# Temperature-Sensitive Pluronic/Poly(ethylenimine) Nanocapsules for Thermally Triggered Disruption of Intracellular Endosomal Compartment

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Pluronic hydrogel nanoparticles cross-linked with poly(ethylenimine) (PEI) were synthesized by a modified emulsification/solvent evaporation method. Pluronic F-127 preactivated at the terminal group with *p*-nitrophenyl chloroformate was dissolved in dichloromethane, and the organic solution was emulsified in deionized water containing PEI by sonication. Primary amine groups of PEI in the aqueous phase were conjugated and/or cross-linked with activated Pluronic F-127 in the vicinity of the water/dichloromethane interface, resulting in the formation of shell-cross-linked Pluronic/PEI nanocapsules. Pluronic/PEI nanocapsules exhibited a volume transition behavior over a temperature range of 24−33 °C. The thermally reversible swelling/deswelling of Pluronic/PEI nanocapsules was caused by temperature-dependent hydrophobic interaction of cross-linked and/or grafted Pluronic polymer chains in the nanocapsules. Pluronic/PEI nanocapsules were utilized to break up intracellular endosomal compartments by swelling-induced destabilization of the endosomal membrane triggered by a cold-shock treatment.

## Introduction

Nanoparticulate polymeric drug carriers have attracted significant interest in the past decade.[1−3] Various polymeric nanoparticles with diverse morphological characters have been synthesized and utilized for target-specific drug delivery, gene therapy, and diagnostic applications including molecular imaging.[4−6] Polymeric hydrogel nanoparticles, in particular, have recently been synthesized by dispersion polymerization or inverse microemulsion polymerization with various ionic/nonionic vinyl monomers.[7,8] An example of a polymeric hydrogel nanoparticle is poly(ethylene glycol)-*cross-linked(cl)*-poly(ethylenimine) (PEO-*cl*-PEI) nanogel (40−300 nm in diameter) prepared by a modified emulsification/solvent evaporation method.[9] These cationic nanogels were utilized for intracellular delivery of negatively charged bioactive drugs such as plasmid DNA and oligonucleotides.[10,11]

Thermosensitive hydrogels exhibiting a lower critical solution temperature (LCST) behavior in an aqueous solution have received much attention recently. Particularly, poly(*N*-isopropylacrylamide, NIPAAm)-based hydrogels have been extensively investigated for their unique temperature-dependent volume transition over a narrow temperature range (31−32 °C).[12−16] More recently, temperature/pH dual-responsive colloidal nanogels were also synthesized by combining poly(NIPAAm) with a pH-sensitive polymer in the core and shell structure, respectively.[17] Pluronics are a series of amphiphilic triblock copolymers composed of poly(ethylene oxide)−poly(propylene oxide)−poly(ethylene oxide) (PEO−PPO−PEO). A distinctive feature of Pluronic block copolymers is its ability to self-assemble in aqueous solution into multimolecular aggregates such as spherical, rodlike, or lamellar structures in a temperature-dependent manner. The size and morphology of Pluronic copolymer aggregates depend on polymer concentration, environmental effects, and hydrophilic/lipophilic balance (HLB). The HLB values of these polymers can be altered by varying their molecular weight and PPO/PEO composition. A number of Pluronic copolymers have been shown to associate in aqueous solution to form micelles consisting of a PPO core and a hydrated PEO corona structure.[18−21] For most Pluronic copolymers, the critical micelle temperature (CMT) values range from ca. 25 to 40 °C, above which they self-assemble to form a spherical micellar structure by dehydration of the PPO middle block within the structure. At concentrations above about 25% (w/v), the Pluronic copolymers exhibit a sol−gel transition behavior around CMT.[22,23] We previously reported that Pluronic-based nanocapsules were prepared using heparin as a shell-cross-linking agent.[24] The Pluronic/heparin nanocapsules had a hollow interior surrounded by a cross-linked Pluronic/heparin shell layer. They exhibited a large extent of volume transition over the temperature range of 25−33 °C.

One of the most useful cationic polymers in gene delivery is poly(ethylenimine) (PEI).[25,26] The branched form of PEI contains 1°, 2°, and 3° amines and exhibits high buffering capacity over an entire pH range. When PEI is mixed with plasmid DNA or oligonucleotides, they immediately form compact polyelectrolyte complexes due to electrostatic interactions between PEI and DNA.

In the present work, thermosensitive cross-linked Pluronic/PEI nanocapsules having a diameter of about 100 nm were produced for intracellular and cytosol-specific delivery of various bioactive agents. Pluronic F-127 preactivated at the terminal with an amine-specific reactive group was dissolved in a dichloromethane phase, which was emulsified under sonication conditions in an aqueous phase containing PEI. The resultant Pluronic/PEI nanocapsules were characterized with an emphasis on thermal volume transition. The morphological features of Pluronic/PEI nanocapsules were studied using transmission electron microscopy (TEM), and their swelling/deswelling characteristics were also evaluated by a spectrofluorescent technique. It was hypothesized that cationic Pluronic/

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PEI nanocapsules could be easily taken up by cells via endocytosis, localized within endosomes, and readily released into the cytosol by an endosomal break-up through temperature-dependent volume transition of the nanocapsules. The destabilization and disruption of the endosomal membrane is known to be a key barrier for intracellular delivery of many bioactive agents including genes and anti-cancer drugs. This study proposes a novel delivery method for cytosol-targeting drugs using nanoscale hydrogel particles that exhibit thermally triggered volume transition behaviors.

