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Technical Note

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# PAMAM Dendrimers with an Oxyethylene Unit-Enriched Surface as Biocompatible Temperature-Sensitive Dendrimers

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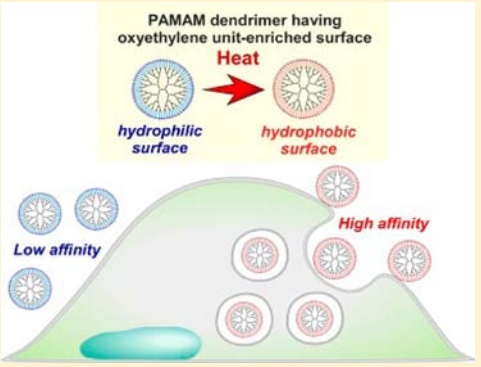
Supporting Information

**ABSTRACT:** A novel type of temperature-sensitive dendrimer was synthesized using one-step terminal modification of polyamidoamine dendrimers (PAMAM) with various alkoxy diethylene glycols such as methoxy diethylene glycol, ethoxy diethylene glycol, and propoxy diethylene glycol. The obtained dendrimers exhibited tunable lower critical solution temperature (LCST), depending on PAMAM generation and terminal alkoxy groups. These dendrimers were shown to be taken up by HeLa cells through endocytosis and were trapped in intracellular compartments such as endosomes and lysosomes. Cellular uptake of the dendrimers was enhanced by increasing their incubation temperature above the LCST. In addition, the in vitro cytotoxicity of temperature-sensitive dendrimers at incubation temperatures below and above LCST was much lower than that of their parent PAMAM dendrimers. Results indicate that the dendrimers with oxyethylene unit-enriched surface might be promising to construct intelligent drug delivery systems.




## INTRODUCTION

Dendrimers have various features that conventional linear polymers do not have.[1] Their size, structure, and surface properties are highly controllable. In addition, their interiors can encapsulate small molecules. Using these features of dendrimers, production of nanosized materials with unique functions is anticipated.

Although many attempts have been made to apply dendrimers in a large variety of fields, applications in the biomedical field, such as drug delivery, are among the most anticipated because the structural uniformity of dendrimers is expected to decrease the uncertainty of their behavior in the body and to increase the accuracy of delivery to the target site.[2,3] Regarding the use of dendrimers for the drug-delivery purposes, temperature-sensitive properties are important to increase their efficacy as carriers.[4,5]

Production of dendrimers with temperature-sensitive properties has been attempted actively. Incorporation of thermosensitive polymers such as poly(*N*-isopropylacrylamide) has been applied to provide thermosensitive properties to dendrimers.[6−8] Indeed, these dendrimers exhibited a lower critical solution temperature (LCST) as the incorporated linear polymers. However, dendrimers obtained using this approach might be regarded more as star polymers and might lose their important features such as their highly defined structure and globular shape.

Thermosensitive dendrimers have also been obtained using surface modification of dendrimers with structural units common with thermosensitive polymers.[9−11] We have incorporated various alkylamide groups, which are common structural units of thermosensitive poly(*N*-alkylacrylamide)s, to chain terminals of PAMAM dendrimers and poly(propyleneimine) dendrimers. Thereby, we demonstrated that these modified dendrimers exhibited a LCST.[9−11] In contrast to dendrimers with thermosensitive polymer grafts, these alkylamide-terminated dendrimers retain structural features of dendrimers such as a highly defined structure and globular shape. In general, their LCSTs change depending on dendrimer generation and the structure of the terminal alkylamide groups, but the LCST of the dendrimers can be adjusted to a desired temperature through modification with different alkylamide groups at an appropriate ratio.[10] Therefore, this strategy is extremely attractive to obtain thermosensitive dendrimers for practical purposes such as drug delivery.

Surface properties of nanoparticles are known to affect their behavior in the body. Especially, surfaces covered with poly(ethylene glycol) chains have been shown to provide highly biocompatible and long-circulating properties in the body.[12,13] Poly(glycidol), which has structural similarity to poly(ethylene glycol), also exhibits excellent biocompatibility, as judged from cytotoxicity, coagulation, and other characteristics.[14,15] These facts indicate the importance of a surface covered with oxyethylene units to achieve high biocompatibility of materials. Recently, we demonstrated that attachment of oxyethylene units, which are common structural units of thermosensitive poly(vinyl ethers),[16] provided temperature-

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sensitivity to hyperbranched poly(glycidol)s.[17] Therefore, oxyethylene units might be useful as another type of functional group for temperature sensitization of dendrimers according to our surface modification strategy, considering the fact that the dendrimer core shows greater potential for use in drug delivery than a hyperbranched poly(glycidol) core.

On the basis of these previous findings, oxyethylene units are highly attractive for surface modification of dendrimers because they can provide both temperature-sensitive properties and nontoxic characteristics to the dendrimer surface simultaneously. In this study, we attempted to use oxyethylene units for the surface modification of PAMAM dendrimers for the first time. We produced a new type of functional dendrimer having both high temperature-sensitivity and noncytotoxic property. Temperature-controlled functions from the viewpoints of water solubility, cellular uptake, and intracellular distribution and low cytotoxicity for the dendrimers with the oxyethylene unit-enriched surface were described.

## MATERIALS

Methoxy diethylene glycol (MDEG), ethoxy diethylene glycol (EDEG), propoxy diethylene glycol (PDEG), chlorpromazine hydrochloride, cytochalasin B, 4,4′-azobis(4-cyanovaleric acid), and 3-(4,5-dimethyl-2-thiazoryl)-2,5-diphenyl-2*H*- tetrazolium bromide (MTT) were obtained from Wako Pure Chemical Industries Ltd. (Osaka Japan). Filipin (≥70%), 4-nitrophenyl chloroformate, PAMAM dendrimers of the third, fourth and fifth generations, and fluorescein isothiocyanate (FITC) were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Hoechst 33342 and LysoTracker DND-99 were obtained from Invitrogen (Eugegne, OR). Tetrahydrofuran (THF), dimethyl sulfoxide, and triethylamine (TEA) were supplied from Kishida Chemical (Osaka, Japan). Tetrahydrofuran (THF) and dimethyl sulfoxide were distilled just prior to use. Poly(*N*-isopropylacrylamide) (the weight average molecular weight = 47000; the number average molecular weight = 32000) were obtained by the free radical polymerization method using 4,4′-azobis(4-cyanovaleric acid) in THF at 70 °C as the initiator.[11]

