-isopropylacrylamide) (PolyNIPAAm) hydrogels have been extensively studied.[3−5] PolyNIPAAm hydrogels in aqueous solution exhibit a sharp volume transition at an LCST value of 32 °C, and its LCST range can be controlled by copolymerizing with other hydrophilic or hydrophobic monomers. Pluronic tri-block copolymers composed of poly(ethylene oxide)−poly(propylene oxide)−poly(ethylene oxide) (PEO−PPO−PEO) also show similar LCST behaviors over a broad temperature range depending on the composition and molecular weight.[6−10] They self-assemble to form a spherical micellar structure above the LCST by hydrophobic interaction of the PPO middle block in the structure.[7,8] At high concentration above about 25% (v/v), they exhibit a sol−gel transition behavior when raising the temperature above the LCST.[9,10]

Nanogels are cross-linked water soluble polymer networks that swell in aqueous solution. They have nanoscale spherical dimensions (less than 1 μm in diameter) with narrow size distribution. They are different from self-assembling polymer micelles that are formed primarily by noncovalent interactions. Various polymeric nanogels with diverse morphological characters have been synthesized and utilized for drug delivery, gene therapy, and diagnostic applications such as molecular imaging.[11−14] Poly(ethylene glycol) nanogels cross-linked with polyethylenimine were used for gene delivery.[15,16] Thermosensitive PolyNIPAAm nanogels showing a reversible swelling/deswelling behavior at the LCST were also produced for intracellular delivery of bioactive agents.[17,18] More recently, dual stimuli-sensitive core/shell nanogels that exhibit volume changes in response to temperature and/or pH were synthesized by incorporating temperature- and pH-sensitive polymer in the core and shell structure, respectively.[19] The nano-structured polymer particles, however, have an average diameter about 100−200 nm, which limits the nanogels for drug and gene delivery applications. Most self-assembling polymeric micelles and polyelectrolyte complex nanoparticles have a much smaller size than 100 nm, but they are inherently unstable, tend to aggregate, or easily disintegrated in the body fluid conditions because their supra-molecular structures are primarily maintained by noncovalent interactions. Thus, it is desirable to synthesize robust and stable nanogels <100 nm that are tolerable to various

* Corresponding author. Phone: +82-42-869-2621. Fax: +82-42-869-2610. E-mail: tgpark@kaist.ac.kr.

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‡ Department of Nuclear and Quantum Engineering.

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physiological conditions. They have definite advantages as carriers for drugs and imaging agents. For example, such nanogels can be exploited more effectively as anti-cancer drug carriers to target solid tumors having a loosened vascular structure (enhanced permeation and retention (EPR) effect).[20]

In this study, thermo-sensitive cross-linked Pluronic F-127/heparin composite nanocapsules having a diameter of about 30 nm at the body temperature were produced. Terminal two hydroxyl groups of Pluronic F-127 were activated with amine reactive groups. The activated Pluronic F-127 was dissolved in a methylene chloride phase, which was emulsified under sonication conditions in an aqueous phase containing heparin that had primary amine residues as impurities. The resultant Pluronic/heparin nanocapsules were characterized with an emphasis on thermal sensitivity and reversibility. Morphological characters were studied by using transmission electron microscopy and small angle neutron scattering techniques below and above the phase transition temperature.

Experimental Section

Materials. Pluronic F-127 was obtained from BASF Corporation (Parsipanny, NJ) and used without additional purification. Its average molecular weight provided by the manufacturer is 12 600. Partially De-N-sulfated heparin sodium (fractionated, $M_w = 6000$) was obtained from Celsus laboratories, Inc. (Cincinnati, OH), which has 20% free amino group per repeating unit measured by fluorescamine assay.[21] Pyrene, *p*-nitrophenyl chloroformate (p-NPC) were from Sigma-Aldrich Corporation (ST. Louis, MO). All other chemical reagents were of analytical grade.