## Materials and Methods

**Materials.** Pluronic F-127 (average $M_w$ 12 600) was obtained from BASF Corp. (Parsipanny, NJ) and used without additional purification. Poly(ethylenimine) ($M_w$ 2000, 50 wt % solution in water), pyrene, *p*-nitrophenyl chloroformate (*p*-NPC), and fluorescein isothiocyanate (FITC) were purchased from Sigma-Aldrich Corp. (St. Louis, MO). LysoTracker Red DND-99 was purchased from Molecular Probes (Eugene, OR). All other chemical reagents were of analytical grade.

**Methods.** *Preparation of Activated Pluronic F-127.* A 2 g amount of Pluronic F-127 was completely dried and dissolved in 6 mL of anhydrous benzene. The solution was slowly added to a stirred solution of 6 mL of 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 ($\epsilon = 1.7 \times 10^4$ $M^{-1}$ $cm^{-1}$).[24]

*Synthesis of Pluronic/PEI Nanocapsules.* Pluronic/PEI nanocapsules were synthesized using a modified emulsification/solvent evaporation method.[11,24] A dichloromethane solution (200 $\mu$L) containing activated Pluronic F-127 (10%, 20%, and 30% w/v) was added dropwise to an aqueous solution (2 mL, pH 9) of PEI (7.5% w/v). The mixture solution was sonicated in a Branson sonifier 450 (20 kHz, duty cycle = 40, output control = 2.5) for 3 min. The oil-in-water emulsion solution was transferred to a rotary evaporator. Residual solvent was removed at 30 °C until the solution became clear. After neutralizing with hydrochloric acid, the solution was dialyzed by a Spectra/Por dialysis membrane $M_w$ cutoff 50 000 against water at pH 4.0.

*Determination of the Pluronic/PEI Ratio in Nanocapsules.* $^1$H NMR data was obtained using a Brucker AVANCE 400 NMR operating at 400.13 MHz using $D_2O$ solvent containing 0.03% (v/v) tetramethylsilane (TMS). Chemical shifts were measured in ppm using TMS as an internal reference.

*Particle Size and Zeta-Potential Measurements.* The effective diameter and surface $\zeta$-potential value of the Pluronic/PEI 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 a 90° detection angle. The concentration of the nanocapsules was 10 mg/mL in a phosphate-buffered saline solution (pH 7.4), and the temperature was controlled. The measurement was carried out in triplicate.

*Transmission Electron Microscopy (TEM).* For TEM, 0.2% (w/v) nanocapsule solution was preequilibrated at 20 or 37 °C and 1 drop of the solution was dried on a Formvar/carbon support grid with 300 mesh for 2 min. To improve images, nanocapsule specimens were stained for 1 min with 1 drop of 2% (w/v) uranyl acetate solution. Negatively stained samples were analyzed using a Zeiss Omega 912 TEM (Carl Zeiss, Oberkochen, Germany) electron microscope.

*Critical Micelle Temperature of Pluronic/PEI Nanocapsules.* The critical micelle temperature (CMT) was measured by a fluorescence probe technique using pyrene as described previously.[24,27] Pyrene dissolved in acetone was added to deionized water to a concentration of $1.2 \times 10^{-6}$ M, and acetone was subsequently removed under reduced pressure for 3 h at room temperature. The final concentration of pyrene was adjusted to $6.0 \times 10^{-7}$ M. The concentration of the nanocapsules was 10 mg/mL, and the temperature was varied from 12 to 45 °C. The combined mixture of pyrene solution and nanogel solution was equilibrated at each temperature for 30 min in a dark room. Fluorescent spectra were monitored using a spectrofluorophotometer (Shimadzu, RF-5301PC, Kyoto, Japan) at an excitation and emission wavelength of 339 and 390 nm, respectively.

*Cell Culture.* HeLa (human epithelial carcinoma cell line) cells were cultured in DMEM supplemented with 10% FBS, penicillin G sodium (10 U/mL), streptomycin sulfate (10 $\mu$g/mL), and amphotericin B (25 ng/mL). Cells ($1 \times 10^5$ cells/well) were placed in a four-chamber culture slide (BD Biosciences, MA) in 1.0 mL of DMEM with 10% FBS and cultivated at 37 °C in a humidified 5% $CO_2$ incubator. After 24 h, the cell culture medium was replaced with serum-free medium prior to addition of FITC-labeled Pluronic/PEI nanocapsules and LysoTracker Red DND-99. FITC was labeled onto the nanocapsules according to a previous method.[28] The cells were incubated with 0.4 mL of 50 $\mu$g/mL nanocapsules and 50 nM LysoTracker RED in DMEM solution for 30 min at 37 °C. After incubation, the cells were washed three times with prewarmed PBS in a 37 °C water bath. The cells for cold-shock treatment were incubated for 15 min at 20 °C. Intracellular images of FITC-labeled nanocapsules and endosomes were examined by a confocal laser scanning microscope (LSM 510, Carl-Zeiss Inc.) with argon laser (excitation wavelength, 488 nm) and HeNe laser (excitation wavelength, 543 nm).