## EXPERIMENTS

**Synthesis of 4-Nitrophenyl Chloroformate Functionalized Alkoxy Diethylene Glycol.** A typical method for the synthesis of propoxy diethylene glycol 4-nitrophenyl carbonate (PDEG-NPC) was as follows: propoxy diethylene glycol (3.077 g, 20.76 mmol) and triethylamine (4.64 mL, 33.38 mmol) were dissolved in 30 mL of dry THF, then 4-nitrophenyl chloroformate (NPC) (5.036 g, 24.99 mmol) was added. The mixed solution was stirred at room temperature overnight. Then the compound was purified by silica gel (ethyl acetate/hexane = 1/1) and dried in vacuum to afford the final product (PDEG-NPC) (2.6738 g, yield 52%). MDEG-NPC and EDEG-NPC were prepared by the same procedure except for the amount of the reagents used for the preparation. MDEG-NPC (yield 62%), MDEG (16.65 mmol), NPC (24.98 mmol), TEA (33.3 mmol); EDEG-NPC (yield 83%), EDEG (20.76 mmol), NPC (24.99 mmol), and TEA (33.38 mmol).

**Synthesis of R-DEG-Modified PAMAM Dendrimers.** A typical synthesis method for the PDEG-modified generation 4 PAMAM dendrimer (PDEG-G4) is as follows: 1 mL of G4 PAMAM methanol solution (10 wt %) was added to a 10 mL flask. Then, the methanol was evaporated completely. The left G4-PAMAM was weighed (81.5 mg, 5.7 μmol) and dissolved in 3 mL of dry DMSO. PDEG-NPC (246 mg, 785 μmol) was dissolved in 2 mL of dry DMSO and was then added to the flask. The mixed solution was stirred at room temperature for 5 days to ensure completion of the reaction of all terminal amine groups of dendrimers with the PDEG-NPC. The synthesized dendrimer was purified using a Sephadex LH20 column [4 cm (diameter) × 70 cm (length)] with methanol as the elution solvent. The eluted dendrimers were detected using an UV detector at a fixed wavelength of 220 nm. About 50 mL fractions were collected and dried in vacuum to afford the final product PDEG-G4 (125.3 mg, yield 86%). EDEG-G3, MDEG-G4, EDEG-G5, and (EDEG-PDEG)-G4 were synthesized using the same procedure, except for the amount of reagents used for the reaction (Supporting Information, Table S1).

**Synthesis of FITC-Labeled (EDEG-PDEG)-G4 PAMAM Dendrimer.** Typically, 0.75 mL of 10 wt % G4-PAMAM methanol solution (60 mg, 4.2 μmol) was added to 5 mL of methanol. Then, FITC (1.64 mg, 4.2 μmol) was added. The mixed solution was stirred in the dark at room temperature for 1 day. Then it was dialyzed against deionized water through a dialysis bag (molecular weight cutoff of 10,000) until free dye could not be detected by thin layer chromatography with methanol as the mobile phase and a 365 nm UV light detector. Finally, it was lyophilized to afford the FITC-labeled generation 4 PAMAM dendrimer (FI-G4) (58 mg, yield 94%). Next, FI-G4 (41.9 mg, 2.85 μmol) was dissolved in 3 mL of dry DMSO. PDEG-NPC (57.5 mg, 183.5 μmol) and EDEG-NPC (54.91 mg, 183.5 μmol) were dissolved in 2 mL of dry DMSO and then added to the flask. The mixed solution was stirred at room temperature for 5 days. The synthesized dendrimer was purified using a Sephadex LH20 column [4 cm (diameter) × 70 cm (length)] with methanol as the elution solvent. The eluted dendrimers were detected using an UV detector at a fixed wavelength of 220 nm. About 30 mL fractions were collected and dried in vacuum to afford the final product of (FI-EDEG-PDEG)-G4 (81.3 mg, yield 83%).

**Turbidity Measurements.** Turbidity of temperature-sensitive dendrimers in 10 mM phosphate and 150 mM NaCl solutions (pH 7.4) was measured at 700 nm with stirring using a spectrophotometer (V-550; Jasco Corp.) equipped with a Peltier type thermostatic cell holder coupled with a controller ETC-505T. The heating rate of sample cells was adjusted at 1.0 °C $min^{-1}$. The cloud point was taken as the initial break point in the resulting transmittance versus temperature curves.

**Cell Culture.** HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO), supplemented with 10% fetal bovine serum (FBS, GIBCO), 50 units/mL penicillin, and 50 μg/mL streptomycin at 37 ° C under 5% $CO_2$ condition. Cells were cultured 2 days to achieve approximately the same confluency before performing all experiments.

**In Vitro Cytotoxicity Assay.** HeLa cells were seeded into a 24-well microplate ($1 \times 10^5$ cells/well) and cultured for 2 days. Then HeLa cells were incubated with G4 PAMAM, (EDEG-PDEG)-G4, and (FI-EDEG-PDEG)-G4 solutions at concentrations of 5.0 and 10.0 mg/mL in 500 μL DMEM at 37 °C for 24 h. After incubation, HeLa cells were gently washed with phosphate-buffered saline (PBS) twice. Then, 470 μL DMEM and 30 μL MTT solution (10 mg/mL in PBS) were added as the incubation media. After 3 h, MTT media was removed, and 450 μL of isopropanol with 50 μL of 1 N HCl solution was added. The absorbance at 490 nm was measured on a VICTOR$^3$ V 1420 Multilabel Counter (PerkinElmer, USA) to check the cells' surviving profile.


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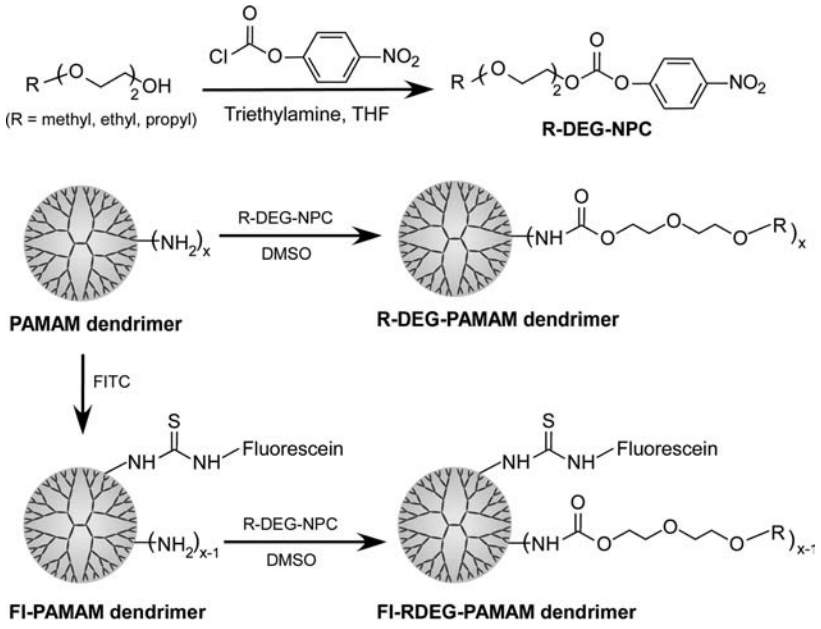
**Scheme 1. Synthesis of Temperature-Sensitive PAMAM Dendrimers**




