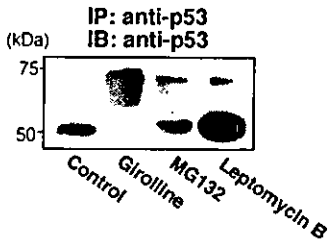


Fig. 3. The Effect of Girolline is Different from the Effect of Leptomycin B

Cell extracts were prepared from FL cells cultured in the presences of 50 $\mu$M girolline, 2 $\mu$M MG132, and 50 ng/ml leptomycin B for 24 h and were then subjected to immunoprecipitation (IP) using anti-p53 antibody-immobilized agarose. The immunoprecipitates thus formed were then subjected to Western blotting with anti-p53 antibody. IB, immunoblotting.

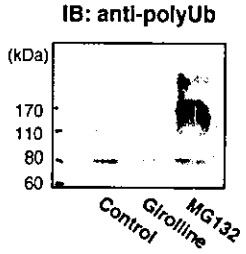


Fig. 4. Girolline Fails to Induce Accumulation of Polyubiquitinated Cellular Proteins

Cell extracts prepared from FL cells cultured in the presence of 50 $\mu$M girolline and 2 $\mu$M MG132 were subjected to SDS-PAGE and then to Western blotting with FK1 antibody, which specifically recognizes polyubiquitin chains (anti-polyUb). IB, immunoblotting.

the cytoplasm and are then polyubiquitinated/degraded.[14,15] To examine the possibility that girolline affects the export of polyubiquitinated p53 from the nucleus, we compared the effects of girolline and leptomycin B, an inhibitor of nuclear export, on accumulation of polyubiquitinated p53 (Fig. 3). Leptomycin B has been reported to completely inhibit MDM2- and E6-AP-dependent proteasomal degradation of p53.[16] In contrast to the effect of girolline, leptomycin B did not induce accumulation of polyubiquitinated p53. These results strongly suggest that girolline does not inhibit the nuclear export of polyubiquitinated p53.

**The Effect of Girolline is Specific for p53** We next carried out an experiment to determine whether treatment with girolline results in accumulation of polyubiquitinated proteins other than p53. Cell extracts prepared from FL cells that had been pretreated with girolline and MG132 (a positive control) were subjected to SDS-PAGE and then to Western blotting with FK1 antibody, which specifically recognizes polyubiquitin chains (Fig. 4).[12] MG132, a proteasome inhibitor, induced accumulation of polyubiquitinated cellular proteins. In contrast, treatment with girolline induced little accumulation of polyubiquitinated cellular proteins. These results strongly suggest that the effect of girolline is specific for p53 and that this compound inhibits the degradation of polyubiquitinated p53 but not that of other polyubiquitinated cellular proteins.

**A Possible Target of Girolline** Girolline has been reported to inhibit protein synthesis.[8] In contrast, in our study, treatment with cycloheximide, a protein synthesis inhibitor, induced little accumulation of polyubiquitinated p53 (data not shown). Since polyubiquitinated proteins are targeted to the proteasome and degraded, all of the above results suggest that girolline inhibits recruitment of polyubiquitinated p53 to the proteasome. Rad23 and Dsk2, ubiquitin-like proteins that have affinities toward polyubiquitinated proteins and also are capable of binding to the proteasome, have been reported to function in the recruitment of polyubiquitinated proteins to the proteasome.[17] It is quite possible that the target of girolline is such a factor functioning in the recruitment of polyubiquitinated p53 to the proteasome. Since girolline failed to induce accumulation of other polyubiquitinated cellular proteins, the candidate factor seems to be specific for p53. Although the target of girolline has not yet been identified, it can be concluded that girolline is a novel-type inhibitor against the ubiquitin-dependent proteplytic pathway.

It is well known that p53-dependent G1 arrest of the cell cycle is an important component of the cellular response to stress, but the mechanism of p53-dependent G2 arrest has not yet been elucidated.[5,6,13] Girolline induces mainly G2/M arrest in FL (wild type p53), HeLa (defective p53), and A549 (wild type p53) cells and also induces the accumulation of polyubiquitinated p53 at least in FL cells. Further study is required to determine whether accumulation of polyubiquitinated p53 causes G2/M arrest.

**Acknowledgments** We thank M. Yoshida for providing leptomycin B. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan, the Hayashi Memorial Foundation for Female Natural Scientists, the Fugaku Trust for Medicinal Research, the Sumitomo Foundation, and the Naito Foundation.

## REFERENCES

1) Hershko A., Ciechanover A., *Annu. Rev. Biochem.*, **67**, 425—479 (1998).
2) Voges D., Zwickl P., Baumeister W., *Annu. Rev. Biochem.*, **68**, 1015—1068 (1999).
3) Pickart C. M., *Annu. Rev. Biochem.*, **70**, 503—533 (2001).
4) Glickman M. H., Ciechanover A., *Physiol. Rev.*, **82**, 373—428 (2002).
5) Alarcon-Vargas D., Ronai Z., *Carcinogenesis*, **23**, 541—547 (2002).
6) Oren M., *Cell Death Differ.*, **10**, 431—442 (2003).
7) Ahond A., Bedoya-Zurita M., Colin M., Fizames C., Laboute P., Lavelle F., Laurent D., Poupat C., Pusset J., Pusset M., Thoison O., Potier P., *C. R. Acad. Sci. Paris 2*, **307**, 145—148 (1988).
8) Lavelle F., Zerial A., Fizames C., Rabault B., Curaudeau A., *Invest. New Drugs*, **9**, 233—244 (1991).
9) Colson G., Rabault B., Lavelle F., Zerial A., *Biochem. Pharmacol.*, **43**, 1717—1723 (1992).
10) Catimel G., Coquard R., Guastalla J. P., Merrouche Y., Le Bail N., Klink Alakl M., Dumortier A., Foy M., Clavel M., *Cancer Chemother. Pharmacol.*, **35**, 246—248 (1995).
11) Tsukamoto S., Tane K., Ohta T., Matsunaga S., Fusetani N., van Soest R. W. M., *J. Nat. Prod.*, **64**, 1576—1578 (2001).
12) Fujimuro M., Sawada H., Yokosawa H., *FEBS Lett.*, **349**, 173—180 (1994).
13) Taylor W. R., Stark G. R., *Oncogene*, **20**, 1803—1815 (2001).
14) Nakayama K., Nagahama H., Minamishima Y. A., Matsumoto M., Nakamichi I., Kitagawa K., Shirane M., Tsunematsu R., Tsukiyama T., Ishida N., Kitagawa M., Nakayama K., Hatakeyama S., *EMBO J.*, **19**, 2069—2081 (2000).
15) Hara T., Kamura T., Nakayama K., Oshikawa K., Hatakeyama S., Nakayama K., *J. Biol. Chem.*, **276**, 48937—48943 (2001).
16) Freedman D. A., Levine A. J., *Mol. Cell. Biol.*, **18**, 7288—7293 (1998).
17) Hartmann-Petersen R., Seeger M., Gordon C., *Trends Biochem. Sci.*, **28**, 26—31 (2003).

