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Research paper

Pluronic decorated-nanogels with temperature-responsive volume transitions, cytotoxicities, and transfection efficiencies

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ABSTRACT

DNA nanogels were prepared by chemically conjugating Pluronic to the surface of cationic polymer/DNA complex in order to prepare thermo-responsive nanogels with endosomal disrupting abilities. Amine-reactive Pluronic was prepared by activating hydroxyl groups of Pluronic and subsequently reacted with pre-formed PEI/DNA complex. The conjugation process was monitored by measuring liberated nitrophenyl groups during the conjugation reaction. The properties of the nanogels (size and ζ-potential) changed significantly when temperature was increased from 20 to 37 °C. The multimodal size distribution of the nanogel also confirmed the variable sizes and distribution of the nanogel upon changing temperatures. Electron microscopy and atomic force microscopy also confirmed the modulated morphologies and sizes of the thermo-responsive nanogel. Confocal microscopy revealed that the nanogel disrupted lysosomes and endosomes at low temperatures, thus confirming endosomal disrupting abilities. The survival rates results showed that the cytotoxicities of the nanogel increased as the temperature decreased from 37 to 20 °C, showing that collapsed Pluronic chain played a role in modulating cytotoxicities. *In vitro* transfection efficiencies of the nanogel were also measured in NIH3T3 cells. Transfection efficiencies increased as the temperature decreased to 20 °C, thus confirming that endosomal disruptions played a significant role in increasing transfection efficiencies.

1. Introduction

Since non-viral gene delivery strategy was first introduced, many cationic polymers have been employed to prepare nano-scaled nucleic acid/polymer complex [1]. Among those polymers, poly(ethylene imine) [PEI] has been widely employed as a DNA-compacting molecule because of high cationic charges and high transfection efficiency. Upon contact with DNA, PEI instantly condenses bulky DNA into a nano-sized complex ranging from 50 to 200 nm by an ionic interaction between amine groups in PEI and phosphates groups in DNA backbones [2,3]. In addition, many studies employed sophisticated strategies to further improve the transfection efficiency of PEI/DNA complex. Incorporating fusogenic peptides to PEI/DNA complex was one of the strategies to overcome an intracellular barrier during the transfection processes [4]. Fusogenic peptides including KALA have been known to accelerate the escape of cationic polymer/DNA complexes from endosomes to the cytosolic area by disrupting endosomes. Because endosomal escape into the cytosol is a major step toward nuclear targeting of the plasmid, fusogenic peptide of the influenza virus was employed to promote the transfection efficiency of Lipofectamine, and a high level of gene delivery was obtained at a very low dose of the membrane-destabilizing peptide [5]. In another study employing poly(L-lysine)/DNA complex, the conjugated KALA peptide significantly enhanced *in vitro* transfection efficiency.

Pluronic, a triblock copolymer composed of poly(ethylene oxide)[PEO]-poly(propylene oxide)[PPO]-[PEO] has been widely applied to drug delivery for its unique thermo-sensitive gelation property. Above lower critical solution temperature (LCST), it forms physical gels by a hydrophobic interaction between hydrophobized PPO blocks in Pluronic. Many studies employed this thermo-gelation property to control the release of many bioactive molecules in response to temperature modulations. Among those, Pluronic was conjugated to PEI to prepare thermo-sensitive gene carriers in an aim to modulate transfection efficiencies according to temperature changes [6]. In another stimuli-responsive strategy, a nano-sized gel was prepared to solubilize DNA in organic solvents [7]. They employed disulfide linkages between thiol groups of branched sulfhydryl poly(ethyleneglycol) to render stimuli-responsiveness in response to external reducing conditions.

In the current study, we chemically modified PEI/DNA complex with amine-reactive Pluronic to develop a thermo-responsive

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nanogel. Pluronic was chemically conjugated on the surface of PEI/DNA complex, and the conjugation process was also investigated. Their thermo-responsive volume transition was monitored by dynamic light scattering, electron microscopy, and atomic microscopy. In addition, the endosomal disrupting ability of the nanogels was monitored within cells by confocal microscopy. Finally, the transfection efficiency of the modified PEI/DNA complex was evaluated *in vitro*.

2. Materials and methods

2.1. Materials

Pluronic F127 was obtained from BASF Corporation (Mount Olive, NJ). Linear Polyethylenimine (LPEI, Mw 25,000) was purchased from Polysciences Inc. (Warrington, PA). *p*-nitrophenyl chloroformate (*p*-NPC) was purchased from Sigma (St. Louis, MO). Dialysis membrane (MW cutoff = 50 kDa) was purchased from Spectrum (Houston, TX). The micro bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Rockford, IL). WST-1 reagent was purchased from Roche (Indianapolis, IN). Dulbecco's modified Eagle's medium (DMEM), streptomycin/penicillin, trypsin, fetal bovine serum (FBS), and all other cell culture supplements were purchased from Invitrogen (Carlsbad, CA).