Preparation of Activated Pluronic F-127. A total of 2 g of Pluronic F-127 were completely dried in vacuo at 80 °C overnight and dissolved in 6 mL of anhydrous benzene. The solution was slowly added to a stirred solution of 6 mL anhydrous benzene containing p-NPC (192 mg, 0.95 mmol) in a dropwise manner. The reaction was carried out for 3 h at room temperature with gentle stirring under nitrogen atmosphere. The activated Pluronic F-127 was precipitated three times in ice-cold diethyl ether and dried under vacuum. To determine the activation extent of Pluronic F-127 with p-NPC, a known amount of activated Pluronic F-127 was treated with 0.2 N NaOH at 22 °C for 2 h. The concentration of *p*-nitrophenoxide released in the aqueous phase was quantified spectrophotometrically at 410 nm ($\epsilon = 1.7 \times 10^4$ $M^{-1}cm^{-1}$).

Synthesis of Pluronic/Heparin Nanocapsules. Pluronic/heparin nanocapsules were synthesized using an emulsification/solvent evaporation method with modifications.[16] Methylene chloride solution (200 μL) containing activated Pluronic F-127 (60 mg) was added dropwise to an aqueous solution (2 mL, pH 9) of heparin (30 mg). The mixture solution was sonicated for 3 min in a Branson sonifier 450 (20 kHz, output control = 2.5). The oil-in-water emulsion solution was quickly transferred to a rotary evaporator and residual methylene chloride was removed at 30 °C until the solution became clear. After neutralizing by hydrochloric acid, the solution was dialyzed by a Spectra/Por dialysis membrane with the Mw cutoff 50,000 against water at pH 4.0.

Heparin Amount Assay of Pluronic/Heparin Nanocapsules. The amount of heparin incorporated in Pluronic/heparin nanocapsules was determined by toluidine blue colorimetric assay with modification.[22] Briefly, a known amount of sample was dissolved in 0.2 mL of water, which was mixed with 2.5 mL of 0.005% (w/v) toluidine blue solution in 0.01 N HCl and 0.2% (w/v) NaCl and vigorously stirred for 30 s. To this heparin/toluidine blue complex was added 5 mL of hexane, and the solution was vortexed for 5 min. This mixture was centrifuged at 1000 rpm for 3 min, and the hexane phase was discharged to remove the heparin/toluidine blue complex. A total of 1 mL of the remaining toluidine blue in aqueous phase was mixed with 9 mL of ethanol and the absorbance value measured at 631 nm with UV−visible spectrophotometer (Shimadzu, UV-1601, Kyoto, Japan). A series of heparin concentrations was used to construct a calibration curve.

Particle Size Measurements. The effective diameter and surface ζ-potential value of the Pluronic/heparin nanocapsules were measured by a dynamic light scattering instrument (ZetaPlus, Brookhaven Instrument Co., NY) equipped with a He−Ne laser at a wavelength of 632 nm at 90° detection angle. The concentration of nanocapsules was 10 mg/mL and the temperature was varied from 7 to 45 °C. The measurement was carried out in triplicate.

Critical Micelle Temperature of Pluronic/Heparin Nanocapsules. The critical micelle temperature was measured by a fluorescence probe technique using pyrene as a fluorescence probe, as described previously.[23] Pyrene dissolved in acetone was added to deionized water to make a concentration of 1.2×10^{-6} M and acetone was subsequently removed under reduced pressure for 3 h at room temperature. The final concentration of pyrene was adjusted at 6.0×10^{-7} M. The concentration of nanocapsules was 10 mg/mL and the temperature was varied from 7 to 45 °C. A combined mixture of the pyrene solution and the nanocapsule solution was equilibrated at each temperature for 30 min in a dark room. Fluorescence spectra were monitored using a spectrofluorophotometer (Shimadzu, RF-5301PC, Kyoto, Japan) at an excitation and an emission wavelength of 339 and 390 nm, respectively.

Transmission Electron Microscopy (TEM) and Atomic Force Microscopy (AFM). For obtaining TEM images, 0.2% (w/v) nanocapsule solution was preequilibrated at 15 °C or 37 °C and one drop of the solution was dried on a Formvar/carbon support grid with 300 mesh for 2 min. To improve images, nanocapsule specimen was stained for 1 min with one drop of 2% (w/v) uranyl acetate solution. Negatively stained samples were analyzed using a Zeiss Omega 912 TEM (Carl Zeiss, Oberkochen, Germany) electron microscopy. For AFM image, the sample equilibrated at 37 °C was air-dried on a clean mica surface and its image was obtained with a 50×50 μm scanner of PSIA XE−100 AFM system (Santa Clara, CA) in a noncontact mode. The scanned images were collected from a 0.7×0.7 μm area.