# Transfection Activity of Polyamidoamine Dendrimers Having Hydrophobic Amino Acid Residues in the Periphery

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We designed poly(amidoamine) dendrimers with phenylalanine or leucine residues at their chain ends. Thereby, we achieved efficient gene transfection of cells through synergy of the proton sponge effect, which is induced by the internal tertiary amines of the dendrimer, and hydrophobic interaction by the hydrophobic amino acid residues in the dendrimer periphery. Dendrimers having 16, 29, 46, and 64 terminal phenylalanine residues were prepared by the reaction of the amine-terminated poly(amidoamine) G4 dendrimer and L-phenylalanine using condensing reagent 1,3-dicyclohexylcarbodiimide. Transfection activity of these phenylalanine-modified dendrimers for CV1 cells, an African green monkey kidney cell line, increased concomitant with the increasing number of the terminal phenylalanine residues, except for the dendrimer with 64 phenylalanine residues, which showed poor water solubility and hardly formed a complex with DNA at neutral pH. However, under weakly acidic conditions, the dendrimer with 64 phenylalanine residues formed a complex with DNA, thereby achieving highly efficient transfection. In contrast, the attachment of L-leucine residues was unable to improve the transfection activity of the parent dendrimer, probably because of the relatively lower hydrophobicity of this amino acid. The phenylalanine-modified dendrimer exhibited a higher transfection activity and a lower cytotoxicity than some widely used transfection reagents. For that reason, the phenylalanine-modified dendrimers are considered to be promising gene carriers.

## INTRODUCTION

Development of safe and efficient nonviral vectors is an important task in the field of gene therapy. Despite many efforts to develop nonviral vectors, their ability to transfect cells has yet to be improved (*1*, *2*). A variety of nonviral systems have been studied for this purpose. Cationic polymers offer some advantages such as a large diversity of molecular structures and freedom of molecular design (*3*, *4*). Cationic polymers can form stable complexes, so-called polyplexes, with DNA through electrostatic interactions. Positively charged polyplexes interact strongly with negatively charged cell membranes and are taken up by the cells through an endocytic pathway. However, many cationic polymers cannot achieve efficient transfection. A major cause is that, when internalized into the cell and taken up in the endosome, a large fraction of their complexes with DNA is delivered to the lysosome and is ultimately degraded there.

Nevertheless, several kinds of cationic polymers can mediate relatively efficient transfection of various animal cells. Poly(ethylenimine) (PEI) and poly(amidoamine) (PAMAM) dendrimers are among these polymers (*5*–*8*). These polymers possess many secondary and tertiary amino groups, which become protonated under weakly acidic conditions. These amino groups suppress the lowering of pH in endosomes and lysosomes by adsorbing protons and prohibiting their degradation in the lysosome. In addition, endosome buffering by these polymers is thought to induce osmotic swelling of the endosome interior, engendering rupture of the endosome and subsequent release of DNA into cytoplasm (*9*, *10*). Such a function of these secondary and tertiary amino groups of polymers is called the proton sponge effect (*6*).

PAMAM dendrimers are unique synthetic macromolecules with highly branched structure and globular shape (*11*, *12*). Molecular size of the dendrimers is increased stepwise via the repetition of a reaction sequence. Consequently, their size and structure are highly controllable and their molecular weight distribution is generally very narrow (*12*). Taking into account the high controllability of structure of the PAMAM dendrimers and their relatively high transfection activity, PAMAM dendrimers are highly attractive materials for the design of gene carriers. For that reason, many studies have been carried out to produce efficient gene carriers using PAMAM dendrimers as base materials (*13*). For example, Haensler and Szoka attempted to improve their transfection activity by conjugation of a pH-sensitive membrane-active peptide that destabilizes the endosomal membrane and promotes the escape of DNA into the cytosol (*5*). Arima and colleagues also prepared conjugates of PAMAM dendrimers with cyclodextrins, concluding that this conjugation greatly enhanced the transfection activity of PAMAM dendrimers (*14*, *15*). In addition, modification of PAMAM dendrimers with various molecules, such as a hydrophobic fluorescent dye (*16*) and poly(ethylene glycol) (*17*), demonstrably improves their efficiency for gene and oligonucleotide delivery.

Many studies have established that hydrophobic moieties affect transfection activity of cationic polymers (*18*–*22*). The hydrophobic nature of gene carriers will influence formation and stability of complexes between the

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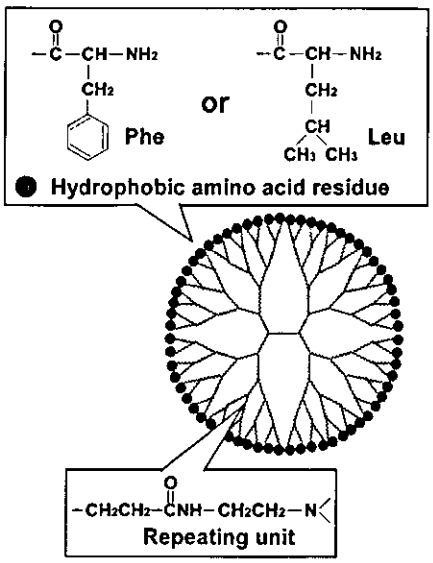


**Figure 1.** Structure of phenylalanine- or leucine-modified PAMAM G4 dendrimer.

carriers and DNA as it enhances interactions between the carriers and cells. In a previous study (*23*), we prepared PAMAM dendrons having two dodecyl groups. That study found that dendrons with hydrophobic moieties showed much higher transfection activity than PAMAM dendrimers of the same generation. That result suggests that introduction of an appropriate amount of hydrophobic moieties to the dendrimers can elevate their transfection efficiency.

In this study, we designed PAMAM G4 dendrimers having hydrophobic amino acid residues to produce efficient nonviral vectors that achieve cell transfection by synergistic actions of hydrophobic interactions and the proton sponge effect. We prepared PAMAM G4 dendrimers having phenylalanine or leucine residues at the dendrimer chain ends (Figure 1). Subsequently, we investigated their abilities to form a polyplex with plasmid DNA and to transfect CV1 cells, an African green monkey kidney cell line. The remarkable improvement of transfection activity of the PAMAM G4 dendrimer achieved by incorporation of phenylalanine is described.

## EXPERIMENTAL PROCEDURES

**General Methods.** PAMAM (starbust) G4 dendrimer with an ethylenediamine core was purchased from Aldrich Chemical (Milwaukee, WI). *N*-(*tert*-Butoxycarbonyl)-L-phenylalanine (Boc-Phe) and *N*-(*tert*-butoxycarbonyl)-L-leucine (Boc-Leu) were obtained from Peptide Institute, Inc. (Osaka, Japan). 1, 3-Dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide were supplied from Tokyo Kasei Kogyo (Tokyo, Japan). Trifluoroacetic acid, triethylamine, 2-amino-2-hydroxymethyl-1,3-propanediol (Tris), and *N*,*N*-dimethylformamide (DMF) were from Kishida Chemical (Osaka, Japan). Dimethyl sulfoxide (DMSO) and ethidium bromide were from Wako Pure Chemical Industries (Osaka, Japan). Fetal calf serum (FCS) was from JRH Biosciences (Tokyo, Japan). Agarose was from Sigma (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) was from Nissui Pharmaceutical (Tokyo, Japan). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) was obtained from Dojin Laboratories (Kumamoto, Japan). Lipofectamine and SuperFect were purchased from Invitrogen (Carlsbad, CA) and Qiagen (Hilden, Germany), respectively. Plasmid DNA pCMV-Luc, which contains the cDNA of firefly luciferase driven by a human cytomegalovirus immediate-early promoter, was a gift from Dr. Kazuo Maruyama, Teikyo University.