**Flow Cytometric Analysis.** HeLa cells were seeded into a 24-well microplate ($1 \times 10^5$ cells/well) and cultured for 2 days. Then, HeLa cells were incubated with FI-G4 and (FI-EDEG-PDEG)-G4 solution with concentrations of 0, 0.01, 0.1, 0.5, and 1.0 mg/mL in 500 $\mu$L of 10 mM phosphate and 150 mM NaCl solution (pH 7.4) at 37 and 41 °C for 15 min. After incubation, HeLa cells were gently rinsed with phosphate-buffered saline (PBS) three times at room temperature (25 °C), followed by treatment with 300 $\mu$L of 0.05% trypsin-EDTA. Then, cellular uptake was determined on an Epics XL flow cytometer (Beckman Coulter, Fullerton, USA), and 10000 events were analyzed.

**Effect of Endocytosis Inhibitors on the Intracellular Uptake of Temperature-Sensitive Dendrimer.** HeLa cells were seeded into a 24-well microplate ($1 \times 10^5$ cells/well) and cultured for 2 days. Then, cells were washed twice with PBS and incubated for 15 min at 37 °C in 500 $\mu$L of DMEM media containing chlorpromazine (30 $\mu$M), cytochalasin B (250 $\mu$M), or filipin (10 $\mu$g/mL). After the endocytosis inhibitor incubation, cells were washed twice with DMEM and one time with PBS, and incubated with (FI-EDEG-PDEG)-G4 solution at a concentration of 1.0 mg/mL in 500 $\mu$L of 10 mM phosphate and 150 mM NaCl solution (pH 7.4) at 41 °C for 15 min. After incubation, HeLa cells were gently rinsed with phosphate-buffered saline (PBS) three times at room temperature (25 °C), followed by treatment with 300 $\mu$L of 0.05% trypsin-EDTA. Then, cellular uptake was determined on an Epics XL flow cytometer (Beckman Coulter, Fullerton, USA), and 10000 events were analyzed.

**Confocal Laser Scanning Microscopy.** HeLa cells ($1 \times 10^5$ cells) cultured for 2 days were washed with phosphate-buffered saline (PBS) and then incubated with FI-G4 and (FI-EDEG-PDEG)-G4 solution at a concentration of 1.0 mg/mL in 500 $\mu$L of 10 mM phosphate and 150 mM NaCl solution (pH 7.4) at 37 and 41 °C for 15 min. After incubation, HeLa cells were gently rinsed with phosphate-buffered saline (PBS) three times at room temperature (25 °C), followed by staining with 15 $\mu$L of LysoTracker Red DND-99 (10 pmol/$\mu$L in water) and 1 $\mu$L of Hoechst33342 (10 mg/mL in water) in 500 $\mu$L of DMEM without serum at 37 °C for 30 min, and then washed with PBS three times. Confocal laser scanning microscopic (CLSM) analysis of these cells was performed on a LSM 5 EXCITER (Carl Zeiss Co. Ltd.). In another group of experiments, after incubation with dendrimers at 41 °C for 15 min, HeLa cells were first gently rinsed with phosphate-buffered saline (PBS) three times at room temperature (25 °C) and then incubated in DMEM/FBS at 37 °C for another 24 h. After incubation, HeLa cells were stained and observed by CLSM using the same method described above.

**Other Measurements.** $^1$H and $^{13}$C NMR spectra were recorded on a JEOL JNM-LA 400 instrument. Differential scanning calorimetry (DSC) was performed on a NANO DSC (TA Instruments, USA). The heating rate was 0.5 °C min$^{-1}$. Zeta potential and particle size of dendrimers at different temperatures were measured using a Zetasizer Nano ZS90 (Malvern Instruments) with a standard He−Ne 633 nm laser and 90° collecting optics. The average values from the intensity statistics of a total of 20 runs of 30 s each were used. Data were analyzed using Malvern Zetasizer Software 6.32. The sample was kept for 5 min at each temperature before the measurement.

**Statistical Analysis.** The statistical analysis was performed using the Student's $t$ test; $p < 0.05$ was considered statistically significant in all analyses.

## RESULTS AND DISCUSSION

**Synthesis and Characterization of Temperature-Sensitive Dendrimers.** For this study, PAMAM dendrimers with oxyethylene unit-enriched surfaces were synthesized by incorporating various alkoxy diethylene glycols (R-DEG), such as MDEG, EDEG, and PDED, to every chain end of the PAMAM dendrimers of different generations (Scheme 1). First, MDEG, EDEG, and PDEG were functionalized with 4-nitrophenyl chloroformate (Supporting Information, Figure S1); then, they were reacted with PAMAM G3, G4, and G5 dendrimers at different ratios (Supporting Information, Table S1). The number of the R-DEG attached to each dendrimer was determined from the integral ratio of the signal at 2.7−2.9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ppm, which corresponds to the protons of methylenes next to the tertiary amine groups of the PAMAM dendrimer, to the signal at 4.1−4.3 ppm, which corresponds to the protons of methylenes adjacent to carbamate groups of the EG unit (Supporting Information, Figures S2, S3, S4, S5, and S7). In the cases of (EDEG-PDEG)-G4 dendrimers, the ratio of EDEG to PDEG was determined from the integral ratio of the signal at 1.0−1.2 ppm (terminal methyls in EDEG) to signal at 0.85−0.95 ppm (terminal methyls in PDEG) (Supporting Information, Figure S5). In addition, the complete modification of R-DEG to PAMAM dendrimers was confirmed using $^{13}C$ NMR, in which the peak corresponding to the carbons next to the terminal amino groups of PAMAM dendrimers disappeared completely after the reaction, indicating that no terminal amino group remained (Supporting Information, Figures S2, S3, S4, S6, and S7).