Small-Angle Neutron Scattering (SANS). To characterize the morphological structure of Pluronic/heparin nanocapsules, small angle neutron scattering (SANS) measurements were performed on 8 m SANS instrument at High-flux Advanced Neutron Application Reactor Center, Korea Atomic Energy Research Institute in Daejeon, Republic of Korea. Neutrons of wavelength $\lambda = 6.38$ Å with a wavelength spread of $\Delta\lambda/\lambda = 10\%$ were incident on samples held in a quartz cell. Scattering intensities were measured using a 2-dimensional He-3 detector positioned at 2 m away from samples and offset by 5 cm to give an overall q range of 0.016 Å$^{-1} < q < 0.28$ Å^{-1} where $q = (4\pi/\lambda)\sin(\theta/2)$ is the magnitude of the scattering vector and θ is the scattering angle. Scattering from samples was corrected for background and empty cell scattering. The corrected data sets were placed on an absolute scale and circularly averaged using standard samples.

SANS Data Analysis. The SANS intensity $I(q)$ from particles in solution can be calculated as

$$I(q) = (\Delta\rho)^2 nP(q)S(q) + b \quad (1)$$

where $\Delta\rho$ is the contrast of the scattering length density (SLD) between the particles and solvent, n is the number density of the particles, $P(q)$ is the intraparticle interference called form factor, $S(q)$ is the interparticle interference called structure factor, and b is the residual incoherent scattering. In this study, model fitting was performed on dilute samples where interparticle interference is negligible ($S(q) \approx 1$). Then, the scattering function can be simplified as

$$I(q) = (\Delta\rho)^2 nP(q) + b \quad (2)$$

In this study, two different models for the form factor, a hard sphere

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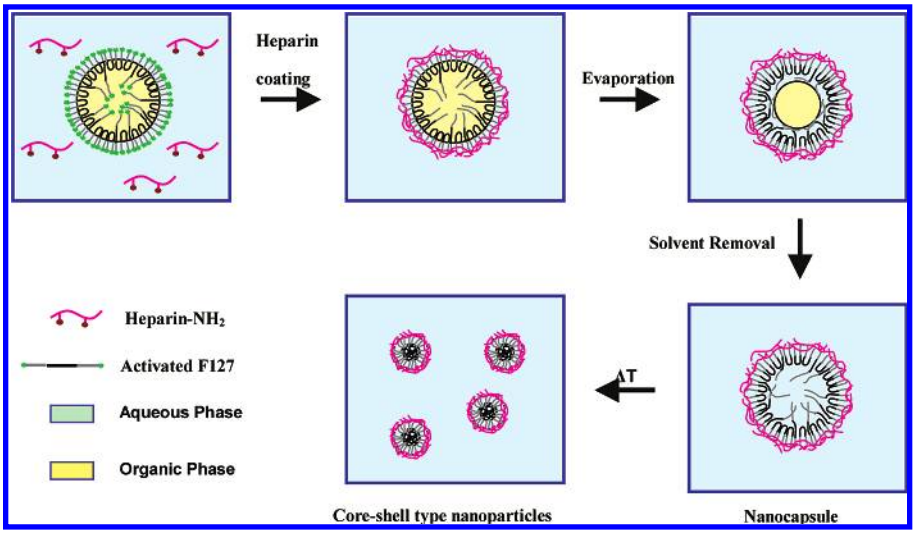

Figure 1. Schematic diagram of Pluronic/heparin nanocapsules.

model and a spherical core−shell model, have been tested to analyze the scattering intensities from the nanocapsule solutions. The hard-sphere model which describes the form factor of a dense spherical particle with a sharp interface is given as[24,25]

$$P(q) = \left[\frac{3(\sin(qR) - qR\sin(qR))}{(qR)^3}\right]^2 \quad (3)$$

where R is the radius of the sphere. The spherical core−shell model for the form factor is given as[24,25]

$$(\Delta\rho)^2 P(q) = \left[\frac{3V_c(\rho_c - \rho_s)J_1(qR_c)}{qR_c} + \frac{3V_s(\rho_s - \rho_{solv})J_1(qR_s)}{qR_s}\right]^2 \quad (4)$$

where V_c and V_s are the volume of core and shell, R_c and R_s are the radii of core and shell, ρ_c, ρ_s, and ρ_{solv} are the SLDs of core, shell and solvent, respectively, and $J_1(x)$ is the first-order spherical Bessel function $J_1(x) = (\sin x - x \cos x)/x^2$.