**Synthesis of Phenylalanine-Modified PAMAM G4 Dendrimers.** The PAMAM dendrimers having varying numbers of phenylalanine residues at the chain terminals were prepared by the reaction of the amine-terminated PAMAM G4 dendrimers with varying amounts of Boc-Phe using *N*-hydroxysuccinimide and DCC and subsequent deprotection of Boc-Phe residues attached to the dendrimers using trifluoroacetic acid and thioanisole. A typical procedure was as follows: *N*-hydroxysuccinimide (44 mg, 0.380 mmol) and DCC (85 mg, 0.412 mmol) were added to Boc-Phe (84 mg, 0.317 mmol) dissolved in DMF (2 mL) and stirred for 7 h. Then, the PAMAM G4 dendrimer (46.9 mg, 3.30 nmol) and triethylamine (0.066 mL, 0.475 mmol) dissolved in DMSO (2 mL) were added to the solution and stirred for 7 days at room temperature. The obtained Boc-Phe-attached G4 dendrimer was purified with a Sephadex LH-20 column using methanol as the eluent. Boc groups of the dendrimer were removed by the treatment with trifluoroacetic acid (4 mL) and thioanisole (1 mL) at room temperature for 8 h. The dendrimer was purified by dialysis against distilled water and recovered by freeze-drying. Yield, 45.6 mg. Other Phe-modified dendrimers were prepared by the same procedure.

**Synthesis of Leucine-Attached PAMAM G4 Dendrimer.** *N*-Hydroxysuccinimide (102 mg, 0.883 mmol) and DCC (197 mg, 0.956 mmol) were added to Boc-Leu (170 mg, 0.735 mmol) dissolved in DMF (2 mL) and stirred for 3.5 h. Then, the PAMAM G4 dendrimer (108.9 mg, 7.66 nmol) and triethylamine (0.153 mL, 1.10 mmol) dissolved in DMSO (4 mL) were added to the solution and stirred for 8 days at room temperature. The obtained Boc-Leu-attached dendrimer was purified with a Sephadex LH-20 column using methanol as eluent. The deprotection of Boc-Leu-attached dendrimer and purification of the resultant dendrimer were carried out by the same procedure. Yield, 87.7 mg.

**Agarose Gel Electrophoresis.** The dendrimer–DNA polyplexes with varying N/P ratios were prepared by mixing plasmid DNA (1 $\mu$g) dissolved in 20 mM Tris-HCl buffer (5 $\mu$L) and the dendrimer dissolved in PBS (5 $\mu$L). After 10-min incubation at room temperature, the samples (10 $\mu$L) were electrophoresed on 0.6 wt % agarose gel in 40 mM Tris, 20 mM sodium acetate, and 2 mM EDTA buffer containing 1 $\mu$g/mL ethidium bromide at 100 V for 30 min. The ethidium bromide-stained bands were visualized and photographed using an Eagle Eye II Still Video System (Stratagene).

**Transfection.** For the preparation of dendrimer–plasmid DNA polyplexes, plasmid DNA (1 $\mu$g) dissolved in 20 mM Tris-HCl buffer (pH4.0–7.4, 50 $\mu$L) was mixed with the dendrimer dissolved in PBS of the same pH (0.2–5 g/l, 50 $\mu$L). The mixed solution was incubated for 10 min at room temperature to afford a polyplex with a given ratio of primary amino group of Phe-G4 to DNA phosphate (N/P ratio). The CV1 cells were seeded in 0.5 mL of DMEM supplemented with 10% FCS in 24-well culture plates at $5.0 \times 10^4$ cells per well the day before transfection. The cells were washed with PBS containing 0.36 mM $CaCl_2$ and 0.42 mM $MgCl_2$ [PBS(+)] and then covered with DMEM (1 mL). The polyplex containing plasmid DNA (1 $\mu$g) was added gently to the cells and incubated at 37 °C for 4 h. Then, the cells were rinsed with PBS(+), covered with DMEM containing 10% FCS,

and incubated at 37 °C. After 40 h, the cells were lysed by adding 50 μL of Luc-PGC-50 detergent (Toyo Ink, Tokyo, Japan). A 20 μL aliquot from each dish was used for one luciferase assay using a kit (Toyo Ink) and a Lumat LB9507 luminometer (Berthold, Bad Wildbad, Germany). The protein content of the lysate was measured by Coomassie Protein Assay Reagent (Pierce, IL) using bovine serum albumin as the standard. Transfection by Lipofectamine and Superfect was performed according to protocols recommended by the manufacturers.

**Cytotoxicity.** The cytotoxycity of complexes of the dendrimer, Lipofectamine, and Superfect with plasmid DNA was assessed by MTT assay (*24*). The cells were seeded in 1 mL of DMEM supplemented with 10% FCS in 12-well culture plates at $1.5 \times 10^5$ cells per well the day before transfection. The cells were washed with PBS (+) and then covered with DMEM (1 mL). The dendrimer−, Lipofectamine−, or SuperFect−DNA complex containing plasmid DNA (3μg) was added gently to the cells and incubated at 37 °C for 4 h. Then, the culture medium was carefully replaced with 0.5 mL of fresh DMEM containing 10% FCS, and 70 μL of MTT dissolved in PBS (5 mg/mL) was added to each well. After 4-h incubation at 37 °C, the medium was removed and the cells were solubilized in 500 μL of 2-propanol containing 0.1 M HCl. The number of viable cells was determined by absorbance at 570 nm. The cytotoxicity of the dendrimer, Lipofectamine, and Superfect without plasmid DNA was also measured by the same procedure.

## RESULTS AND DISCUSSION

**Design and Synthesis of Phenylalanine-Modified Dendrimers.** The PAMAM dendrimers are currently considered to be extremely potent and promising nonviral vectors. These polymers can achieve efficient transfection of cells by the proton sponge effect, which induces suppression of gene degradation by lysosomal enzymes and promotes the transfer of DNA into the cytosol, resulting in transfection of the cells. This study prepared PAMAM dendrimers having hydrophobic amino acid residues because such dendrimers are expected to achieve an efficient transfection by a synergy of the proton sponge effect and hydrophobic interactions with cellular membranes.

We used phenylalanine for modification of PAMAM G4 dendrimers because this amino acid has a highly hydrophobic side group. We have already shown that amino acid molecules can be attached to terminal amino groups of the PAMAM dendrimers using condensing agent, DCC (*25*). Therefore, in this study, we combined phenylalanine with the dendrimer chain ends by the same method. Figure 2 depicts $^1$H NMR spectra for the PAMAM G4 dendrimer (A), phenylalanine (B), and the dendrimer after reaction with 96 equiv of phenylalanine (C). Figure 2C shows that the spectrum of the dendrimer after the reaction exhibits signals corresponding to the PAMAM dendrimer and signals corresponding to phenylalanine, indicating that the amino acid residues are combined with the dendrimer. The number of phenylalanine residues attached to the dendrimer can be evaluated using the integral ratio of the signal at 2.2 ppm to 7−7.2 ppm. Table 1 lists the numbers of amino acid molecules added per dendrimer molecule for the reaction and the numbers of the amino acid residues combined to the dendrimer after the reaction. The number of phenylalanine residues combined to the dendrimer was controllable by adjusting the quantities of amino acids added for the condensation