## Results and Discussion

Temperature-sensitive nanocapsules were synthesized by conjugating and cross-linking branched PEI ($M_w$ 2000) with *p*-NPC-activated Pluronic F-127. A schematic representation of Pluronic/PEI nanocapsules is described in Figure 1. The organic phase, *p*-NPC-activated Pluronic F-127 solution in dichloromethane, was ultrasonically emulsified in an aqueous phase containing PEI. The branched PEI used in this study had 22.1% primary amine group in the backbone structure as characterized by $^{13}$C NMR spectroscopy.[29] During sonication the primary amine groups of PEI reacted with the activated terminal groups in the Pluronic triblock copolymer in the vicinity of the interface between organic and aqueous phases, resulting in shell cross-linking.

Pluronic/PEI nanocapsules prepared at a Pluronic concentration of 30% (w/v) exhibited a volume transition behavior with increasing temperature as shown in Figure 2 A. The nanocapsules showed a temperature-dependent volume change over the temperature range of 20−37 °C. The average diameter was decreased from 332.5 ± 65.4 to 95.2 ± 8.8 nm with increasing temperature from 20 to 37 °C, which represents a 42.7-fold volume change (see the DLS results in Figure 2B and 2C). The volume transition was fully reversible when the temperature was cycled between 20 and 37 °C, which was similarly observed for the Pluronic/heparin nanocapsules (data not shown). In our previous study, de-*N*-sulfated heparin was used instead of PEI to prepare Pluronic/heparin nanocapsules using a similar method.[24] The Pluronic/heparin nanocapsules changed the diameter from 335.7 ± 42.4 to 32.4 ± 1.9 nm with about a 1000-fold volume transition over the same temperature range. The dramatic change of volume was attributed to the hollow morphology and the hydrophobic interaction of cross-linked/grafted Pluronic polymer chains in response to increasing temperature. It was previously shown that a fragile and soft shell layer of Pluronic/heparin nanocapsule was completely collapsed to produce a monolithic core/shell micelle when