Results and Discussion

A schematic illustration of Pluronic/heparin nanocapsules was depicted in Figure 1. Organic (methylene chloride) phase emulsion droplets containing high concentration (30% w/v) of p-NPC activated Pluronic F-127 were ultrasonically dispersed in an aqueous phase containing fractionated heparin. The fractionated De-N-sulfated heparin has about 2.2 primary amine groups in the backbone structure as an impurity, as determined by fluorescamine assay. The amount of primary amine groups in heparin is sufficient enough to cross-link activated Pluronic copolymers. The amine reactive p-NPC groups at the Pluronic copolymer ends were conjugated to the primary amine groups in heparin, thereby cross-linking Pluronic tri-block copolymers, vice versa. Since heparin was not soluble in the methylene chloride droplet phase, the cross-linking reaction primarily took place at the interface between oil and water phases, where heparin and activated Pluronic met together. As methylene chloride in the emulsion droplets was gradually extracted into the aqueous phase and evaporated in the air, the size of organic emulsion droplets was slowly decreased. At the same time, p-NPC activated Pluronic copolymers in the organic phase would continuously diffuse out into the aqueous phase, whereas heparin present in the aqueous phase concurrently cross-linked out-fluxing Pluronic copolymers at the interface. Since the cross-linking reaction would occur predominantly at the surface of initially formed emulsion droplets, it was likely that the resultant nanoparticles had a Pluronic/heparin cross-linked shell structure on the surface and an aqueous fluid-filled hollow cavity in the interior. As shown in Figure 1, the structure of nanoscale reservoir capsules is different from those of previously reported nanogels that are homogeneously cross-linked in the polymer matrix.[15,16] The Pluronic/heparin nanocapsules contained 29.2% (w/v) heparin in the structure on a dry weight basis as determined by toluidine blue assay.

Pluronic/heparin nanocapsules exhibited an LCST behavior with increasing temperature, as shown in Figure 2A. They showed a sharp volume transition behavior over a temperature range of 25∼33 °C. Their average diameter was 335.7 ± 42.4 nm at 25 °C but was drastically reduced to 32.4 ± 1.9 nm at 37 °C. About a 10-fold decrease in diameter indicates that the volume was reduced about 1000 times. The greatly collapsed volume of nanocapsules over the LCST region was attributed to the self-association of Pluronic copolymers with increasing the temperature. In the structure of nanocapsules, both of the two terminal groups in the activated Pluronic copolymer were reacted with heparin molecules inter- and intramolecularly for cross-linking. However, it was also possible that only one terminal group in the activated Pluronic copolymer was conjugated to a heparin molecule, resulting in the formation of a freely mobile Pluronic copolymer grafted heparin structure. Thus, it is reasonable to postulate that cross-linked and grafted Pluronic copolymers were self-aggregated with increasing the temperature via hydrophobic interaction between PPO middle blocks in the tri-block Pluronic copolymer structure. It is well-known that Pluronic copolymers self-associate in aqueous solution to form spherical micelles.[6−10] PPO middle blocks hydrophobically interacted to form an inner core, while two PEO side blocks surround it.[7−9] Likewise, Pluronic/heparin nanocapsules were collapsed and shrunken with increasing the temperature above 25 °C due to the micellization of grafted and cross-linked Pluronic copolymers. Figure 2B shows the critical micelle temperature (CMT) of Pluronic/heparin nanocapsules, as determined by using pyrene as a fluorescent probe. The CMT value, as determined from the first inflection point, was about 24 °C. The excitation fluorescent intensity ratio determined at 339 and 334 nm changed over the temperature range from 24 to 30 °C. The change of fluorescent intensity ratio profile was exactly matched with that of effective diameters over the same temperature range. This directly reveals that the