To investigate the cellular uptake and intracellular distribution of temperature-sensitive R-DEG-PAMAM dendrimers, fluorescein isothiocyanate (FITC) was used to label the (EDEG-PDEG)-G4 dendrimers (Scheme 1). The characteristic proton peaks related to FI are apparent in the aromatic region at 6.47 and 7.07 ppm in the $^1H$ NMR spectra, and the quantities of FI, EDEG, and PDEG units attached on each PAMAM dendrimer were determined based on $^1H$ NMR (Supporting Information, Figure S7). The isolated dendrimers were shown to be free of residual diethylene glycols by $^1H$ NMR and $^{13}C$ NMR spectra. For example, the $^1H$ NMR spectrum of the ($EDEG_{22.1}$-$PDEG_{41.9}$)-G4 dendrimer contains no signal from free EDEG or free PDEG (Supporting Information, Figure S8). Detailed structural information of synthesized dendrimers is presented in Table 1.

**Table 1. Characterization of Various R-DEG-PAMAM Dendrimers**

| sample[a] | feed R-DEG per dendrimer | attached R-DEG per dendrimer |
|---|---|---|
| EDEG-G3 | 64.05 | 32.0 |
| MDEG-G4 | 128.0 | 64.0 |
| EDEG-G5 | 257.6 | 128.0 |
| EDEG-G4 | 128.8 | 64.0 |
| PDEG-G4 | 137.7 | 64.0 |
| (EDEG-PDEG)-$G4_1$ | 42.9/85.8[b] | 22.1/41.9[b] |
| (EDEG-PDEG)-$G4_2$ | 64.4/64.4[b] | 33.0/31.0[b] |
| (EDEG-PDEG)-$G4_3$ | 85.8/42.9[b] | 43.5/20.5[b] |
| (FI-EDEG-PDEG)-G4 | 1.0/64.4/64.4[c] | 1.0/32.8/30.2[c] |

[a]G3, G4, and G5 mean generations 3, 4, and 5 PAMAM dendrimers with, respectively, 32, 64, and 128 terminal amine groups. [b]Number of EDEG and PDEG, respectively. [c]Number of FI, EDEG, and PDEG, respectively.

**Temperature-Induced Transitions of R-DEG-Modified Dendrimers.** The temperature-sensitive properties of synthesized R-DEG-modified PAMAM dendrimers were first investigated by the detection of the LCST of their aqueous solutions. The influences of structural factors such as dendrimer generation and terminal alkoxy groups on the LCST were examined. We have already shown that the LCST of IBAM-terminated PAMAM dendrimers increases with decreasing pH below pH 7 but, above that pH, the dendrimers exhibit a constant LCST value because protonation of tertiary amines in the PAMAM dendrimer interior takes place below pH 7.[9]

Figure 1 shows the optical transmittances of various R-DEG-modified PAMAM dendrimers dissolved in 10 mM phosphate and 150 mM NaCl solution (pH 7.4, 10 mg/mL) as a function of temperature. Figure 1A shows the temperature dependence of transmittance for the solutions of EDEG-G3, EDEG-G4, and EDEG-G5 dendrimers. These dendrimer solutions exhibited a sudden decrease in transmittance at a specific temperature, which is designated as the cloud point and is indicative of LCST. Unmodified PAMAM dendrimers have no thermosensitive properties.[9] Therefore, this result indicates that the EDEG units on the dendrimer periphery provide temperature-sensitive properties to temperature-insensitive PAMAM dendrimers and that these dendrimers change their surface between hydrophilic and hydrophobic depending on the ambient temperature. The cloud points of EDEG-G3, EDEG-G4, and EDEG-G5 dendrimers were, respectively, 78.3, 64.4, and 60.0 °C, indicating that the cloud point decreases concomitantly with increasing dendrimer generation. Considering that all the thermal sensitive EDEG units are attached on the surface of the dendrimer, the density of EDEG units on the dendrimer surface increases with the dendrimer generation, which enables more efficient interactions of EDEG units to each other and make the dendrimer surface more prone to dehydration and phase transition.[9]

The influence of the terminal alkoxy group on the phase transition temperature was then investigated using methoxy, ethoxy, and propoxy end DEG-G4 dendrimers (Figure 1B). The cloud points of EDEG-G4 and PDEG-G4 dendrimers were respectively 64.4 and 26.9 °C, whereas the MDEG-G4 dendrimers show no phase transition at all, which means that the hydrophobicity of the terminal groups plays an important role in the generation of thermosensitive properties of the dendrimer surface and that the increase in hydrophobicity of the terminal group causes a decrease in the cloud point.

The cloud points of these dendrimers were either much higher or lower than body temperature; therefore, these dendrimers were unsuitable for biomedical use. Reportedly, the LCST of the temperature-dependent copolymer was influenced strongly by the ratio of hydrophobic units to hydrophilic units.[18] In general, the hydrophobic units decrease LCST, and the hydrophilic units increase LCST. On the basis of the same principle, the transition temperatures of R-DEG-modified PAMAM dendrimers through the body temperature were attempted by adjusting the composition of R-DEGs with different hydrophobicity. (EDEG-PDEG)-G4 dendrimers containing both PDEG and EDEG at different ratios were synthesized and characterized (Table 1). As Figure 2A shows, the solutions of these dendrimers exhibited cloud points at different temperatures depending on their compositions. Figure 2B depicts the relationship between the cloud point and EDEG content of the dendrimer terminal EDEG/PDEG groups. The cloud point increased proportionally with the EDEG content. The result indicates that the transition temperature of the (EDEG-PDEG)-G4 dendrimers can be controlled from 26.9 to 64.4 °C by changing the composition of the terminal EDEG/PDEG units. Especially, the cloud point of the ($EDEG_{33.0}$-$PDEG_{31.0}$)-G4 dendrimer was 40.8 °C, which was higher than the body temperature (37.0 °C). Therefore, it is useful to deliver drugs to tumor tissues controlled by heating the tumor tissues to a temperature around its cloud point. The ($EDEG_{22.1}$-$PDEG_{41.9}$)-G4 dendrimer with a cloud point of 35.9 °C, which was lower than body temperature, can be used to deliver drugs to tumor tissues by local injection.