2.2. Preparation of PEI/DNA-Pluronic F127

The PEI/DNA complex surface-modified with Pluronic F127 was prepared by conjugating activated Pluronic F127 to the residual amine groups of the PEI/DNA complex. The hydroxyl groups of Pluronic F127 were activated by *p*-NPC to prepared amine-reactive Pluronic F127. Pluronic F127 (2 g) was completely dried in a vacuum and dissolved in methylenechloride (6 ml). The solution was slowly added to methylenechloride (6 ml) containing *p*-NPC (192 mg). The reaction progressed at room temperature in a nitrogen atmosphere for 24 h, and the product was precipitated in ice-cold diethyl ether, recovered by filtration, and dried. The activated Pluronic F127 was conjugated on the surface of PEI/DNA complexes (nitrogen/phosphate ratio = 8) at 5% or 10% of free nitrogen content in PEI. PEI (104.2 μg) and plasmid DNA (100 μg) added to PBS (5 ml), and the solution was incubated for 20 min. After adjusting the pH of the solution to pH 8.2, the activated Pluronic F127 was slowly added to the PEI/DNA complex solution in a drop-wise manner and further reacted for 12 h. The molar ratios of the activated Pluronic F127 to the uncomplexed amine groups of the PEI/DNA complex were 0.05 and 0.1. In order to remove unreacted Pluronic F127, the reaction mixture was extensively dialyzed against phosphate-buffered saline (PBS) at pH 7.4 using a Spectra/Por 6 membrane (MW cutoff = 50,000). For FITC-labeled PEI/DNA complex, FITC in acetone at 5% or 10% of free nitrogen content in PEI was mixed with the Pluronic decorated complex.

2.3. Particle size and ξ-potential measurements

The effect of the conjugated Pluronic F127 on the particle sizes and surface charges of the PEI/DNA complex was investigated by measuring the hydrodynamic volumes and ξ-potential of the PEI/DNA complex modified with Pluronic F127 (PEI/DNA-Pluronic). The particle sizes and ξ-potential values of the PEI/DNA-Pluronic complexes were measured by dynamic light scattering (90Plus, Brookhaven Instrument, Holtsville, NY). After incubating the complex solution at 20 and 37 °C, the measurement was carried out using the thermo-static module of the instrument to keep the constant temperatures at 20 and 37 °C. The measurement was carried out in triplicate.

2.4. Atomic force microscopy (AFM)

The morphology of the PEI/DNA-Pluronic was examined by AFM using a contact mode, as previously described in the literature [6,8]. To prepare the samples for AFM, the PEI/DNA-Pluronic solution was dropped on a cover slip and dried. In order to examine the morphology of the PEI/DNA-Pluronic at different temperatures, one PEI/DNA-Pluronic was air-dried at 37 °C and the other sample was dried in a refrigerator at 4 °C. The completely-dried sample was placed on an atomic force microscope and examined using contact mode on a Multimode NanoScope IIIa at the Central Laboratory in Kangwon National University, Korea (Digital Instruments, Santa Barbara, CA).

2.5. Cryo-transmission electron microscopy (TEM)

For cryo-TEM images, the PEI/DNA-Pluronic complexes at 37 and 4 °C were incubated for 1 h in a water bath and refrigerator, respectively. After incubation, each PEI/DNA-Pluronic complex solution was slowly added to liquid nitrogen in a drop-wise manner, and the sample was freeze-dried. The sample was placed on a Formvar/carbon support grid with 200 mesh. The morphology of sample was examined using a High Resolution Transmission Electron Microscope (JEM-2011, JEOL, Japan)

2.6. Confocal laser microscopy

NIH3T3 cells at a density of 1×10^5 cells/well were plated in a 4-chambered culture slide in DMEM (0.5 ml) with 10% FBS. After 24 h, the cell culture medium was replaced with serum-free medium prior to the addition of FITC-labeled PEI/DNA-Pluronic and LysoTracker Red DND-99. The cells were incubated with PEI/DNA-Pluronic solution containing 6 μg/well of DNA and 50 nM of LysoTracker RED in serum-free DMEM solution for 30 min at 37 °C. After 30 min, the cells were washed three times with PBS. The cells to be used for a cold-shock treatment were incubated in 2.5% formaldehyde for 30 min at 20 °C. Intracellular images of FITC-labeled PEI/DNA-Pluronic complexes and endosomes were taken by a confocal laser microscope equipped with an argon laser (excitation wavelength = 488 nm) and a HeNe laser (excitation wavelength = 543 nm). Emission filters were set for detecting FITC and Lysotracker at 530 and 590 nm, respectively.

2.7. WST-1 assay

The cytotoxicities of the nanogel were assessed by incubating NIH3T3 cells with each nanogel. NIH3T3 cells at logarithmic growth phase were seed on 96 well culture plates at a density of 2×10^5 cells/ml in DMEM supplemented with 10% FBS, penicillin G sodium (10 U/ml) streptomycin sulfate (10 μg/ml). After 24 h, various amounts of the nanogel (plasmid DNA = 5 μg/well) were added to each well, and the cell was incubated at 20 or 37 °C for 6 h. After moving the nanogel into fresh medium, 10 μl of WST-1 solution (5 mg/ml) was added to each well. After 3 h at 37 °C, absorbance at 560 nm was measured using a microplate reader (Bio-Tek EL800, Winooski, VT). As a control group, cells with no treatment were incubated at each temperature, and survival rates were calculated with respect to the control groups.