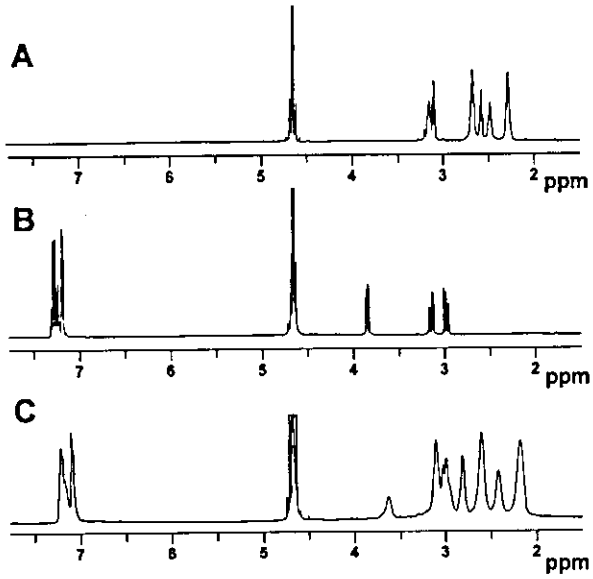


**Figure 2.** $^1$H NMR spectra for PAMAM G4 dendrimer (A), L-phenylalanine (B), and (Phe)64-G4 dendrimer (C) in $D_2O$.

**Table 1. Preparation of Phe- and Leu-Modified PAMAM G4 Dendrimers**[a]

| | number of amino acids per dendrimer | |
|---|---|---|
| dendrimer | in feed (mol/mol) | product (mol/mol) |
| $(Phe)_{16}$-G4 | 19.2 | 16.4 |
| $(Phe)_{29}$-G4 | 32.0 | 29.2 |
| $(Phe)_{46}$-G4 | 48.0 | 46.3 |
| $(Phe)_{64}$-G4 | 96.0 | 64.5 |
| $(Leu)_{63}$-G4 | 96.0 | 63.2 |

[a] Evaluated by $^1$H NMR spectra.

reaction. When 96 equiv quantities of phenylalanine were reacted with the dendrimer, 64 phenylalanine residues were combined to the dendrimer, indicating that this Phe-modified dendrimer has a phenylalanine residue at every chain end. Consequently, we prepared four kinds of phenylalanine-modified dendrimers, (Phe)16-G4, (Phe)-29-G4, (Phe)46-G4, and (Phe)64-G4, which possessed 16, 29, 46, and 64 phenylalanine residues, respectively (Table 1).

**Evaluation of Complex Formation with Plasmid DNA.** The PAMAM dendrimers can form complexes efficiently with plasmid DNA through electrostatic interaction between the protonated primary amine of the dendrimer and negatively charged phosphodiester groups of DNA (*8*, *26*). Nevertheless, attachment of phenylalanine to the primary amine may affect the dendrimers' ability to form the complex with DNA. For that reason, we examined the influence of attachment of phenylalanine residues to the dendrimer on its ability of complex formation using agarose gel electrophoresis (Figure 3). In this experiment, the plasmid DNA was incubated with the dendrimer having varying numbers of phenylalanine residues for 10 min at pH 7.4, and electrophoresed on an agarose gel. Figure 3 shows that the free DNA band was not seen at the N/P ratio of 1 for the unmodified PAMAM dendrimer, indicating that all plasmid DNA molecules were associated with the dendrimer. However, for dendrimers having phenylalanine residues, the free DNA band can be observed at N/P ratios higher than 1. In addition, dendrimers having more phenylalanine residues needed a higher N/P ratio to complete their complex formation with DNA. For the fully phenylala-

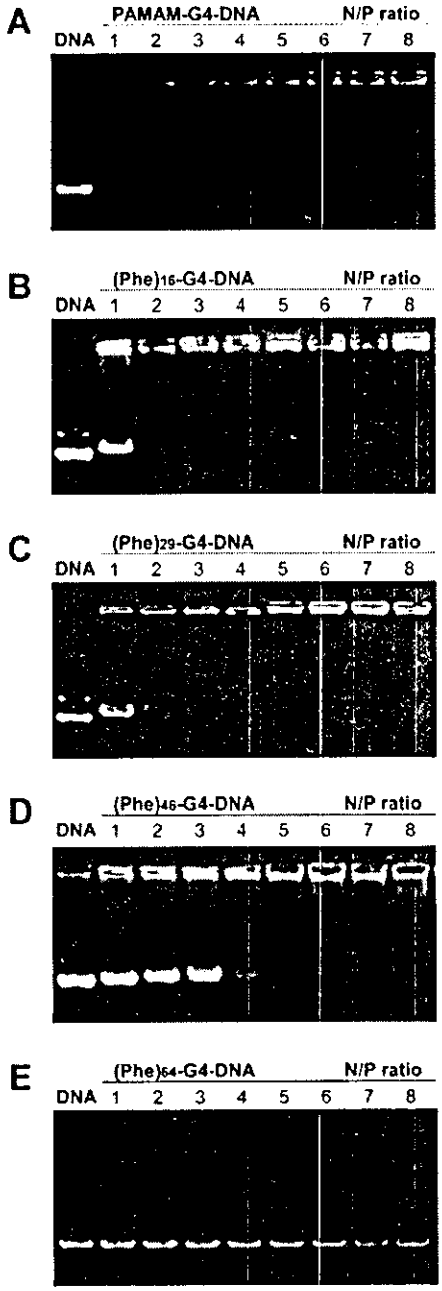


**Figure 3.** Agarose gel electrophoretic analysis for polyplex formation of PAMAM G4 dendrimers with varying numbers of phenylalanine residues: A, unmodified G4; B, (Phe)16-G4; C, (Phe)29-G4; D, (Phe)46-G4; E, (Phe)64-G4. Dendrimers and plasmid DNA were mixed at varying charge ratios and incubated for 10 min at room temperature. Then, the mixed solution was applied to the agarose gel and electrophoresed at 100 V for 30 min.

nine-substituted dendrimer, the free DNA band did not disappear, even at the N/P ratio of 8. These results emphasize that attachment of phenylalanine residues showed less ability to form a complex with DNA. Considering that protonation of the primary amino groups of the dendrimer occurs between pHs 7 and 10 (27) and the $pK_a$ of α-amino group of phenylalanine is 9.1, these amino groups possess similar basicity. However, when combined to the dendrimer chain terminal, phenylalanine

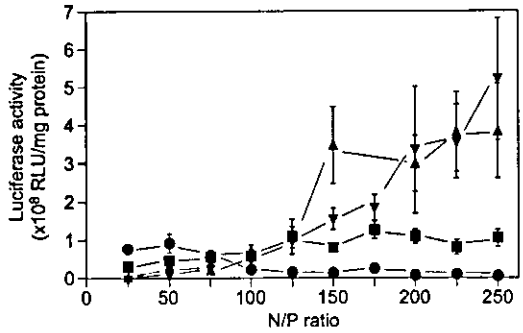


**Figure 4.** Luciferase activities of CV1 cells treated with polyplexes consisting of plasmid DNA and PAMAM G4 dendrimers with varying amounts of phenylalanine residues with varying N/P ratios: ■, unmodified G4; ●, (Phe)16-G4; ▲, (Phe)-29-G4; ▼, (Phe)46-G4 dendrimers. The cells ($5 \times 10^4$) were treated with the polyplexes containing 1 μg DNA. Each point represents the mean ± SD ($n = 3$).

residues were able to form a hydrophobic environment in the periphery of the dendrimer, reducing protonation of their α-amino groups. Consequently, the phenylalanine-modified dendrimer showed smaller ability to form a complex with DNA.