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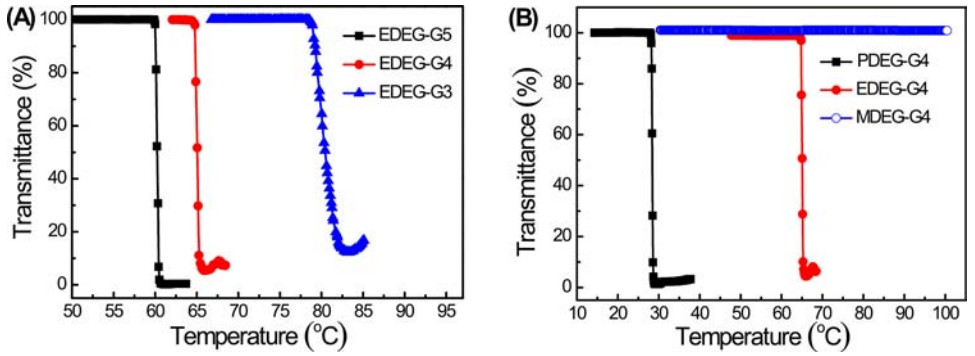


**Figure 1.** Temperature dependence of transmittance for EDEG-G3, EDEG-G4, and EDEG-G5 dendrimers (A), and MDEG-G4, EDEG-G4, and PDEG-G4 dendrimers (B) dissolved in 10 mM phosphate and 150 mM NaCl solution (pH 7.4, 10 mg/mL).

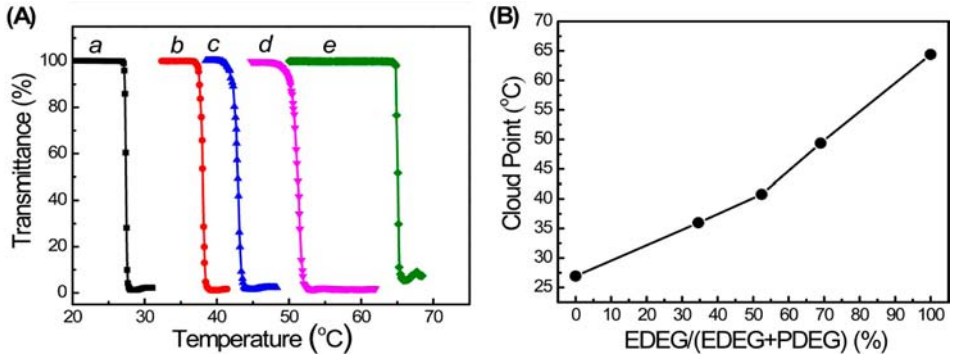


**Figure 2.** (A) Effect of temperature on transmittance for solutions of (a) PDEG-G4, (b) ($EDEG_{22.1}$-$PDEG_{41.9}$)-G4, (c) ($EDEG_{33.0}$-$PDEG_{31.0}$)-G4, (d) ($EDEG_{43.5}$-$PDEG_{20.5}$)-G4, and (e) EDEG-G4 dendrimers. (B) Cloud point of (EDEG-PDEG)-G4 dendrimer solutions as a function of EDEG content. All dendrimers were dissolved in 10 mM phosphate and 150 mM NaCl solution (pH 7.4, 10 mg/mL).

Dehydration of the polymer is known to occur at the phase transition, which can be detected as an endothermic peak in DSC analysis.[19] We demonstrated previously that the phase transition of temperature-sensitive N-isopropylamide (NIPAM)-terminated PAMAM dendrimers can be detected using DSC: we observed an endothermic peak around their phase transition temperature.[11] Subsequently, DSC was performed to determine the transition process of (EDEG-PDEG)-G4 dendrimers (Supporting Information, Figure S9). The temperature at the peak maximum ($T_{max}$) and the transition enthalpies ($\Delta H$) for these dendrimer are presented in Table 2 along with the LCST estimated from turbidity measurements. As shown in Supporting Information, Figure S9, endothermic peaks were observed at temperatures around their cloud point for all (EDEG-PDEG)-G4 dendrimer solutions. In addition, $T_{max}$ for all dendrimers was higher than their cloud point, which indicates that the dendrimers lose water-solubility in the initial stage of their transition process and that their dehydration continues several degrees above the LCST.[13]

**Table 2. Cloud Point and DSC Analysis of Polymers Used in This Study**

| sample | cloud point[a] (°C) | $T_{max}$[b] (°C) | $\Delta H$[b] (J/g) | $\Delta H$[b] [KJ/(mol RDEG)] |
|---|---|---|---|---|
| PDEG-G4 | 26.9 | 28.2 | 7.48 | 2.97 |
| ($EDEG_{22.1}$-$PDEG_{41.9}$)-G4 | 35.9 | 38.0 | 6.06 | 2.38 |
| ($EDEG_{33.0}$-$PDEG_{31.0}$)-G4 | 40.8 | 44.2 | 5.77 | 2.25 |
| ($EDEG_{43.5}$-$PDEG_{20.5}$)-G4 | 49.4 | 52.1 | 2.67 | 1.03 |
| EDEG-G4 | 64.4 | 70.1 | 1.37 | 0.53 |
| PNIPAM | 31.1 | 31.0 | 70.4 | 7.97[c] |

[a]Determined by turbidity measurement. [b]Determined by DSC. [c]The $\Delta H$ value for PNIPAM was calculated by KJ/(mol NIPAM unit).

The phase transition of linear polymer poly(*N*-isopropylacrylamide) (PNIPAM) was measured by DSC as a control. As expected, the $\Delta H$ value of PNIPAM was greater than those of the (EDEG-PDEG)-G4 dendrimers (Table 2). $\Delta H$ for PNIPAM was 7.97 KJ/(mol NIPAM unit), whereas the (EDEG-PDEG)-G4 dendrimers showed $\Delta H$ between 0.53 and 2.97 KJ/(mol RDEG group). The result indicates that the thermosensitive dendrimers show smaller $\Delta H$ than PNIPAM based on the number of thermosensitive moieties. The endotherm of PNIPAM at the LCST is explained from the release of structured water around hydrophobic isopropyl groups upon the transition from a hydrated coil to a dehydrated globule at the LCST.[19,20] However, the unique globular shape of dendrimers with dense packing of temperature-sensitive groups in the periphery led to inefficient hydration below the LCST and inefficient dehydration above the LCST, thereby resulting in the extremely small transition enthalpy.[11] Therefore, the small transition endotherm for the (EDEG-PDEG)-G4 dendrimers means that a lower extent of dehydration was induced during the transition, which indicates that only the surface of the dendrimer undergoes the phase transition from hydrophilic to hydrophobic upon heating. It is particularly interesting that the $\Delta H$ increased gradually with the greater PDEG proportion in (EDEG-PDEG)-G4 dendrimers, which means that the hydrophobic PDEG enhances dehydration of the dendrimer surface.