2.8. In vitro transfection

NIH3T3 cells (Mouse embryonic fibroblast cell line) were cultured in DMEM supplemented with 10% FBS. The cells (2×10^5 cells/well) seeded on a 6-well tissue culture plate were cultured 24 h before transfection. After 24 h, the culture medium was exchanged with serum-free medium prior to the addition of

a PEI/DNA-Pluronic solution. Each PEI/DNA-Pluronic solution containing 5 μg of DNA was then added to each well and incubated for 3 h. Cells to be used for the cold-shock treatment were incubated for 15 min at 20 °C and were further incubated in a fresh complete medium for 45 h. After 48 h of incubation, cells were lysed using 200 μl of a Glo lysis buffer (Promega, Madison, WI). Luciferase activity was measured using a Steady-Glo luciferase assay system (Promega, Madison, WI), and the luminescence light was measured by a plate-type luminometer (Molecular Devices, CA). Peak light emission was measured at room temperature for 30 s. The luminescence units were normalized with respect to the protein content of the cells.

3. Results and discussion

Fig. 1 schematically shows the intracellular delivery of the PEI/DNA complex surface-modified with Pluronic. A major characteristic of this system is that Pluronic was chemically conjugated on the surface of PEI/DNA complex after DNA and PEI were fully complexed. Thus, the nanogel composed of PEI/DNA complex and Pluronic could be prepared without disturbing ionic complexation processes between PEI and DNA. The PEI/DNA-Pluronic complex significantly affected an intracellular delivery of nano-sized gene delivery vehicles compared to that without Pluronic because of a huge volume transition according to temperature modulations. Upon treating transfected cells with a short cold-shock (Fig. 1B), the endosomes inside the cells were disrupted by fully-extended Pluronic moieties of the PEI/DNA-Pluronic complex as well as proton sponge effects between PEI and DNA. On the other hand, the complex without Pluronic could escape from the endosomes only by proton sponge effects. This effect could be compared to endosomal disrupting effects of fusogenic peptides increasing transfection efficiencies of endocytosed complex. Compared to the similar study employing Pluronic grafted PEI, the surface-modification of the complex is advantageous in reducing cytotoxicities of Pluronic because smaller amount of Pluronic was enough for thermosensitive properties of the complex.

After preparing PEI/DNA complex, amine-reactive Pluronic reacted to the amine groups in the complex. The amount of amine-reactive Pluronic was determined based on the amount of free amine groups in PEI, which did not bind to DNA by ionic interactions. When nitrogen in PEI/phosphate in the DNA (N/P) ratio was kept at 8, 7 out of 8 amine groups were considered as free amine groups in the complex. In order to validate the reaction ratios between amine-reactive Pluronic and PEI/DNA complex, the molar ratio of amine-reactive Pluronic to free amine groups in the PEI/DNA complex changed from 0% to 200% (Fig. 2). As the molar ratio increased, the liberated *p*-nitrophenyl groups also increased, confirming that the number of conjugated Pluronic also increased. However, when the ratio increased to over 100%, the reaction mixture did not generate any more nitrophenyl groups, which showed that no more amine-reactive was conjugated to the PEI/DNA complex. This result clearly confirmed that 7 out of 8 amine groups in the complex were free to react with amine-reactive Pluronic in accordance with our hypothesis. For the remaining study, we, therefore, employed a reaction ratio of 5% and 10% to prepare PEI/DNA complex with Pluronic.

The thermo-responsive properties of the PEI/DNA complex with Pluronic on the surface were monitored by dynamic light scattering, as shown in Table 1. The PEI/DNA complex surface-modified with Pluronic exhibited significant size modulation behaviors according to temperature changes while unmodified PEI/DNA complex showed no changes in diameters. At 20 °C, the diameters of the modified PEI/DNA complex with 5% and 10% of Pluronic were approximately 1 μm while that of PEI/DNA complex was 265 nm. At 37 °C, however, the diameter and volume of PEI/DNA complex with 5% Pluronic dramatically decreased by 4.3- and 79.5-fold, respectively. This result clearly demonstrated that conjugating Pluronic on the surface rendered a significant thermo-sensitivity to the complex, and only 5% of Pluronic was enough to prepare a ther-