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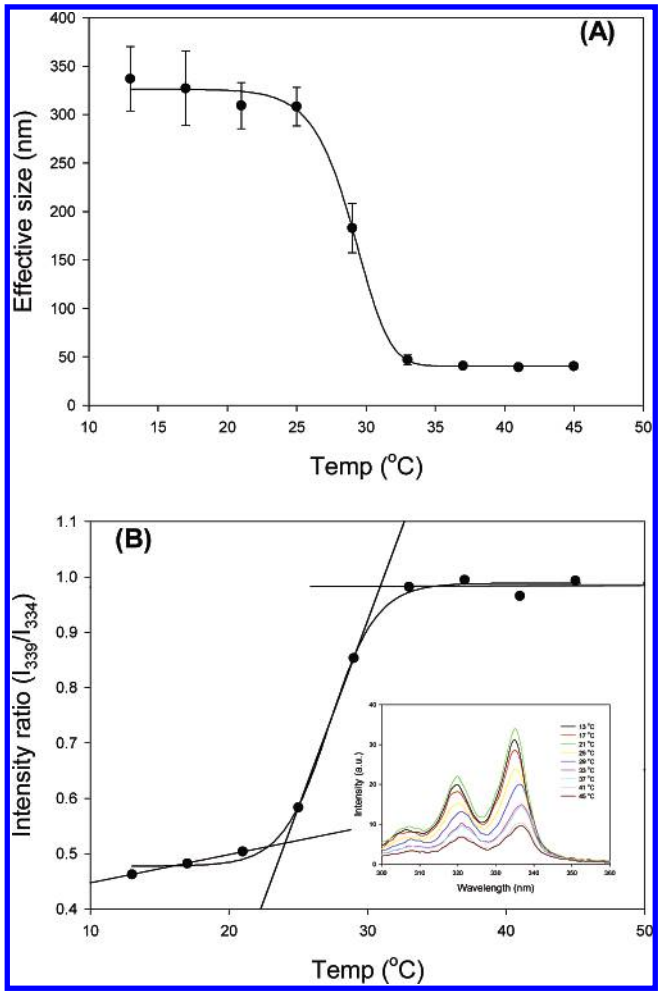

Figure 2. (A) Size change of Pluronic/heparin nanocapsules measured by DLS over a temperature range of 13−45 °C, and (B) the critical micelle temperature determined from the relative fluorescence intensity ratio (I_{339}/I_{334}) of pyrene in the excitation spectra. The inserted figure is fluorescence spectra as a function of temperature.

thermal collapse of Pluronic/heparin nanocapsules was caused by self-association of free mobile grafted or cross-linked Pluronic copolymers abundantly present inside of the nanocapsules in a cooperative manner. Thus, the observed deswelling behavior of nanocapsules above the LCST can be attributed to the collapse of a fragile and soft nanocapsule structure by inward hydrophobic interaction of Pluronic copolymers pendant onto the Pluronic/heparin shell layer. The formation of a spherical Pluronic micellar structure within the interior might drive the shrinkage of nanocapsules. The average size of collapsed nanocapsules in aqueous solution was around 30 nm, similar to that of a single Pluronic micelle.[9] This indirectly implies that the collapsed micelles might contain a single Pluronic copolymer micelle inside. Figure 3 shows reversible swelling and deswelling of Pluronic/heparin nanocapsules when thermally cycled between 25 and 37 °C. It can be seen that the Pluronic/heparin nanocapsules exhibit similar values of effective diameter at each temperature even after several thermal cycles, suggesting that they maintained structural integrity during the repeated swelling−collapse processes. The kinetic rates of swelling and deswelling processes in response to thermal stimuli were very fast on the order of msec because the Pluronic/heparin nanocapsules had a nanoscale dimension. It is known that swelling/deswelling kinetics of the hydrogel volume transition are highly dependent on the their

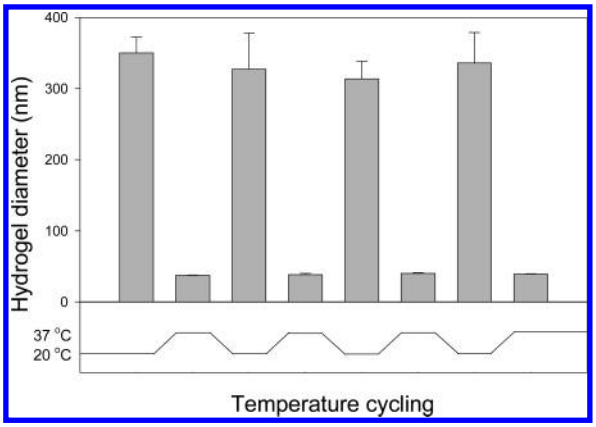

Figure 3. Reversible swelling and deswelling behavior of Pluronic/heparin nanocapsules when the temperature was cycled between 20 and 37 °C.