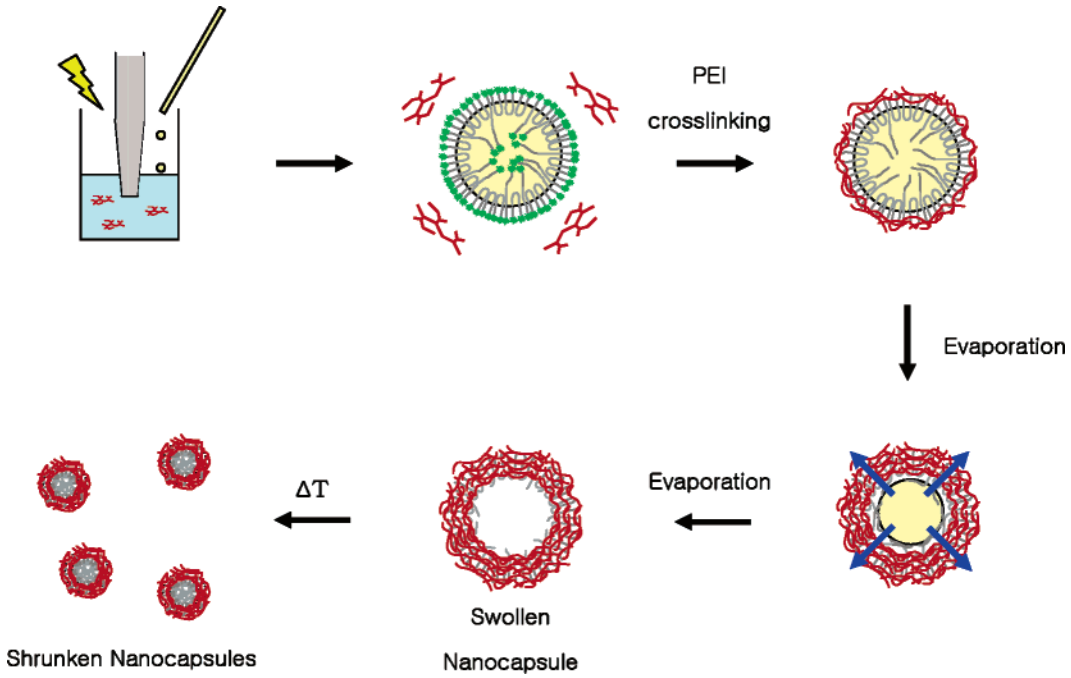


**Figure 1.** Schematic diagram for the preparation of Pluronic/PEI nanocapsules.

raising the temperature above the LCST. In the current study, Pluronic/PEI nanocapsules showed a relatively smaller extent of volume transition than that of Pluronic/heparin nanocapsules. The difference in the extent of volume transition was possibly caused by the different shell cross-linking density and the high positively charged nature of PEI chains within the shell layer. Heparin is only soluble in the aqueous phase, so the cross-linking reaction of heparin with Pluronic copolymers occurred predominantly at the interface between organic and aqueous phases. This would lead to production of nanocapsules having a hollow interior cavity surrounded by a thin Pluronic/heparin shell layer. On the other hand, Pluronic/PEI nanocapsules were likely to have a relatively thicker shell layer than Pluronic/heparin nanocapsules. Because PEI is soluble in both organic and aqueous phases, PEI could diffuse into the organic phase, while the activated Pluronic copolymers simultaneously diffused out into the aqueous phase during the emulsification and solvent evaporation step. Penetration of PEI into the organic phase would result in formation of a dense and thick Pluronic/PEI cross-linked shell layer. Consequently, a nanocapsule structure with a thicker Pluronic/PEI outer layer was formed compared to that of Pluronic/heparin nanocapsules. Additionally, the Pluronic/PEI shell had a much higher charge density than the Pluronic/heparin shell due to the presence of a more highly charged branched PEI. Thus, the Pluronic/PEI nanocapsules were likely to exhibit a smaller change in diameter and a lesser extent of volume transition than the Pluronic/heparin nanocapsules. The change in surface zeta potential values as shown in Figure 2A provides direct evidence of an increase in charge density with increasing temperature. This was obviously caused by the collapse of highly charged nanocapsules with temperature. The surface charge of the Pluronic/PEI nanocapsules greatly increases from 11.7 ± 3.1 mV at 20 °C to 44.2 ± 7.0 mV at 37 °C. Becoming more positively charged on the surface with increasing temperature resulted in stronger charge repulsions between PEI chains, which was likely to hamper collapse of the Pluronic/PEI shell layer. In other words, hydrophobic interaction of the PPO middle block within the Pluronic triblock copolymer was likely to be counter-balanced with the charge repulsion of strongly cationic PEI in the Pluronic/PEI shell layer. As a result, the size of shrunken Pluronic/PEI nanocapsules at 37 °C was larger than the size of a single Pluronic micelle, ~30 nm.[24]

In Figure 3A the excitation spectra of pyrene are shown at various temperatures. The band shifts in pyrene excitation spectra occurred as a result of partitioning pyrene into the micellar hydrophobic core, which was used to determine the critical micelle temperature (CMT) of Pluronic/PEI nanocapsules.[27] In Figure 3B it can be seen that the fluorescence intensity ratio begins to increase considerably from a critical temperature, indicating partitioning of pyrene into the micellar hydrophobic core. The CMT value was about 21 °C as determined from the reflection point in Figure 3B. The CMT value was exactly matched with the temperature at which the effective diameter of nanocapsules started to decrease, as shown in Figure 2A. This reveals that the thermal collapse of Pluronic/PEI nanocapsules was caused by increased hydrophobic interaction between Pluronic copolymers cross-linked or grafted in the shell layer. It is well known that Pluronic copolymers self-associate in aqueous solution to form spherical micelles above the critical micelle concentration and critical temperature. The PPO middle block hydrophobically interacts to form an inner core, while the two PEO side blocks form a surrounding corona. Likewise, Pluronic/PEI nanocapsules were collapsed and shrunken with increasing temperature above 21 °C, most probably due to self-association of cross-linked Pluronic copolymers in the shell layer.[24]

Figure 4 shows effective diameters of Pluronic/PEI nanocapsules prepared with changing Pluronic concentration in the organic phase. The structural properties of the Pluronic/PEI nanocapsules described in Figure 4 are summarized in Table 1. As the Pluronic concentration increased, the size of swollen Pluronic/PEI nanocapsules was increased accordingly. At 20 °C, the average diameter of swollen Pluronic/PEI nanocapsules prepared at Pluronic concentrations of 10%, 20%, and 30% (w/v) was 201.8 ± 23.1, 267.2 ± 67.2, and 332.5 ± 65.4 nm, respectively. Increasing the concentration of activated Pluronic in the organic phase might lead to more rapid diffusion of Pluronic copolymer into the aqueous phase, which could produce larger nanocapsules. At 37 °C, the collapsed Pluronic/PEI