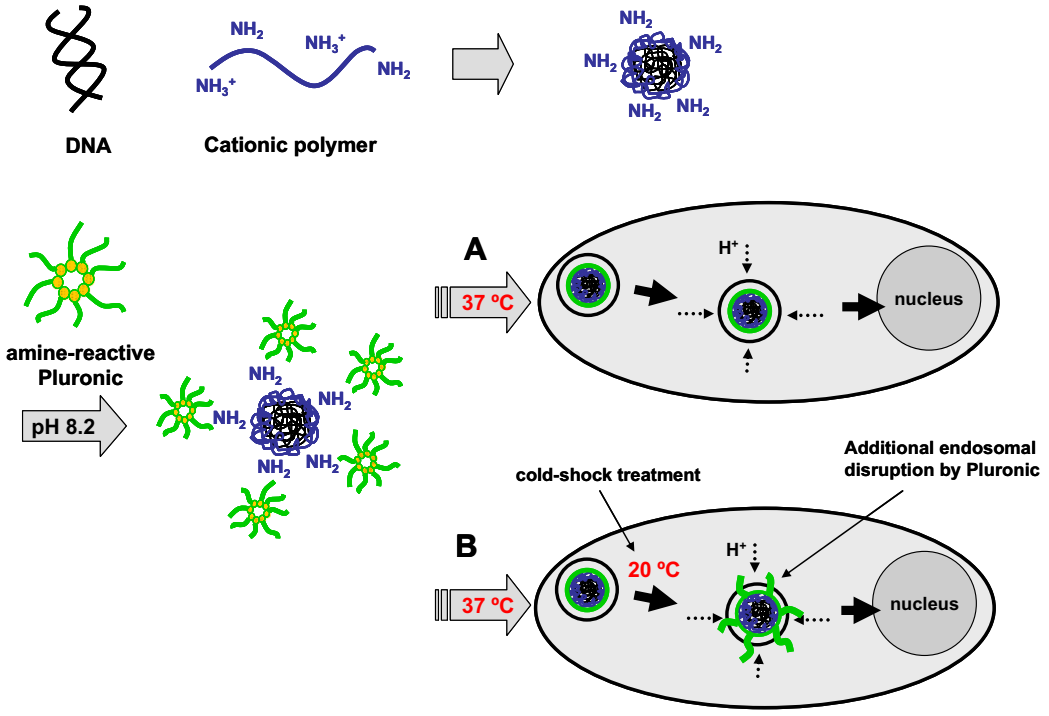

Fig. 1. Intracellular delivery of polycation/DNA complex modified with Pluronic. (A) Non-cold-shock treatment (only proton sponge effects), (B) cold-shock treatment (proton sponge effect + disruption by extended Pluronic).

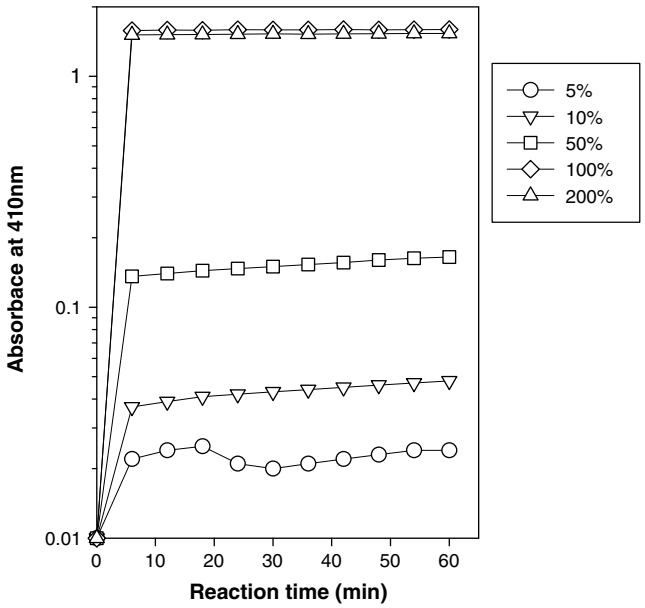

Fig. 2. Monitoring liberated nitrophenyl groups during the conjugation reaction between free amine groups in PEI/DNA complex and amine-reactive Pluronic at various molar ratios from 5% to 200%.

Table 1
Sizes and ζ-potential change of PEI/DNA complex and nanogels with Pluronic at different temperatures

	20 °C		37 °C	
	Diameter (nm)	ζ-Potential	Diameter (nm)	ζ-Potential
PEI/DNA	265.8 ± 2.7	+30.6 ± 1.7	216.5 ± 4.1	+31.6 ± 3.1
Nanogel (5% Pluronic)	1001.5 ± 13.2	N/D[a]	231.6 ± 16.6	+18.7 ± 1.6
Nanogel (10% Pluronic)	1011.7 ± 7.3	N/D[a]	772.2 ± 16.2	+18.4 ± 8.2

[a] N/D, not determined.

mo-sensitive complex when the Pluronic was conjugated on the surface of the complex. When ξ-potentials of the PEI/DNA complex were measured at 20 and 37 °C, no change was observed for unmodified PEI/DNA complex while significant changes were detected for PEI/DNA complex with 5% and 10% Pluronic. At 20 °C, ξ-potentials could not be measured because of the unstable signals. This could be attributed to PEO chains of Pluronic on the surface. PEI/DNA complexes with Pluronic at 20 °C would not expose their surface charges on the surface; thus, ξ-potentials could not be measured. Numerous studies employing PEO for surface-modifications of nanoparticles indicated that ξ-potentials could not be measured for the same reason [9,10]. On the contrary, the Pluronic chains on PEI/DNA complex with 5% and 10% Pluronic were collapsed at 37 °C. Therefore, ξ-potentials could be measured at 37 °C; however, the values were lower than that of unmodified PEI/DNA complex because 5% or 10% amine groups of PEI reacted with Pluronic. This result also confirmed that Pluronic was conjugated to the PEI/DNA complex along with the result from Fig. 2.