dimension.[26] To ensure that the reversible swelling/deswelling behavior was indeed caused by covalently linked Pluronic copolymers, Pluronic/heparin nanocapsules were freeze-dried and extracted with methylene chloride to thoroughly remove unconjugated Pluronic fraction. There was no difference in reversible swelling/deswelling behavior after the extraction, suggesting that the thermo-sensitive property was due to the cooperative formation of a single Pluronic micelle from each Pluronic/heparin nanocapsule.

Figure 4 shows transmission electron microscopy (TEM) picture of nanocapsules taken after preequilibrating at 15 and 37 °C. The average sizes of nanocapsules at 15 and 37 °C were 325.6 ± 39.8 and 48.3 ± 10.8 nm, respectively, which are well corroborated with the results shown in Figure 3. The TEM pictures in Figure 4A show clear evidence that the Pluronic/heparin nanocapsules have a nanoreservoir structure below the LCST. They exhibit an aqueous fluid-filled hollow interior with a surrounding shell layer. The shell structure was likely to be composed of a Pluronic grafted or cross-linked heparin network. Above the LCST, the reservoir-type nanocapsule structure was collapsed and became a void-free matrix-type nanoparticular structure (Figure 4B) From the TEM results, it is evident that, with increasing temperature above the LCST, grafted or cross-linked Pluronic copolymers were locally interacted in the vicinity of the shell layer, and they further self-associated to form a spherical micellar structure by pulling the soft and malleable cross-linked heparin shell layer around it. An atomic force microscopic image as shown in Figure 4C also reveals the formation of hard nanospheres above the LCST.

To further probe the morphological structure of collapsed Pluronic/heparin nanocapsules above the critical temperature, SANS measurement was performed for a 1% (w/v) Pluronic/heparin nanocapsule solution at 37 °C, above the LCST. For this measurement, D_2O was used as a solvent to enhance the contrast of scattering length density between the solvent and the nanocapsules. The scattering length densities of D_2O, PEO, and PPO are 6.48×10^{10}, 0.547×10^{10}, and 0.325×10^{10} cm^{-2}, respectively. Figure 5 shows the SANS intensity and model fittings using a spherical core−shell model or a hard sphere model, respectively. In these model fittings, the structure factor was not included. The spherical core−shell model reasonably well matched to the obtained SANS intensities over the entire q range, whereas the hard sphere model failed. The radius of the core and the thickness of the shell obtained from the spherical core−shell

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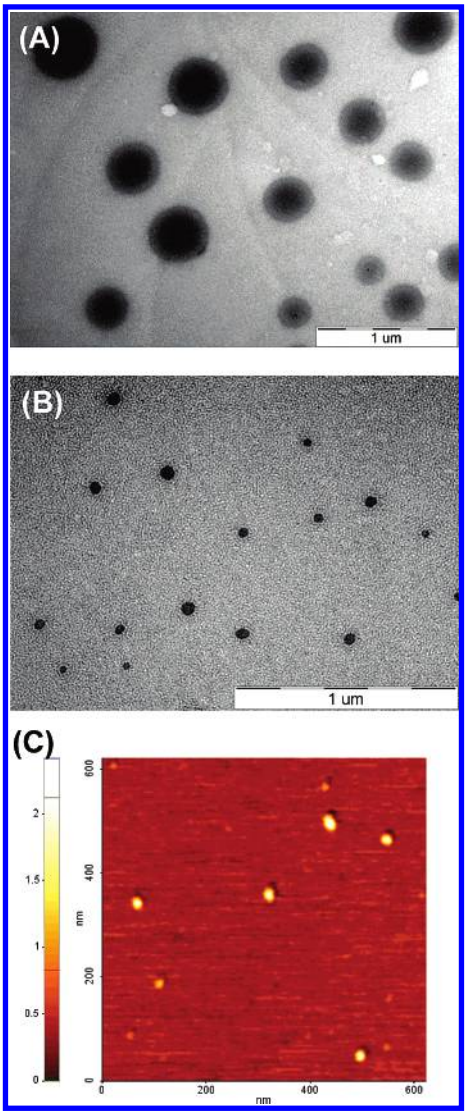