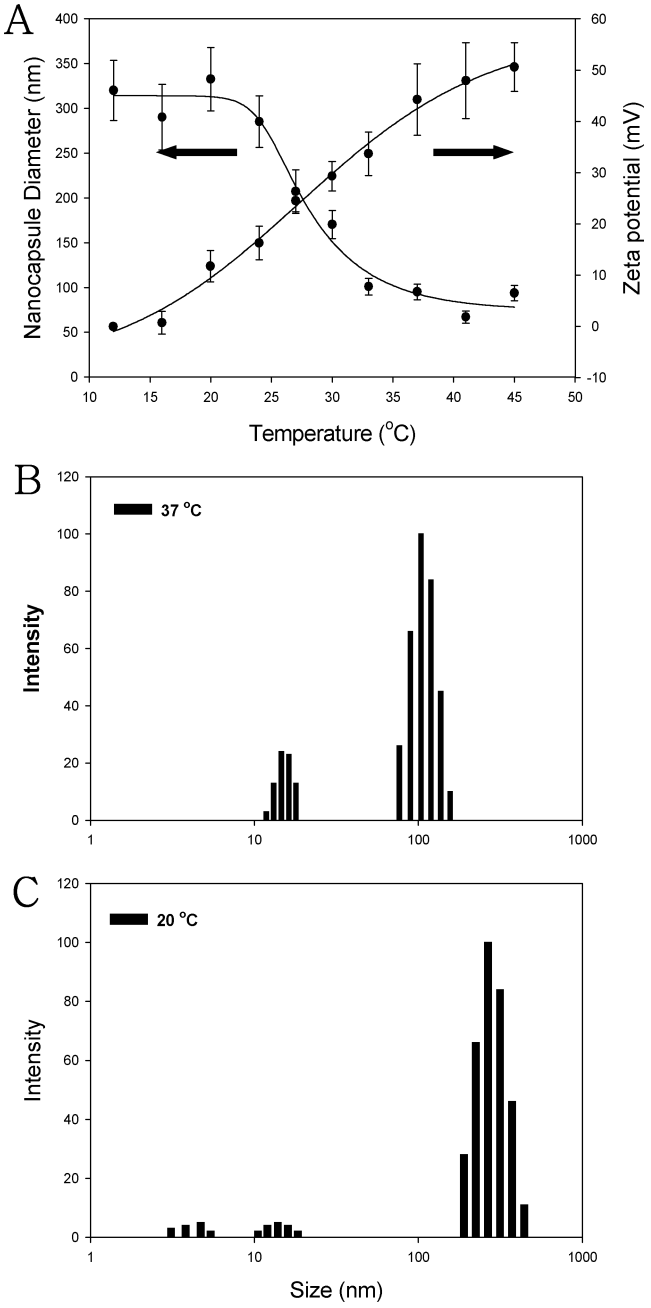


**Figure 2.** (A) Size and surface charge values for Pluronic30/PEI nanocapsules measured by DLS and a zeta potential analyzer over a temperature range of 13−45 °C. (B and C) DLS profiles of Pluronic30/PEI nanocapsules at 20 and 37 °C, respectively.

nanocapsules prepared with 10%, 20%, and 30% (w/v) Pluronic concentrations were 88.2 ± 7.5, 94.1 ± 23.3, and 95.2 ± 8.8 nm, respectively. The amount of PEI within the nanocapsules was 12.0%, 17.3%, and 22.9% (w/w), on the basis of dry weight as determined by $^1$H NMR, at Pluronic concentrations of 10%, 20%, and 30% (w/v), suggesting that Pluronic/PEI cross-linking density in the shell and shell thickness could be controlled by increasing Pluronic concentration in the formulation.

The morphological characteristics of Pluronic/PEI nanocapsules were expected to be different from those of previously reported Pluronic/heparin nanocapsules that were cross-linked only at the interface. Figure 5 shows transmission electron microscopy (TEM) pictures of Pluronic/PEI nanocapsules prepared at a Pluronic concentration of 30% (w/v) taken after equilibrating above and below the CMT. The average sizes of nanocapsules at 20 and 37 °C were 350.6 ± 59.3 and 135.3 ± 15.3 nm, respectively, which are well corroborated with the results shown in Figure 2. Each nanocapsule exhibited an aqueous fluid-filled hollow interior with a surrounding cross-linked Pluronic/PEI shell layer (~100 nm) at 20 °C. The shell structure was likely to be composed of a Pluronic cross-linked PEI network. It can be visualized that a reservoir-type nanocapsule structure is still retained at 37 °C (Figure 5B), which exhibits a fluid-filled interior with a surrounding thick shell layer even at high temperatures above the CMT. The TEM pictures additionally support that highly charged Pluronic/PEI nanocapsules were more resistant from collapse above the CMT, as compared to Pluronic/heparin nanocapsules that had a less charged and thinner shell layer (~70 nm).

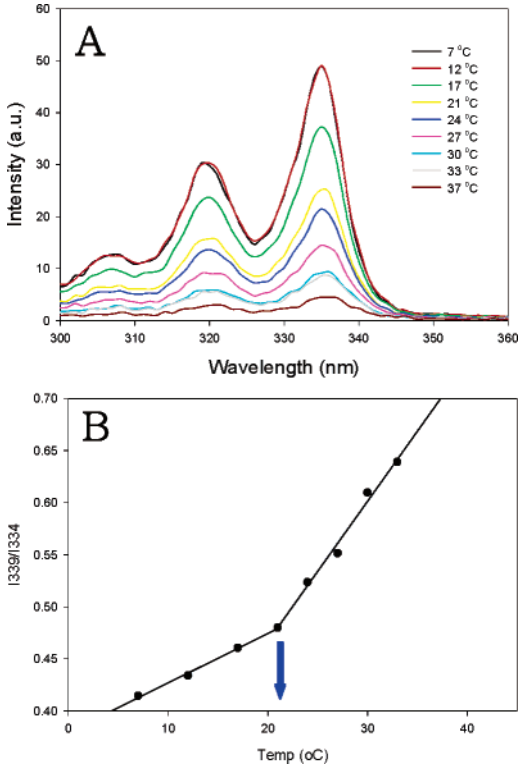


**Figure 3.** Excitation spectra (A) and intensity ratio ($I_{339}/I_{334}$) of pyrene in the excitation spectra (B) according to the change of temperature.