It was noteworthy that PEI/DNA complex with 10% Pluronic was less sensitive to temperature changes. In fact, its size only decreased by 1.3-fold in diameter and 2.2-fold in volume. In order to investigate in detail what happened to the complex during temperature changes, the multimodal size distributions of the complexes were also investigated, as shown in Fig. 3. At 20 °C, PEI/DNA complex with 10% Pluronic showed a narrower distribution of the particles (panel B) compared to that with 5% Pluronic (panel A), although average diameters were almost the same for those two complexes. The reason for this could be that more Pluronic was attached to the complex with 10% Pluronic compared to that with 5%, thus decreasing aggregation among the complexes. At 37 °C, Pluronic chains in PEI/DNA complex with Pluronic collapsed because of hydrophobized PPO blocks in the conjugated Pluronic. However, for PEI/DNA complex with 10% Pluronic, wide distributions of particles were observed as well as a small decrease in size compared to that with 5% Pluronic. This result clearly showed that aggregations among the complex occurred for the complex with 10% Pluronic. Thus, the size of the complex with 10% Pluronic apparently increased because of the inter-complex association among hydrophobized Pluronic chains at 37 °C. However, the complex with 5% Pluronic did not aggregate at the same temperature, thus exhibiting narrow distributions of particles. Therefore, this result strongly suggests that the number of Pluronic chains attached to the complex should be also optimized in order to maintain significant size modulations of the complex in responses to tempera-

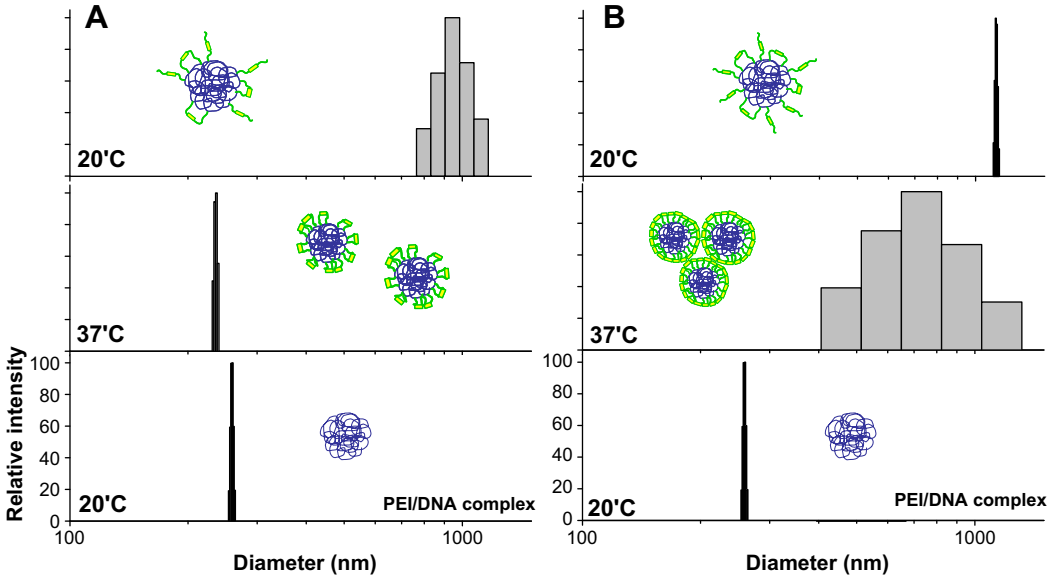

Fig. 3. Multimodal size distributions of PEI/DNA complex surface-modified with Pluronic 5% (A) and 10% (B) at 20 and 37 °C. Distribution of the PEI/DNA complex without Pluronic at 20 °C is shown in the bottom panels (A and B) for comparison.

ture changes. In addition, it should be also stressed that smaller amount of Pluronic decoration in PEI/DNA complex was enough for thermo-sensitivity compared to the similar study employing Pluronic grafted PEI complex [6].

The morphologies of the nanogels were investigated by AFM and TEM, as shown in Fig. 4. In order to investigate morphological differences according to the temperature changes, the solution containing the nanogel was dried at 4 and 37 °C, respectively. Although morphological changes of Pluronic occurred in aqueous solutions, dried nanogels were still expected to memorize the morphologies of Pluronic in the solution. As shown in Fig. 4A–D, the dried nanogel at 4 °C (Fig. 4A and C) showed significant differences in morphology and size compared to that at 37 °C (Fig. 4B and D). The nanogel at 37 °C was round-shaped, and the diameter was approximately 200 nm while the nanogel at 4 °C was rough-surfaced and the diameter was approximately 500 nm. The transmit-