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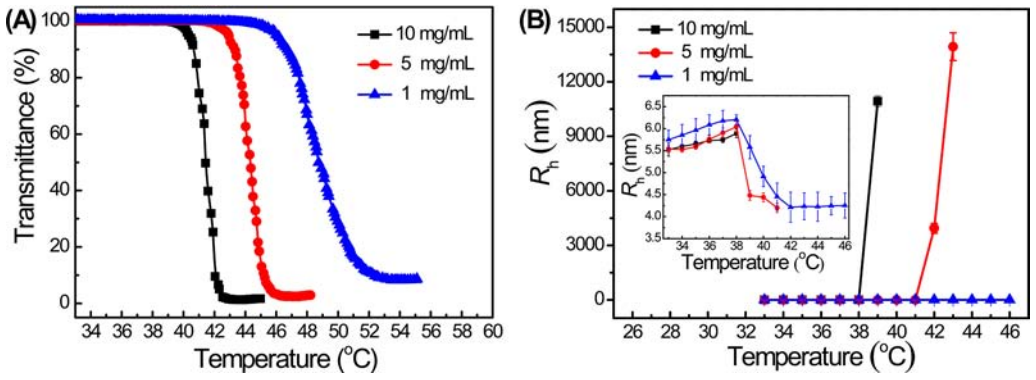


**Figure 3.** (A) Effect of temperature on the transmittance of (FI-EDEG-PDEG)-G4 dendrimer solutions at different concentrations. (B) Size change of the (FI-EDEG-PDEG)-G4 dendrimer at different concentrations during the heating process. The inset shows the size of dendrimers before aggregation. Data are given as the mean ± SD. All samples were measured in 10 mM phosphate and 150 mM NaCl solution (pH 7.4).

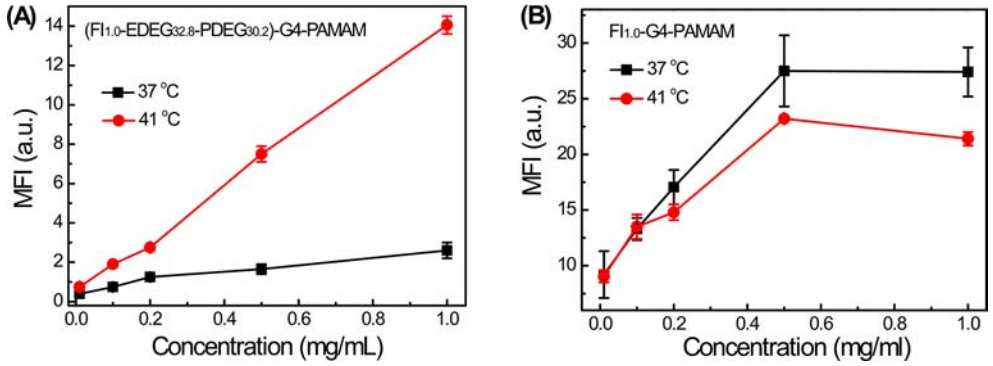


**Figure 4.** Cellular uptake of (FI-EDEG-PDEG)-G4 (A) and FI-G4 (B) dendrimers at 37 °C (black) and 41 °C (red) at varying dendrimer concentrations. Data are given as the mean ± SD of triplicate experiments.

**Cellular Uptake and Intracellular Distribution of FITC-Labeled Temperature-Sensitive Dendrimers.** The FITC-labeled temperature-sensitive dendrimer (FI-EDEG-PDEG)-G4 was synthesized and used for the cellular uptake investigation (Supporting Information, Figure S7). Figure 3A shows the temperature dependence of transmittance for the solutions of (FI-EDEG-PDEG)-G4 dendrimers at different concentrations. The cloud point is 39.2 °C with a sharp phase transition at the concentration of 10 mg/mL. However, the cloud point increases along with a broader phase transition with the decrease of dendrimer concentration. It is noteworthy that the cloud point of FITC-labeled (EDEG-PDEG)-G4 dendrimers was about 1.0 °C lower than that of dendrimers without FITC conjugation because the FITC molecule was hydrophobic.

In addition to turbidity measurements, the phase transition of (FI-EDEG-PDEG)-G4 dendrimers was measured using DLS. Figure 3B shows the hydrodynamic radius ($R_h$) of dendrimers during heating at different concentrations. A significant increase in $R_h$ indicative of dendrimer aggregation was observed for the dendrimer solutions of 10 mg/mL and 5 mg/mL, respectively, at 39 and 42 °C, which are consistent with cloud points for the corresponding dendrimer solutions. Aggregation was not observed for dendrimers at the concentration of 1.0 mg/mL at 46 °C, which is the cloud point of the dendrimer at this concentration. Probably, the very low concentration of dendrimers suppresses aggregation of dendrimers without stirring for DLS measurement, even though the dendrimer becomes hydrophobic at that temperature. It is noteworthy that, irrespective of the dendrimer concentration, the dendrimer retained the hydrodynamic radii ($R_h$) of 5.5−6 nm below 39 °C but exhibited a sudden change in size above this temperature, indicating the occurrence of phase transition of the dendrimer. Importantly, the $R_h$ of dendrimer at 1.0 mg/mL and 5 mg/mL shrank to 4.0−4.5 nm above 39 °C before aggregation. This phenomenon is consistent with those found in an earlier study, where thermosensitive hyperbranched polymer first shrank and then mutually aggregated above the cloud point during heating.[21] Results show that the temperature-sensitive dendrimers begin to dehydrate and shrink at a particular temperature (39 °C) independent of dendrimer concentration; then, the dendrimer molecules with a dehydrated surface form an aggregate above the cloud point, which changes depending on the dendrimer concentration.

Cellular uptake of temperature-sensitive dendrimers (FI-EDEG-PDEG)-G4 by HeLa cells was investigated using flow cytometry. Both concentration and incubation temperature effects on cellular uptake were investigated (Figure 4A). The fluorescent intensity derived from FI associated with the cell increased with the increase of incubation dendrimer concentrations at both 37 and 41 °C. However, the enhanced cellular uptake of dendrimers was observed at higher incubation temperature (41 °C) in the whole concentration range from 0.01 to 1.0 mg/mL. Particularly, the fluorescence intensity was 5.5 times stronger at the dendrimer concentration of 1.0 mg/mL with an incubation temperature of 41 °C. These results indicate that the cellular uptake of the (EDEG-PDEG)-G4 dendrimers is controllable by temperature. The cellular uptakes of the FI-G4 dendrimer at different concentrations and temperatures were investigated as controls. As Figure 4B


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depicts, the amine-terminated FI-G4 dendrimer exhibited relatively stronger cellular uptake at incubation temperature of 37 °C compared with 41 °C, meaning that a higher temperature was not helpful to increase the cellular uptake of dendrimers.