Cellular uptake and localization of Pluronic/PEI nanocapsules in HeLa cells were examined by laser confocal fluorescent microscopy. Cationic nanoparticular substances are taken up by cells mostly by endocytosis and sequestered within endosomes.[25] It was postulated that temperature-sensitive hydrogel nanoparticles such as Pluronic/PEI nanocapsules could be utilized as novel disruptive agents of the endosomal membrane for releasing their cargo into the cytoplasm. After endocytosis, a temperature drop below the CMT may enable the shrunken Pluronic/PEI nanocapsule to abruptly expand within a restricted volume of the early endosome normally having a critical size of about 150−200 nm.[30,31] It is conceivable that endocytosed Pluronic/PEI nanocapsules within the endosome compartment were initially in a collapsed state at 37 °C but rapidly swelled by a cold shock, resulting in the break-up of endosomal membrane by a physical swelling force. For intracellular delivery of various bioactive agents using nanoparticulate carriers, early destabilization, and disruption of endosome membrane are highly desirable to transport their cargos into the cytosol. Otherwise, the bioactive agents travel through a series of subcellular organelles and eventually degrade in the lysosome compartment.[32] In these experiments, FITC-labeled Pluronic/PEI nanocapsules and LysoTracker RED were used to visualize

**Table 1.** Structural Properties of Pluronic/PEI Nanocapsules

| | feed ratio (Pluronic/PEI, mg) | shrunken size (at 20 °C, nm) | swollen size (at 37 °C, nm) | swelling ratio | PEI (% w/w) in nanocapsule |
|---|---|---|---|---|---|
| Pluronic10/PEI | 20/15 | 88.2 ± 7.5 | 201.8 ± 23.1 | 14.8 | 12.0 |
| Pluronic20/PEI | 40/15 | 94.1 ± 23.3 | 267.2 ± 67.2 | 22.9 | 17.3 |
| Pluronic30/PEI | 60/15 | 95.2 ± 8.8 | 332.5 ± 65.4 | 42.7 | 22.9 |

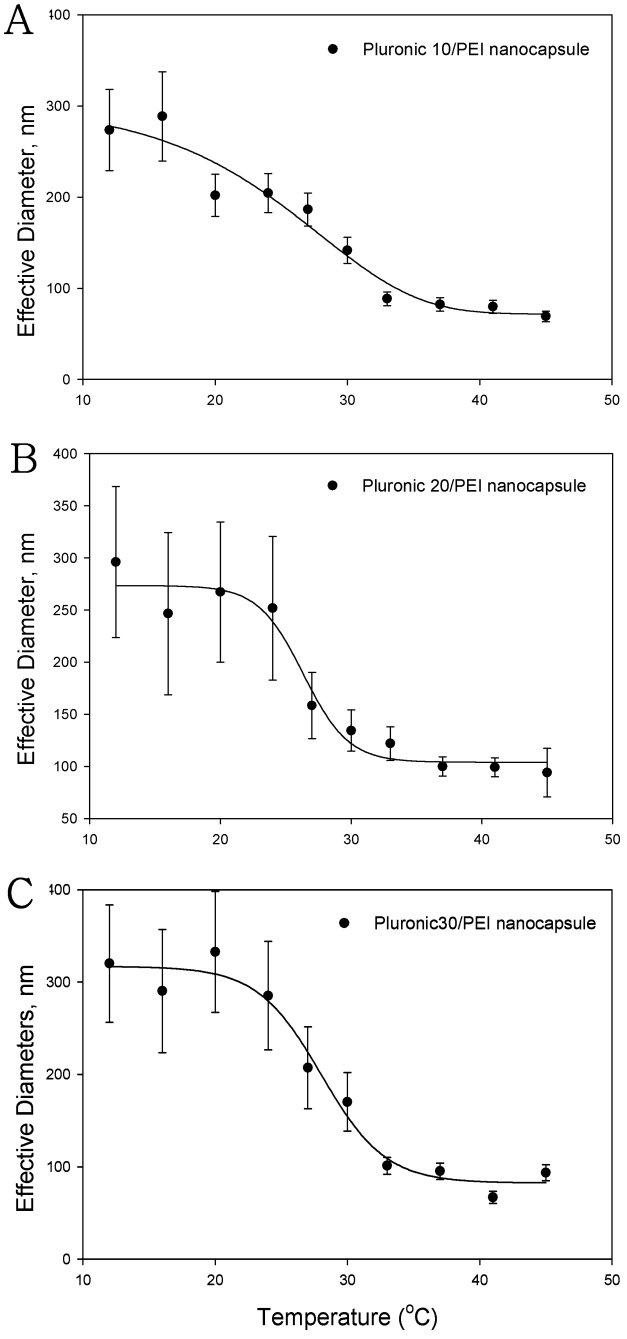


**Figure 4.** Size change profiles of Pluronic/PEI nanocapsules over a temperature range of 13−45 °C: (A) 10% (w/v) Pluronic concentration in dichloromethane, (B) 20% (w/v) Pluronic concentration, and (C) 30% (w/v) Pluronic concentration.