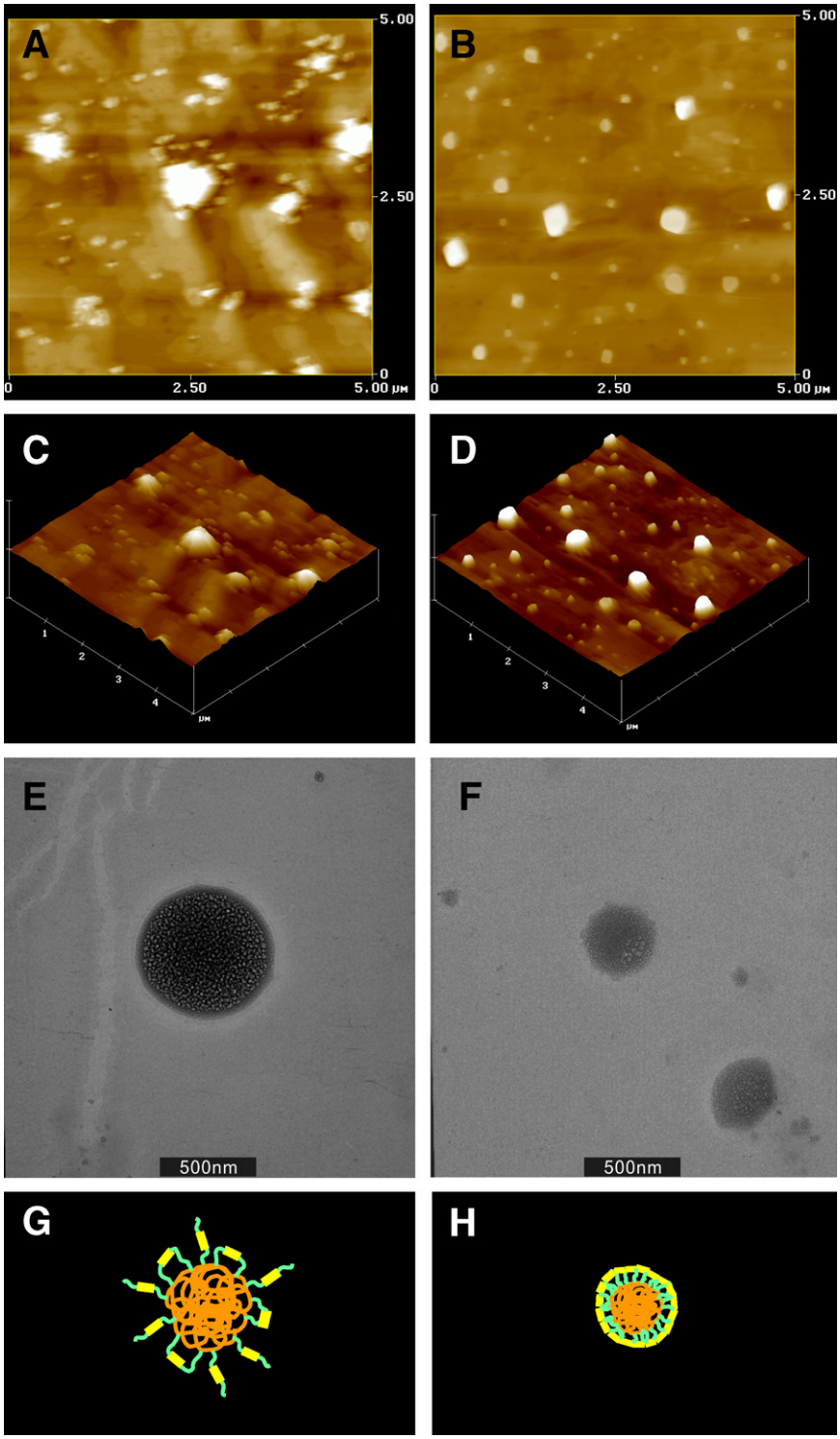

Fig. 4. AFM and cryo-TEM of nanogels with 10% Pluronic at 4 °C (A, C, E and G) and 37 °C (B, D, F and H). (A and B) AFM-2D, (C and D) AFM-3D, (E and F) cryo-TEM, (G and H) an estimated morphology.

ted images of the nanogels were also investigated by TEM. In order to minimize morphological changes during the temperature changes and drying, the nanogel solutions at 4 and 37 °C were instantly dropped in liquid nitrogen and freeze-dried. Although morphological changes occurred upon being frozen in liquid nitrogen, the freeze-dried nanogel should retain its morphology at each temperature. As shown in Fig. 4E and F, TEM images confirmed the significant difference in size between the nanogels at 4 and at 37 °C. The diameters of the nanogel at 4 °C (Fig. 4E) and at 37 °C (Fig. 4F) were approximately 600 and 300 nm, respectively. Therefore, from the results from Fig. 4A–F, significant size changes were confirmed between the nanogels at 4 and at 37 °C, which coincides with the result from Table 1. However, it should be mentioned that the overall sizes measured by AFM and TEM were smaller than those measured by dynamic light scattering. Dynamic light scattering measures hydrodynamic volumes of the nanogel while AFM and TEM monitor the particles in a dried state.

Because the nanogel showed temperature-responsiveness according to the temperature changes, it was expected that the endosomal disruption of transfected cells could be enhanced by modulating incubation temperatures. As shown in Fig. 5, NIH3T3 cells were transfected with the nanogels and endosomal disruption was monitored by confocal microscopy. The transfected cells with cold-shock showed significant differences in the degree of transfection and endosomal disruptions. Considering that lysosomes and endosomes were stained with Lysotracker, a pH sensitive dye, the disappearance of the red color indicates disruptions of lysosomes or endosomes. When cells are transfected with the nanogel with Pluronic, a cold shock treatment decreased the intensities of the red color (Fig. 5C and E) compared to those without a cold shock treatment (Fig. 5D and F). This result suggests that endosomes were disrupted by swollen nanogels with Pluronic in the endosome when the cells were incubated at 20 °C for a short period. At 20 °C, extended Pluronic chains of the nanogel disrupted endosomal membrane and made the endosome unload the nanogel into cytosolic area. Therefore, endosomal escapes of the endocytosed nanogels were facilitated by temperature-responsiveness of the nanogel as well as the proton sponge effect of PEI/DNA complex, as described in the previous studies [11,12].