Temperature-induced enhancement of cellular uptake has been reported for thermosensitive micelles and liposomes, and the morphology, size, and intracellular uptake pathways are all important factors for the enhancement of the cellular uptake of temperature-sensitive nanomaterials.[22−26] Therefore, the zeta potential of (FI-EDEG-PDEG)-G4 dendrimers at a concentration of 1.0 mg/mL during heating was measured. As shown in Figure S10 (Supporting Information), the zeta potentials of dendrimers were −4.83 ± 0.48 mV and −8.21 ± 1.62 mV at 37 and 41 °C, respectively. Considering the negative charge of the cell surface, the increased negative zeta potential might affect the cellular uptake of dendrimers only slightly. In addition, as shown in Figure 3B, the diameter of dendrimers were 6.18 ± 0.24 nm and 4.45 ± 0.31 nm at 37 and 41 °C, respectively, and the 1.73 nm size reduction was caused by the dehydration and shrinking of the dendrimer surface when the temperature was above 39 °C. Therefore, the (FI-EDEG-PDEG)-G4 dendrimer surface change from hydrophilic to hydrophobic showed the main contribution to the enhanced cellular uptake. Because the relatively hydrophobic character of the cell surface, the dendrimers with the hydrophobic character strongly interacted with cells, and they were taken up efficiently by the cells.

Subsequently, the possible endocytosis pathways for enhanced internalization of temperature-sensitive dendrimers into HeLa cells were investigated using endocytosis inhibitors. Chlorpromazine (inhibitor of clathrin-mediated endocytosis),[27] cytochalasin B (inhibitor of macropinocytosis),[28] and filipin (inhibitor of caveolae-mediated endocytosis)[29] were incubated with HeLa cells for 15 min. The cells showed healthy morphology after the inhibitor incubation observed by fluorescence images (data not shown). Figure 5 presents

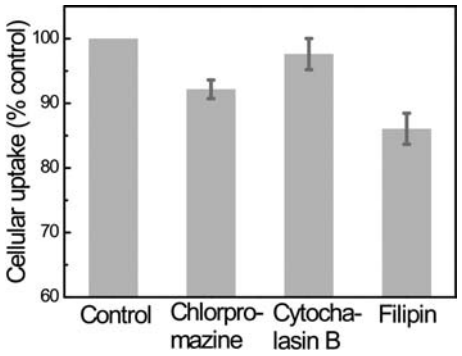


**Figure 5.** Effects of endocytosis inhibitors on the intracellular uptake of (FI-EDEG-PDEG)-G4 dendrimers to Hela cells incubated at 41 °C with a concentration of 1.0 mg/mL. Intracellular uptake of dendrimers to untreated cells was set as the control. Data are given as the mean ± SD of triplicate experiments.

effects of endocytosis inhibitors on the intracellular uptake of temperature-sensitive dendrimers with a concentration of 1.0 mg/mL at 41 °C. Compared with the untreated control cells, cytochalasin B decreased the intracellular uptake by only 2.4%, which means that cytochalasin B has little effect on the intracellular uptake of the dendrimers. In contrast, chlorpromazine and filipin induced 7.9% and 14% decrease of intracellular uptake, respectively, suggesting that the cellular internalization of temperature-sensitive dendrimers might occur via both clathrin-mediated endocytosis and caveolae-mediated endocytosis pathways.

Temperature effects on intracellular distribution of temperature-sensitive and temperature-insensitive dendrimers were investigated using confocal laser scanning microscopy (CLSM) with (FI-EDEG-PDEG)-G4 and FI-G4 dendrimers. For the CLSM experiments, the cells were incubated with the dendrimers at 37 or 41 °C for 15 min and then washed thoroughly with PBS to investigate intracellular distribution of dendrimers, and lysosomes were stained with LTD DND-99 to red color. In the CLSM images of the cells treated with the temperature-sensitive dendrimers at 37 °C (Figure 6A), the green fluorescence derived from the FITC-labeled dendrimer was weak. The dots with green color are located on the red color-stained places in the overlay image, suggesting that the dendrimers existed in lysosomes. However, the green fluorescence was increased for the cells treated with the same dendrimer at 41 °C, indicating that the cellular uptake of the dendrimers was enhanced above the LCST. Although a few yellow dots, which suggest the existence of the dendrimers in lysosomes, were observed around the cell nuclei, several green dots were also apparent around the peripheral region of the cells. Cells treated with the dendrimers were washed thoroughly with PBS below the LCST. Therefore, the noninternalized dendrimers on the surface of the cell were able to become hydrophilic and were thereby removed from the cell surface. The fact that the cells exhibited punctate and intensive fluorescence of FI-dendrimers suggests that the dendrimers were internalized into the cells and trapped in some intracellular compartments with neutral or very weakly acidic environments such as early endosomes. When the cells were additionally incubated in the culture medium for 24 h, then the green dots of FI-dendrimers were completely located on the red color-stained places in the overlay image (Supporting Information, Figure S11), suggesting that the dendrimers were transferred into lysosomes in 24 h. We also examined the effect of the incubation temperature on intracellular distribution of the temperature-insensitive amine-terminated G4 PAMAM dendrimer labeled with FITC. The cells treated with the dendrimers at 37 °C (Figure 6C) and 41 °C (Figure 6D) displayed similarly intensive green color dots derived from FITC-labeled dendrimers in the cells, indicating the similar dendrimer uptake at both temperatures, and the incubation temperature did not affect the cellular uptake of the temperature-insensitive dendrimer.