**Transfection Activity of Phenylalanine-Modified Dendrimers.** Transfection activity of cationic polymer–DNA complexes varies depending on their composition. Therefore, we prepared dendrimer–DNA complexes at various N/P ratios and examined their transfection activity for CV1 cells. Figure 4 depicts luciferase activity of the cells treated with complexes consisting of plasmid DNA containing firefly luciferase gene and the dendrimers having varying numbers of phenylalanine residues. The (Phe)16-G4 dendrimer showed a similar or smaller transfection activity compared to the unmodified dendrimer. However, the dendrimer having 29 or 46 phenylalanine residues exhibited higher transfection activity. This result suggests that increased hydrophobicity enhances the transfection activity of the dendrimer. For that reason, it is expected that a fully phenylalanine-substituted dendrimer may exhibit the highest transfection activity among phenylalanine-modified dendrimers. Nevertheless, this dendrimer exhibited poor ability to form a complex with plasmid DNA, as mentioned above.

**Transfection Activity of Phenylalanine-Modified Dendrimers at Acidic pH.** Although the (Phe)64-G4 dendrimer hardly formed a complex with DNA at pH 7.4, it is possible that this dendrimer exhibits a higher ability to form the complex under acidic conditions because such conditions would enhance protonation of the amino groups contained in the (Phe)64-G4 dendrimer. Agarose gel electrophoresis was performed for mixtures of the dendrimer and DNA at various N/P ratios at pH 4–6 to examine the influence of pH on the ability of the dendrimer to form the complex. As shown in Figure 5, the N/P ratio at which the free DNA band disappears decreases with decreasing pH. This indicates that the ability of the dendrimer to perform complex formation increases with decreasing pH.

We next examined transfection activity of complexes prepared by mixing the (Phe)64-G4 dendrimer and plasmid DNA at varying N/P ratios at mildly acidic pHs. Figure 6A depicts luciferase expression of CV1 cells treated with these complexes. Transfection activity of these complexes was affected markedly by their composition. However, complexes with N/P ratios of 50–60 exhibited remarkably high transfection activity. We

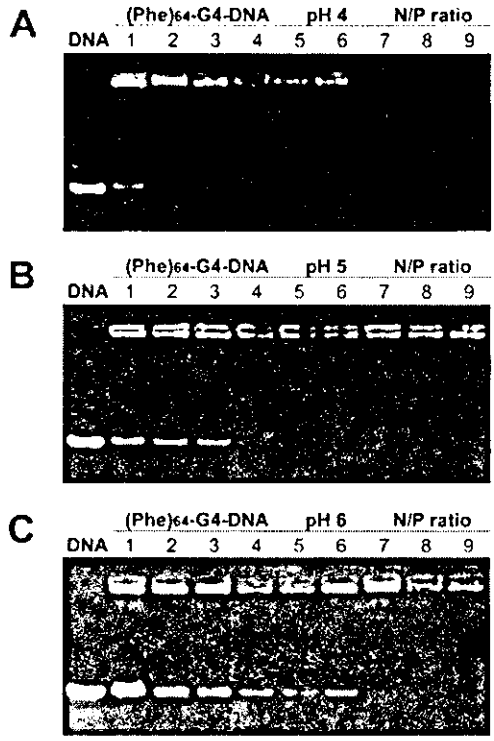


**Figure 5.** Agarose gel electrophoretic analysis for polyplex formation of (Phe)64-G4 dendrimer at pHs 4.0 (A), 5.0 (B), and 6.0 (C). The dendrimer and plasmid DNA were mixed at varying charge ratios and incubated for 10 min at room temperature. Then, the mixed solution was applied to the agarose gel and electrophoresed at 100 V for 30 min.

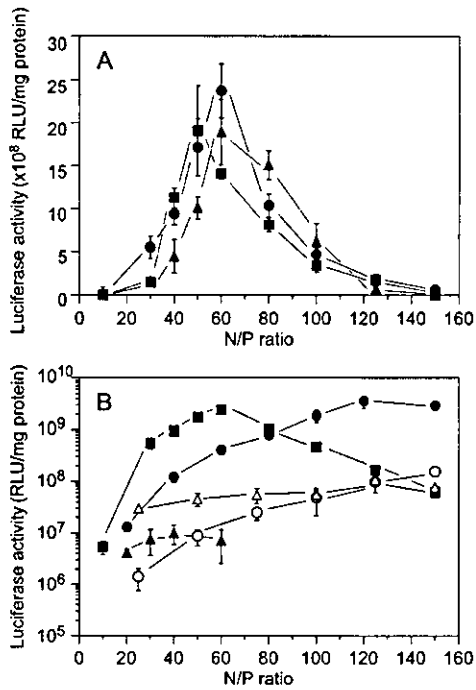


**Figure 6.** Influence of preparation pH on transfection activity of polyplex. (A) Luciferase activities of CV1 cells treated with (Phe)64-G4 polyplex with varying N/P ratios prepared at pHs 4.0 (■), 5.0 (●), and 6.0 (▲). (B) Luciferase activities of CV1 cells treated with (Phe)64-G4 (squares), (Phe)46-G4 (circles), and unmodified G4 (triangles) dendrimer polyplexes prepared at pHs 7.4 (open symbols) and 5.0 (closed symbols). The cells ($5 \times 10^4$) were treated with the polyplex containing 1 $\mu$g DNA. Each point represents the mean ± SD ($n = 3$).

compared the (Phe)46-G4 dendrimer–DNA complex with the N/P ratio of 250 prepared at pH 7.4, which shows the highest activity among the polyplexes prepared at pH 7.4 (Figure 4), and the (Phe)64-G4 dendrimer–DNA polyplexes with the N/P ratio of 60 prepared at pH 5.0. The latter exhibited five times higher expression of luciferase gene, although the amount of the dendrimer contained in the latter polyplex was about one-fourth of that contained in the former polyplex. In addition, compared with the polyplex of the unmodified dendrimer, the (Phe)64-G4 dendrimer–polyplex exhibited about 30 times higher activity.

We further examined cell transfection by the polyplexes of (Phe)46-G4 or unmodified G4 dendrimer prepared at pH 5.0 and compared the result with that of the (Phe)64-G4 dendrimer-containing polyplex (Figure 6B). Thereby, we determined the influence of the pH of polyplex formation on transfection activity. The polyplex of the unmodified dendrimer showed decreased activity when prepared at pH 5.0. Because the charge density of the dendrimer is very high, DNA molecules might be bound very tightly to the dendrimer, resulting in the reduced transfection activity. However, regarding the (Phe)46-G4 dendrimer-containing polyplex, when prepared at pH 5.0, its activity increased 1 order of magnitude over that of the polyplex prepared at pH 7.4. A mildly acidic condition appears to moderately increase protonation of amino groups with reduced basicity in the dendrimer periphery; the resultant polyplex might show high transfection activity because of its hydrophobic nature and proton sponge effect. In comparison with the (Phe)64-G4–dendrimer polyplex, the (Phe)46-G4–dendrimer polyplex required a much higher N/P ratio. Consequently, that polyplex required a much higher amount of the dendrimer to achieve maximum activity, even though these polyplexes with the optimized compositions show approximately the same transfection activity.

**Comparison between Phe-Modified and Leu-Modified Dendrimers.** We first examined the effect of the attachment of L-phenylalanine on transfection activity of the PAMAM dendrimer. Next, L-leucine was used to modify the dendrimer as another hydrophobic amino acid. An excess of leucine was reacted with the PAMAM G4 dendrimer using DCC. The resultant dendrimer contained 63 leucine residues per dendrimer, as evaluated by $^1$H NMR (Table 1). The ability of the (Leu)63-G4 dendrimer to form polyplexes was examined using agarose gel electrophoresis. The plasmid DNA was incubated with the leucine-modified dendrimer for 10 min at pH 7.4; then it was electrophoresed on an agarose gel. In contrast to the case of the (Phe)64-G4 dendrimer, no free DNA band was observed even at the N/P ratio of 1, indicating that the (Leu)63-G4 dendrimer has a much higher ability to form a polyplex with DNA (results not shown).