The disappearance of endosomes was more prominent for the nanogel with 10% Pluronic (Fig. 5E and F), suggesting that the temperature-responsiveness of the nanogel increased as more Pluronic was attached to the surface of the complex. It is also interesting that the nanogel with 10% Pluronic was more efficiently endocytosed by cells, which was confirmed by measuring the light intensities of green colors in the images compared to those with 5% Pluronic. This could be attributed to increased uptakes by cells because the cellular membrane was disrupted by Pluronic on the

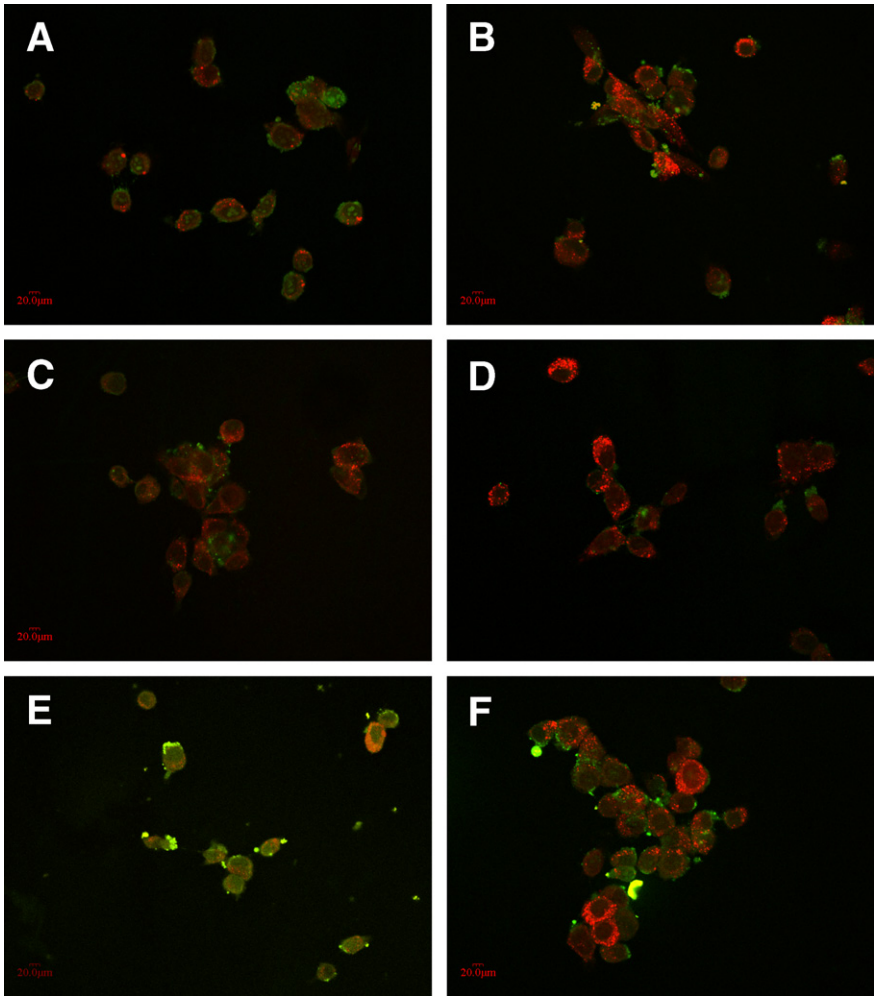

Fig. 5. Confocal laser microscopy of NIH3T3 cells after 3 h-incubation with FITC-labeled PEI(green)/DNA-Pluronic complex and LysoTracker (red). (A and B) PEI/DNA complex, (C and D) nanogel with 5% Pluronic, (E and F) nanogel 10% Pluronic. Cold-shock treatment (A, C and E), Non cold-shock treatment (B, D and F). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

nanogel. Because Pluronic is a non-ionic surfactant and thus loosens the cell membrane, it played an additional role in increasing cellular uptakes by cells.

In order to measure the cytotoxicities of the nanogels according to temperature changes, WST-1 assay was performed. PEI is generally known to be cytotoxic even when it was associated with DNA due to high density of cationic groups. Therefore, the cytotoxicity of nanogels with Pluronic on NIH3T3 cells was measured at 20 and 37 °C. Table 2 shows the survival rates of NIH3T3 cells with or without a cold shock in the presence of various nanogels. It should be noticed that the amount of DNA in each nanogel was fixed, thus, the amount of polymer was different for each nanogel. Compared to PEI/DNA, the nanogel was less cytotoxic when cells were incubated both at 20 and 37 °C. However, the extent of the cytotoxicity difference was more significant at 20 °C. This can be attributed to the differences in surface charges of the nanogels. At 20 °C, the cationic charges of nanogels significantly decreased because of the fully-extended Pluronic chains as shown in Table 1. Considering that PEI exerts cytotoxicity due to the high densities of cationic charges, the low cationic charges of the nanogel decreased the cytotoxicity of PEI/DNA complex [3]. At 37 °C, however, the cytotoxicity of the nanogel showed little difference compared to the PEI/DNA complex. Collapsed Pluronic chains at 37 °C exposed the cationic charges of the PEI/DNA complexes on the surface of the nanogel, subsequently increasing the surface charges of the nanogel. Thus, this increased the cytotoxicity of the nanogels to a similar level to the PEI/DNA complex. Although Pluronic is generally regarded as a safe excipient, several studies indicated a certain cytotoxicity [13,14]. In the current study, the nanogel with 10% Pluronic was more cytotoxic than that with 5% Pluronic at both temperatures, which confirmed the cytotoxicity of Pluronic. However, it should be noticed that Pluronic in the complex did not increase the cytotoxicities of the nanogels compared to the PEI/DNA complex because the amount of Pluronic was relatively low.