Figure 4. Size change of Pluronic/heparin nanocapsules below/above a critical temperature (A) TEM image at 15 °C, (B) TEM image at 37 °C, and (C) AFM image at 37 °C.

model fitting were 5.19 ± 0.03 and 6.36 ± 0.03 nm, respectively. This result clearly indicates that the collapsed Pluronic/heparin nanocapsules were spherical core−shell matrix-type particles which consisted of a hydrophobic PPO core surrounded by a hydrated PEO/heparin shell. The overall diameter (23.1 ± 0.08 nm) of the nanocapsules measured by SANS are slightly smaller than those values observed from DLS and TEM analysis. This discrepancy was due to the different techniques including different assumptions and conditions for size determination, but the observed values were reasonably close.

The Pluronic/heparin nanocapsules are expected to have many interesting applications in the field of drug delivery. The primary reason for selecting heparin as a Pluronic cross-linking material in this study was because heparin is recently known to induce apoptosis for a variety of cancer cells.[27] Thus, it is conceivable that the Pluronic/heparin nanocapsules would exhibit antitumor effects if they were administered in the body. Enhanced permeation and retention (EPR) effect might play a role in passively targeting the nanocapsules in the tumor site.[28] Our preliminary results revealed that they indeed showed a significant

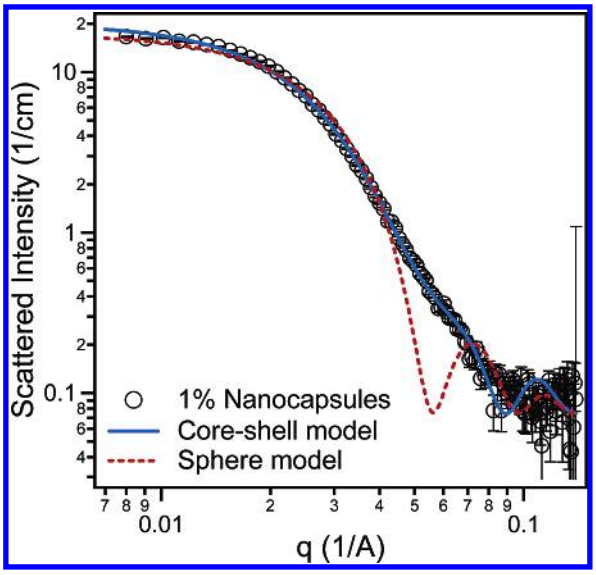

Figure 5. SANS results for 1% Pluronic/heparin nanocapsules solution in D_2O at 37 °C. The SANS data were fitted to the monodisperse spherical core−shell model or to the monodisperse hard sphere model.

antitumor effect in a tumor-bearing animal model study. It should be also mentioned that similar Pluronic based nanocapsules can be readily fabricated by using other multifunctionalized water soluble synthetic and natural polymers instead of using heparin. It can be surmised that such Pluronic-based nanocapsules can encapsulate various bioactive agents in the hollow reservoir and they can release them in a temperature-responsive manner. Although there have been many studies on thermally triggered drug release using temperature-sensitive hydrogels,[29,30] temperature-sensitive nanocapsules with temperature triggered "on/off" drug release characters have never been reported to our knowledge. We are now investigating the thermally modulated drug release with the Pluronic/heparin nanocapsules, and the results will be reported soon.

Conclusions

In this study, novel Pluronic/heparin nanocapsules were successfully synthesized, and they exhibit thermo-responsive LCST properties. They exhibited a very sharp volume transition over a temperature range of 25∼33 °C and a reversible swelling and deswelling behavior when the temperature was cycled between 20 and 37 °C. The reversible volume transition of Pluronic nanocapsules in response to temperature was caused by micellization and demicellization of grafted and cross-linked Pluronic polymer chains pendant on the nanocapsule structure. The TEM analysis showed that the Pluronic/heparin nanocapsules had a hollow interior structure with a surrounding shell layer below the critical temperature. The SANS result indicated that Pluronic/heparin nanocapsules have a core−shell structure above the critical temperature. Pluronic/heparin nanocapsules can be potentially utilized to a wide range of targeted drug delivery applications for anti-cancer agents, proteins, peptides, genes, and imaging agents.

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