We examined the effect of attachment of leucine residues on transfection activity of the PAMAM dendrimer. CV1 cells were treated with the (Leu)63-G4 dendrimer polyplexes prepared at pH 7.4 and pH 5.0. Expression of luciferase in the treated cells was estimated, and the results were compared with the case of (Phe)64-G4 dendrimer polyplexes. We expected that incorporation of leucine residues to the chain ends of the dendrimer would elevate its transfection activity. How-

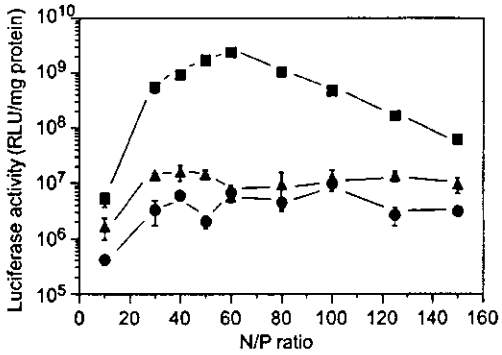


**Figure 7.** Comparison of transfection activity of luecine- and phenylalanine-modified dendrimers. Luciferase activities of CV1 cells treated with (Phe)64-G4 polyplex prepared at pH 5.0 (■) and (Leu)63-G4 polyplexes prepared at pHs 7.4 (▲) and 5.0 (●) containing 1 μg DNA with varying N/P ratios were shown in the figure. Each bar represents the mean ± SD ($n$ = 3).

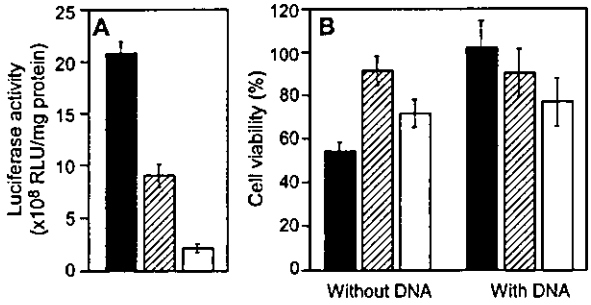


**Figure 8.** Comparison of transfection activity and cytotoxicity of (Phe)64-G4 dendrimer with widely used vectors. (A) Luciferase activities of CV1 cells treated with complexes of (Phe)-64-G4 dendrimers (closed bars), Lipofectamine (hatched bars), and SuperFect (open bars) containing 1 μg of DNA. Each bar represents the mean ± SD ($n$ = 4). (B) Viability of CV1 cells treated with (Phe)64-G4 dendrimers (closed bars), Lipofectamine (hatched bars), and SuperFect (open bars) with or without plasmid DNA. Viability was expressed as the percentage of the untreated control cells. Each bar represents the mean ± SD ($n$ = 3). The (Phe)64-G4 dendrimer polyplex with N/P ratio of 60 prepared at pH 5.0 was used.

ever, as shown in Figure 7, leucine residues did not increase its transfection activity at either pH compared with the unmodified dendrimer. Consequently, the (Leu)-63-G4 dendrimer–polyplexes exhibited a significantly lower activity than the (Phe)64-G4 dendrimer polyplexes.

As mentioned previously, we designed phenylalanine-modified and leucine-modified PAMAM dendrimers because we expected that these dendrimers express high transfection activity by synergistic actions of the proton sponge effect and hydrophobic interactions with cells. In fact, the attachment of phenylalanine increased the transfection activity of the parent dendrimer by about 2 orders of magnitude over that of the parent dendrimer (Figure 6). In contrast, the attachment of leucine showed no remarkable enhancement of dendrimer activity even though this amino acid is also hydrophobic. As a measure of hydrophobicity of the amino acid, Nozaki and Tanford used differences in free energy between a hydrophobic moiety of an amino acid in ethanol (or dioxane) and in water and showed that the free energies of transfer for phenylalanine and for leucine are 2500 and 1800 cal/mol, respectively (*28*), indicating that phenylalanine is more hydrophobic. We observed that (Phe)64-G4 dendrimer showed poor water solubility at pH 7.4, whereas (Leu)-63-G4 dendrimer was soluble in water at the same pH, although both dendrimers have essentially the same number of amino acid residues. This fact implies that the attachment of phenylalanine to the dendrimer lent a highly hydrophobic character to the dendrimer periphery. In fact, Meijer and co-workers reported that phenylalanine residues, when combined to the chain ends of poly(propylenimine) dendrimers, form a densely packed shell on the dendrimer surface (*29*, *30*). Therefore, the highly hydrophobic surface of densely packed phenylalanine residues of the dendrimer might enhance interaction of the dendrimer–DNA polyplexes and cellular membranes, engendering efficient transfection.

Recently, Shibata et al. reported that a random copolymer of L-lysine and L-phenylalanine translocates phospholipid membranes much faster than a poly(L-lysine) (*31*). This rapidity is probably attributable to the random incorporation of hydrophobic phenylalanine residues into the positively charged poly(lysine) chain. Those residues lowered the potential barrier in the hydrophobic region of the lipid membrane for the polypeptide translocation (*31*). Considering that observation, it is possible that the hydrophobic shell of phenylalanine on the dendrimer surface interacts strongly with the hydrophobic region of the cellular membranes and causes their destabilization, thereby enhancing translocation of the gene through the membrane.

**Transfection Activity of Phe-modified Dendrimers and Some Vectors.** We compared the respective activities of Lipofectamine and SuperFect, both of which are widely used and commercially available vectors, to evaluate the potential of phenylalanine-modified dendrimers as a gene carrier. We treated CV1 cells with (Phe)64-G4 dendrimer–DNA polyplex, Lipofectamine–DNA complex, and SuperFect–DNA complex; then we evaluated the cells' expression of luciferase. Figure 8A shows that the (Phe)64-G4 dendrimer polyplex induced about 2.3-fold higher and about 10-fold higher expression of luciferase, respectively, than Lipofectamine and SuperFect complexes that contained identical amounts of plasmid DNA.

The potential use of the phenylalanine-modified dendrimer as a gene carrier was also estimated from the viewpoint of cytotoxicity. Figure 8B depicts the viability of the cells treated with the (Phe)64-G4 dendrimer, Lipofectamine, and SuperFect, and their complexes with the plasmid DNA. The (Phe)64-G4 dendrimer itself was shown to be relatively toxic compared to these commercially available reagents. It is likely that the phenylalanine-modified dendrimer interacts strongly with the cell membranes and damages the cells because of the hydrophobic nature of the phenylalanine. However, in the form of the complex with DNA, the toxicity of the phenylalanine-modified dendrimer decreased remarkably. This decrease is attributable to the weakening of their strong interaction with cellular membranes by complexation with the hydrated DNA molecule. These results indicate that the phenylalanine-modified dendrimer can be considered as an efficient vector.

## CONCLUSION

In this study, we prepared PAMAM G4 dendrimers having hydrophobic amino acid residues, which achieve an efficient transfection of cells through synergy of the proton sponge effect induced by the dendrimer tertiary amines and hydrophobic interaction by the amino acid residues. Transfection activity was not improved when leucine was attached to the dendrimer. However, the attachment of phenylalanine greatly increased the trans-

fection activity of the parent dendrimer. Activity of the fully phenylalanine-substituted dendrimer was shown to be higher than for some widely used vectors. Transfection activity of the PAMAM dendrimers is known to increase with generation (*5*, *8*). Therefore, the phenylalanine-modified dendrimers with higher generations may exhibit still higher activity than the present dendrimers. For this reason, the findings obtained in this study will engender the development of efficient gene carriers. We are currently studying the effect of dendrimer generation on transfection activity of phenylalanine-modified dendrimers.