Table 2
Survival rates of NIH3T3 cells incubated with nanogel at 20 and 37 °C

Incubation temperature	20 °C	37 °C
PEI/DNA	51 ± 6.8%[a,b]	57 ± 6.2%
Nanogel (5% Pluronic)	87 ± 7.4%	71 ± 8.1%
Nanogel (10% Pluronic)	79 ± 6.3%	68 ± 7.3%
Control	100%	100%

Details of incubation schedules are described in Section 2. Average values and standard deviation were obtained when the assay was repeated three times.
[a] Survival rate with respect to the controls at each temperature.
[b] Shown in average value ± standard deviation.

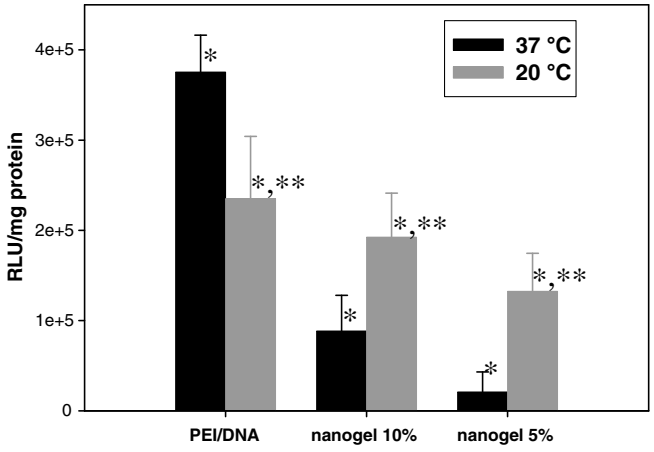

Fig. 6. *In vitro* transfection efficiency of the PEI/DNA complex, the nanogel with 10% Pluronic (nanogel 10%), and the nanogel with 5% Pluronic (nanogel 5%) in NIH3T3 cells. Relative luminescence light (RLU) was normalized with respect to the amount of protein in each cell. For a cold-shock treatment, transfected cells were incubated at 20 °C for 15 min (20 °C) after transfection. Student *t*-test was performed to determine statistical significances of the result using SigmaPlot 9.0 software. $P < 0.05$ was considered statistically significant (*,**).

The temperature-responsiveness of the nanogel in terms of transfection efficiency was investigated, as shown in Fig. 6. Nanogels with Pluronic showed inferior transfection efficiency to PEI/DNA complex at 37 °C, where the nanogels had collapsed Pluronic chains on the surfaces. In cases of the nanogels with 10% and 5% Pluronic, the transfection efficiencies decreased by 4.2 and 17.9 times compared to the PEI/DNA complex, respectively. The proton sponge effect of PEI/DNA complex could be hindered by the conjugated Pluronic on PEI/DNA complex when the nanogels were administered and incubated at 37 °C. A proton sponge effect is caused by the buffering capacity of PEI in the endosome, where protons are abundant to maintain a low pH (pH 5.5) [2]. Because PEI had many amine groups, they acted as buffers to encompass many protons, thus increasing salt concentrations inside endosomes. Consequently, this effect broke endosomal membranes, and endosomes then unloaded their cargos, PEI/DNA complex. However, the nanogel with Pluronic had limitations in the proton sponge effect because some amine groups of PEI were substituted by amine reactive Pluronic. Another explanation for the low transfection efficiency at 37 °C is that associations of the nanogels with cellular membranes were interrupted by the conjugated Pluronic of the nanogels disturbed before endocytosis. It is generally accepted that the transfection efficiency of PEI/DNA complex is decreased by conjugation hydrophilic chains, including PEG and PEO [15]. Many researchers argue that the hydrophilic chains kept PEI/DNA complexes from associating with cellular membranes with a steric hindrance. Therefore, this effect explains the decreased transfection efficiency of the nanogel at 37 °C. However, upon treating cells with a cold shock (20 °C), the transfection efficiency result was astonishing compared to the result at 37 °C, considering the transfection efficiency of the PEI/DNA complex decreased by 1.6-fold. In the case of the nanogel with 10% and 5% Pluronic, the transfection efficiency increased by 2.2- and 6.3-fold. At low temperatures, transfection efficiencies of cells decrease because endosomal disruptions decreased by cells are lowered by the decreased fluidity of cellular membranes. Therefore, in the case of the PEI/DNA complex in Fig. 6, the transfection efficiency decreased due to this effect. However, in case of the nanogels with 5% and 10% Pluronic, the transfection efficiency significantly increased. This could be attributed to the disruption of endosomal membranes by the extended Pluronic chains at 20 °C. In accordance with the confocal microscopy in Fig. 5, the swelling of the nanogel significantly broke down endosomal membranes and helped escapes of the nanogels from endosomes.

4. Conclusion

PEI/DNA complex was surface-modified with Pluronic to prepare thermo-responsive nanogels. The nanogel showed dramatic size and ζ-potential changes according to temperature changes. Cytotoxicity and transfection efficiency of the nanogel were also affected by temperature changes. These results clearly indicate that surface-modification of nanogels can be potentially applied to thermo-responsive gene carriers, where temperature-responsive cytotoxicities or transfection efficiencies are required.

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