**In Vitro Cytotoxicity Measurement.** We finally measured in vitro cytotoxicity of the temperature-sensitive (EDEG-PDEG)-G4 and (FI-EDEG-PDEG)-G4 dendrimers by determining the viabilities of HeLa cells. The unmodified G4 PAMAM dendrimer was used as a control. The HeLa cells were cultured with all dendrimers in DMEM at 37 °C, which is higher than the cloud points of PDEG-G4 (26.9 °C) and ($EDEG_{22.1}$-$PDEG_{41.9}$)-G4 (35.9 °C) and lower than the cloud points of ($EDEG_{33.0}$-$PDEG_{31.0}$)-G4 (40.8 °C), ($EDEG_{43.5}$-$PDEG_{20.5}$)-G4 (49.4 °C), EDEG-G4 (64.4 °C), and (FI-EDEG-PDEG)-G4 (39.2 °C) dendrimers. As Figure 7 shows, the viabilities of HeLa cells incubated with various (EDEG-PDEG)-G4 dendrimers remained at ca. 100% at concentrations of 5 mg/mL and 10 mg/mL. In addition, (FI-EDEG-PDEG)-G4 dendrimers showed no conspicuous toxic effects on HeLa cells compared with (EDEG-PDEG)-G4 dendrimers, meaning that the FI conjugation does not cause additional cell toxicity


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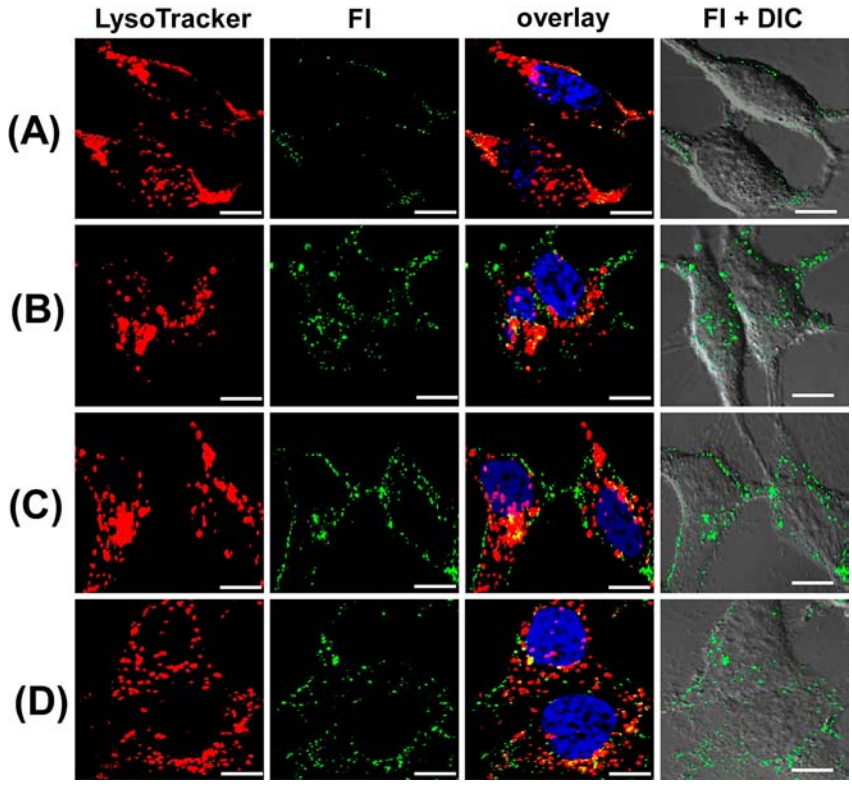


**Figure 6.** CLSM images for HeLa cells treated with the (FI-EDEG-PDEG)-G4 dendrimer at 37 °C (A) and 41 °C (B), and HeLa cells treated with the FI-G4 dendrimer at 37 °C (C) and 41 °C (D). The scale bar represents 10 $\mu$m.

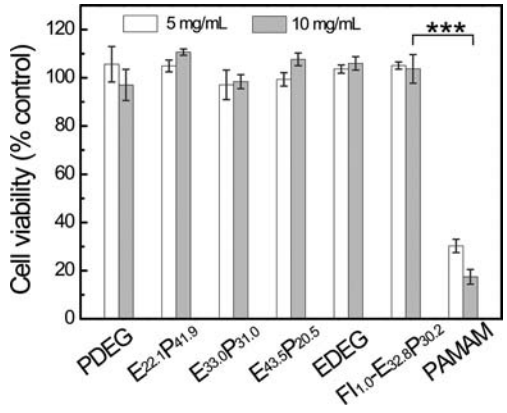


**Figure 7.** In vitro toxicity of various (EDEG-PDEG)-G4, (FI-EDEG-PDEG)-G4, and unmodified G4 PAMAM dendrimers to HeLa cells with dendrimer concentrations of 5 mg/mL and 10 mg/mL at 37 °C after 24 h of incubation. The cell without treatment of dendrimers was set as 100%. Data are given as the mean ± SD of triplicate experiments. *** $p < 0.001$.

on HeLa cells. In contrast, for cells treated with the unmodified dendrimer, the cellular viability decreased significantly at both 5 mg/mL and 10 mg/mL. Therefore, the (EDEG-PDEG)-G4 dendrimers generally exhibited much lower cytotoxicity than the amine-terminated dendrimers did. These results indicate that the R-DEG-modified PAMAM dendrimers were temperature-sensitive dendrimers with extremely low cytotoxicity.

## CONCLUSIONS

Through surface modification of PAMAM dendrimers with alkoxy diethylene glycols, we produced a novel type of temperature-sensitive dendrimer with tunable LCST. These dendrimers showed sharply changed surface properties, between hydrophilic and hydrophobic, at the LCST. The transition temperature can be adjusted approximately to human body temperature by controlling the ratios of different alkoxy diethylene glycols in the dendrimer periphery. Furthermore, the dendrimers exhibited low cellular toxicity below and above the LCST. Excellent noncytotoxic and highly temperature-controllable surface properties of R-DEG-modified PAMAM dendrimers can engender the development of intelligent drug-targeting tools of a new type, which can achieve precision drug delivery through combinations of size-control-induced passive targeting and temperature-sensitive surface property-induced active targeting with local hyperthermia. Further studies for application of these temperature-sensitive dendrimers to drug and nanoparticle delivery are now in progress.

## ASSOCIATED CONTENT

### Supporting Information

Synthesis conditions, $^{1}H$ and $^{13}C$ NMR spectra for various R-DEG-modified PAMAM dendrimers and (FI-EDEG-PDEG)-G4 dendrimers, microcalorimetoric endotherms for (EDEG-PDEG)-G4 dendrimers, zeta potential change for (FI-EDEG-PDEG)-G4 dendrimers during the heating process, and CLSM images of HeLa cells treated with (FI-EDEG-PDEG)-G4 and FI-G4 dendrimers at 41 °C after 24 h. This material is available free of charge via the Internet at http://pubs.acs.org.

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## Notes

The authors declare no competing financial interest.

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