ACKNOWLEDGMENT

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LITERATURE CITED

(1) Mountain, A. (2000) Gene therapy: the first decade. *Trends Biotechnol. 18*, 119−128.
(2) Crystal, R. G. (1995) Transfer of genes to humans: early lessons and obstacles to success. *Science 270*, 404−410.
(3) Brown, M. D., Schätzlein, A. G., and Uchegbu, I. F. (2001) Gene delivery with synthetic (non viral) carriers. *Int. J. Pharm. 229*, 1−21.
(4) Behr, J. P. (1993) Synthetic gene-transfer vectors. *Acc. Chem. Res. 26*, 274−278.
(5) Haensler, J., and Szoka, F. C., Jr. (1993) Polyamidoamine cascade polymers mediate efficient transfection of cells in culture. *Bioconjugate Chem. 4*, 372−379.
(6) Boussif, O., Lezoualc'h, F., Zanta, M. A., Mergny, M. D., Scherman, D., Demeneix, B., and Behr, J. P. (1995) A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: Polyethylenimine. *Proc. Natl. Acad. Sci. U.S.A. 92*, 7297−7301.
(7) Tang, M., Redemann, C. T., and Szoka, F. C., Jr. (1996) In vitro gene delivery by degraded polyamidoamine dendrimers. *Bioconjugate Chem. 7*, 703−714.
(8) Kukowska-Latallo, J. F., Bielinska, A. U., Johnson, J., Spindler, R., Tomalia, D. A., and Baker, J. R., Jr. (1996) Efficient transfer of genetic material into mamalian cells using Starburst polyamidoamine dendrimers. *Proc. Natl. Acad. Sci. U.S.A. 93*, 4897−4902.
(9) Zou, S. M., Behr, J. P., Goula, D., and Demeneix, B. (2000) Gene delivery with polyethylenimine. In *Gene Therapy: Therapeutic mechanisms and strategies* (Templeton, N. S., Lasic, D. D., Eds.) pp 131−139, Marcel Dekker, New York.
(10) Sonawane, N. D., Szoka, F. C., Jr., and Verkman, A. S. (2003) Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine-DNA polyplexes. *J. Biol. Chem. 278*, 44826−44831.
(11) Tomalia, D. A., Baker, H., Dewald, J., Hall, M., Kallos, G., Martin, S., Roeck, J., Ryder, J., and Smith, P. (1985) A new class of polymers: starburst-dendritic macromolecules. *Polym. J. 17*, 117−132.
(12) Tomalia, D. A., Naylor, A. M., and Goddard, W. A. (1990) Starburst dendrimers: molecular-level control of size, shape, surface chemistry, topology, and flexibility from atoms to macroscopic matter. *Angew. Chem., Int. Ed. 29*, 138−175.
(13) Kubasiak, L. A., and Tomalia, D. A. (2004) Cationic dendrimers as gene transfection vectors: dendri- poly(amidoamines) and dendri- poly(propyleneimines). In *Polymeric Gene Delivery: Principles and Applications* (Amiji, M. M., Eds.) Chapter 9, pp 133−157, CRC Press, Boca Raton, FL.
(14) Arima, H., Kihara, F., Hirayama, F., and Uekama, K. (2001) Enhancement of gene expression by polyamidoamine dendrimer conjugates with α-, β-, and γ-cyclodextrins. *Bioconjugate Chem. 12*, 476−484.
(15) Kihara, F., Arima, H., Tsutsumi, T., Hirayama, F., and Uekama, K. (2002) Effects of structure of polyamidoamine dendrimer on gene transfer efficiency of the dendrimer conjugate with α-cyclodextrin. *Bioconjugate Chem. 13*, 1211−1219.
(16) Yoo, H., and Juliano, R. L. (2000) Enhanced delivery of antisense oligonucleotides with fluorofore-conjugated PAMAM dendrimers. *Nucleic Acids Res. 28*, 4225−4231.
(17) Luo, D., Haverstick, K., Belcheva, N., Han, E., and Saltzman, W. M. (2002) Poly(ethylene glycol)-conjugated PAMAM dendrimer for biocompatible, high-efficiency DNA delivery. *Macromolecules 35*, 3456−3462.
(18) Zhou, X., Klibanov, A. L., and Huang, L. (1991) Lipophilic polylysines mediate efficient DNA transfection in mammalian cells. *Biochim. Biophys. Acta 1065*, 8−14.
(19) Remy, J. S., Sirlin, C., Vierling, P., and Behr, J. P. (1994) Gene transfer with a series of lipophilic DNA-binding molecules. *Bioconjugate Chem. 5*, 647−654.
(20) Kurisawa, M., Yokoyama, M., and Okano, T. (2000) Transfection efficiency increases by incorporating hydrophobic monomer units into polymeric gene carriers. *J. Controlled Release 68*, 1−8.
(21) Han, S., Mahato, R. I., and Kim, S. W. (2001) Water-soluble lipopolymer for gene delivery. *Bioconjugate Chem. 12*, 337−345.
(22) Wang, D., Narang, A. S., Kotb, M., Gaber, A. O., Miller, D. D., Kim, S. W., and Mahato, R. I. (2002) Novel branched poly(ethylenimine)-cholesterol water-soluble lipopolymers for gene delivery. *Biomacromolecules 3*, 1197−1207.
(23) Takahashi, T., Kono, K., Itoh, T., Emi, N., and Takagishi, T. (2003) Synthesis of novel cationic lipids having polyamidoamine dendrons and their transfection activity. *Bioconjugate Chem. 14*, 764−773.
(24) Mosmann, T. (1983) Rapid colorimetric assay for cellular grouth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods 65*, 55−63.
(25) Kojima, C., Haba, Y., Fukui, T., Kono, K., and Takagishi, T. (2003) Design of biocompatible dendrimers with environment-sensitivity. *Macromolecules 36*, 2183−2186.
(26) Tang, M. X., and Szoka, F. C., Jr. (1997) The influence of polymer structure on the interactions of cationic polymers with DNA and morphology of the resulting complexes. *Gene Ther. 4*, 823−832.
(27) Niu, Y., Sun, L., and Crooks, R. M. (2003) Determination of the intrinsic proton binding constants for poly(amidoamine) dendrimers via potentiomentric pH titration. *Macromolecules 36*, 5725−5731.
(28) Nozaki, Y., and Tanford, C. (1971) The solubility of amino acids and two glycine peptides in aqueous ethanol and diaxane solutions. *J. Biol. Chem. 246*, 2211−2217.
(29) Jansen, J. F. G. A., de Brabander-van den Berg, E. M. M., and Meijer, E. W. (1994) Encapsulation of guest molecules into a dendritic box. *Science 266*, 1226−1229.
(30) Jansen, J. F. G. A., and Meijer, E. W. (1995) The dendritic box: Shape-selective liberation of encapsulated guests. *J. Am. Chem. Soc. 117*, 4417−4418.
(31) Shibata, A., Murata, S., Ueno, S., Liu, S., Futaki, S., and Baba, Y. (2003) Synthetic copoly(Lys/Phe) and poly(Lys) translocate through lipid bilayer membranes. *Biochim. Biophys. Acta 1616*, 147−155.