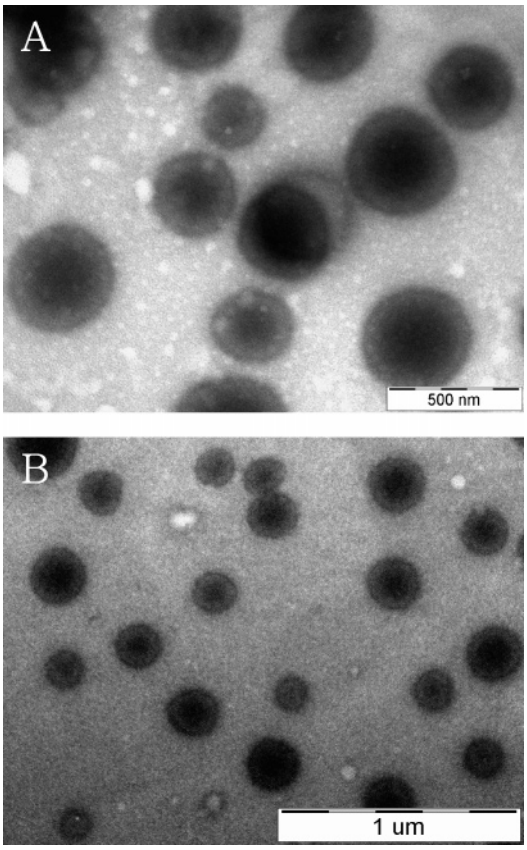


**Figure 5.** Size change of Pluronic30/PEI nanocapsules below and above a critical temperature: TEM image at (A) 20 and (B) 37 °C.

nanocapsules and acidic subcellular organelles such as endosomes and lysosomes, respectively. Figure 6A−D shows typical confocal images of endocytosed Pluronic/PEI nanocapsules. After incubation at 37 °C for 30 min, Pluronic/PEI nanocapsules were located predominantly at endosome compartments within HeLa cells. Figure 6B shows the localization of FITC-labeled Pluronic/PEI nanocapsules. The scattered green spots indicate the position of endocytosed FITC−Pluronic/PEI nanocapsules that were in a collapsed state. Figure 6C shows the location of endosomes and lysosomes stained by LysoTracker RED. The LysoTracker probes are freely permeable to cell membrane and accumulate within the cellular compartments with low internal pH. Figure 6D is a superimposed image of Figure 6B and 6C in which both spots of the subcellular organelles and the collapsed nanocapsule are clearly overlapped, presenting yellow-colored spots. This means that the Pluronic/PEI nanocapsules and endosomes were co-localized at 37 °C. It is likely that the endocytosed nanocapsules are still within early or late endosomes without further traveling to lysosomes because the confocal images were obtained 30 min after transfection.[33,34] The right panels in Figure 6E−H show images of HeLa cells after a cold-shock treatment at 20 °C for 15 min. Figure 6F shows the localization of swollen FITC-labeled Pluronic/PEI nanocapsules in which the green spots were smeared and the cytoplasm was evenly stained with a faint green color. In Figure 6G, in contrast to Figure 6C, the cytoplasmic area was also stained with LysoTracker RED to a much lesser extent. In the superimposed image in Figure 6H it is clear that the nanocapsules and endosomes were not co-localized any more after the cold shock because the nanocapsules were released in the cytosolic area. When fluorescently labeled polystyrene latex particles or PEI/DNA polyplexes were used, it was found that

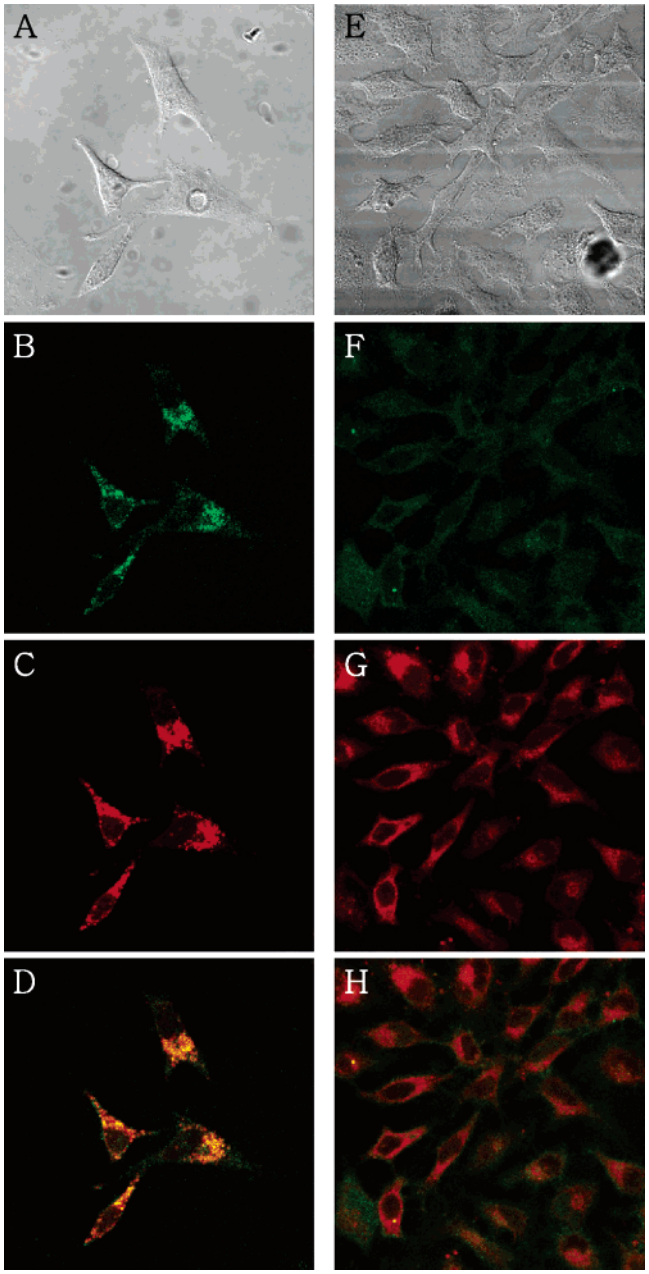


**Figure 6.** Confocal fluorescent microscopy images of HeLa cell line after incubation for 1 h with FITC-labeled Pluronic30/PEI nanocapsules and LysoTracker RED. (A and E) Optical microscopic images of HeLa cells; (B and F) images of FITC-labeled Pluronic/PEI nanocapsules after transfection; (C and G) images stained with LysoTracker RED; (D) superposed image of B and C; (H) superimposed image of F and G. A−D were incubated at 37 °C and no cold-shock treatment. E−H were cold-shock treated at 20 °C for 15 min.

there was no detectable change in their intracellular distribution before and after the cold-shock treatment, revealing that the temporal cold shock for the nanoparticles not having thermosensitivity did not elicit disruption of the endosome compartment. The confocal images strongly suggest that the endosomes in HeLa cells could be physically disrupted by an abrupt swelling of Pluronic/PEI nanocapsules triggered by the cold-shock treatment.

It should be noted that the Pluronic/PEI nanocapsules also exhibited pH-dependent volume transition as well since they have positively charged amine groups in the shell layer. Since PEI has a broad range of p$K_a$ values due to the presence of primary, secondary, and tertiary amine groups, the Pluronic/PEI nanocapsules would show a pH-dependent volume transition over an entire pH range. In this study, the temperature-dependent sizes of Pluronic/PEI nanocapsules were determined at pH 7.4. In the endosomal compartment having a more acidic environment (pH $\approx$ 5.5), the size would be larger than that determined at pH 7.4 due to protonation of the amine groups in the PEI-enriched shell layer. The dual-pH/temperature sensitivity will be separately reported soon elsewhere.

In view of these results, it may be conceptually possible that by controlling temperature cytosol-specific delivery of various bioactive agents including anti-cancer drugs could be achieved using Pluronic/PEI nanocapsules. At 37 °C, shrunken nanocapsules around 100 nm in size could be accumulated in the tumor region by the enhanced permeation and retention (EPR) effect (passive targeting).[35] After being endocytosed within cancerous cells, Pluronic/PEI nanocapsules would be transported primarily into the endosomal compartment. By a brief cold-shock treatment below the CMT, the efficient cytosolic delivery of a drug could be achieved by a sudden swelling of nanocapsules that could physically destabilize and rupture the enclosed endosomal membrane. Furthermore, cell recognizable ligands such as folate could be conjugated to the PEI in the shell layer to promote the extent of cellular uptake with cell specificity.[36,37]

However, a thermally-controlled endosome destabilization strategy will be more useful and realistic for in vitro and ex vivo gene delivery systems for plasmid DNA, antisense oligonucleotides, and small interfering RNA (siRNA) in which the cold-shock strategy could be practically applied to transfected cells. In our preliminary study we indeed observed far enhanced gene silencing effect of vascular endothelial growth factor (VEGF) expression in tumor cells after a cold shock when VEGF siRNA was delivered with Pluronic/PEI nanocapsules.

## Conclusions

In this study temperature-responsive Pluronic/PEI nanocapsules were successfully synthesized and exhibited thermoresponsive swelling/deswelling properties. The Pluronic/PEI nanocapsules exhibited a volume transition behavior over the temperature range of 20−37 °C. The size of nanocapsules was about 100 nm at 37 °C and about 330 nm at 20 °C. The volume transition extent of Pluronic/PEI nanocapsules was controlled by changing the concentration of Pluronic copolymer. TEM analysis showed that the Pluronic/PEI nanocapsule had a hollow interior structure with a surrounding shell layer below and above the critical temperature. Pluronic/PEI nanocapsules could be delivered intracellularly within HeLa cells. The endocytosed Pluronic/PEI nanocapsules broke the endosome compartments when a cold-shock treatment was given below the CMT. The Pluronic/PEI nanocapsules are expected to be useful delivery carriers for anti-cancer agents including antisense oligonucleotide and siRNA.

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