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COMMUNICATIONS

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# Rendering Poly(amidoamine) or Poly(propylenimine) Dendrimers Temperature Sensitive

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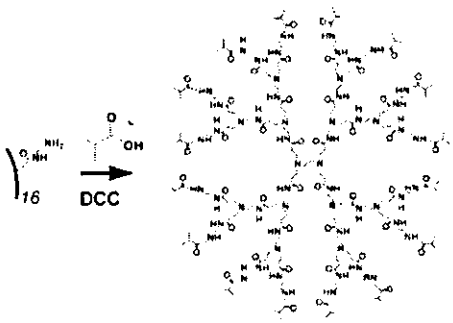
Many efforts have been made to provide functionalities to dendrimers to increase their usefulness.[1] While the functionalization of dendrimers has been attempted by various strategies, one of the most effective strategies is the modification of the terminal groups at the outer surface of the dendrimer molecule.[2] In this study, we attempted to provide dendrimers with temperature-sensitive properties using a surface modification strategy. Here, we show that the introduction of isobutyramide (IBAM) groups to the chain ends of poly(amidoamine) (PAMAM) or poly(propylenimine) (PPI) dendrimers could successfully produce a temperature-sensitive water solubility to the dendrimers.

We designed the PAMAM dendrimers having IBAM groups at all the chain ends, because the IBAM group is contained in a temperature-sensitive polymer, poly(*N*-vinylisobutyramide), which exhibits a lower critical solution temperature (LCST) around 39 °C, and is considered to play an important role in the temperature-dependent solubility change in water.[3]

The PAMAM G2, G3, G4, and G5 dendrimers having an IBAM group at every chain end, which are designated IBAM-G2, IBAM-G3, IBAM-G4, and IBAM-G5, were synthesized according to Scheme 1. The IBAM-G2, IBAM-G3, IBAM-G4, and IBAM-G5 dendrimers possess 16, 32, 64, and 128 terminal IBAM groups, respectively. Figure 1A shows the temperature dependence of the light transmittance of the IBAM-terminated dendrimers dissolved in phosphate-buffered solution of pH 9.0 at 500 nm. The dendrimers were highly soluble in the buffer under low-temperature conditions. The solution of the IBAM-G2 did not change the turbidity over the experimental temperature range. However, for the solutions of the other dendrimers, the turbidity change took place at a specific temperature as the temperature was raised, indicating that the dendrimers became water-insoluble at that temperature. Indeed, no change in the transmittance was observed for the solutions of the unmodified PAMAM dendrimers over the experimental temperature range. In addition, we observed that these dendrimer solutions became transparent again when the temperature of the solutions

***Scheme 1.*** Preparation of IBAM-Terminated PAMAM Dendrimers[a]




[a] Synthetic route for IBAM-G2 was shown. Other dendrimers were prepared by the same method. DCC, 1,3-dicyclohexylcarbodiimide.

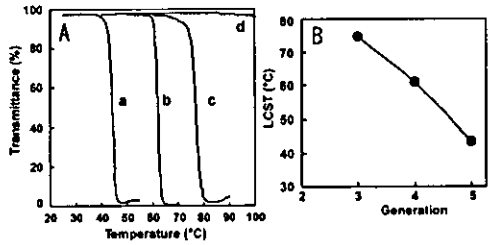



***Figure 1.*** LCST of IBAM-bearing PAMAM dendrimers. (A) Effect of temperature on transmittance of IBAM-G5 (a), IBAM-G4 (b), IBAM-G3 (c), and IBAM-G2 (d) dissolved in 10 mM phosphate-buffered solution (10 mg/mL, pH 9.0). (B) LCST of the IBAM-bearing PAMAM dendrimer as a function of generation.

was decreased below their LCST. Therefore, it is considered that the attachment of the IBAM groups to the chain terminals of the dendrimers provided them a temperature-sensitive water solubility.

Figure 1B represents the LCST of the IBAM-terminated dendrimers as a function of the dendrimer generation. Their LCST remarkably decreased with increasing dendrimer generation. It is known that the LCST of thermosensitive polymers is dependent on the molecular weight. However, the extent of the alteration of the LCST is generally not very significant. For example, Aoshima et al. have shown that the LCST of poly(methoxyethyl vinyl ether) decreased by ca. 5 °C as the number average molecular weight increased from 10 000 to 22 000, but further increase in the molecular weight hardly affected the LCST.[4] Also, Schild and Tirrell demonstrated that the increase in the average molecular weight of poly(*N*-isopropylacrylamide) from 5400 to 160 000 results in a decrease in LCST by ca. 2 °C.[5] In contrast, the IBAM-terminated dendrimers exhibit a more remarkable molecular weight dependence; an increase in the molecular weight of ca. 9150 to ca. 37 800 caused a 30 °C decrease in the LCST. It is considered that the overall shape of the PAMAM dendrimers exhibits a structural change from very open, domed shapes at lower generations to a more dense spheroid-like topologies at higher generations.[6] For the PAMAM dendrimers with the ethylenediamine core, the onset of this structural transition is thought to be at generation 2, and above this generation, the outer dendrimer surface begins to close upon itself with increasing generation, because the terminal groups of the dendrimers are more concentrated on the periphery.[7] Therefore, it is likely that the density of the terminal IBAM groups near the periphery progressively increases with the increasing generation of the dendrimer. Thus, the interaction of the peripheral IBAM groups would take place more efficiently with the increasing generation. As a result, the dehydration of the terminal IBAM groups might be induced at a lower temperature with increasing generation.

Figure 2 shows the influence of the dendrimer concentration on the LCST of the dendrimer. It is apparent that the IBAM-G5 dendrimer exhibits a reduced concentration dependence of LCST,



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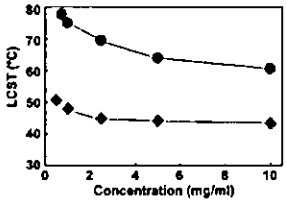


***Figure 2.*** Influence of dendrimer concentration on LCST of IBAM-G4 (●) and IBAM-G5 (◆) in 10 mM phosphate-buffered solution (pH 9.0).

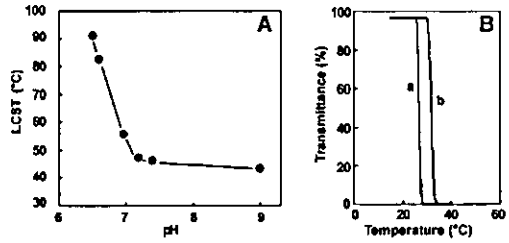


***Figure 3.*** (A) Influence of pH on LCST of IBAM-G5 dissolving in 10 mM phosphate-buffered solution of varying pHs (10 mg/mL). (B) Effect of temperature on transmittance of aqueous solutions of IBAM-terminated DAB-Am-64 (a) and -32 (b) PPI dendrimers (10 mg/mL, pH 12.0).

compared with that of the IBAM-G4 dendrimer. The more densely packed IBAM groups in the periphery of the G5 dendrimer probably enhanced the dehydration caused by the intramolecular interaction of the IBAM groups in addition to the dehydration caused by their intermolecular interaction.

It is expected that the nature of the dendrimer interior may affect the hydrophobicity alteration of the dendrimer surface. Since the PAMAM dendrimers contain tertiary amino groups in their interior, its charge density can be tuned by varying the pH of the solution. Thus, we examined the influence of the pH on the LCST of the IBAM-bearing dendrimer. Figure 3A depicts the pH dependence of LCST of the IBAM-G5 dendrimers. The dendrimer exhibited approximately the same LCST above pH 7.2. However, the LCST significantly increased upon decreasing the pH below 7.2. It has been shown that, in the amine-terminated PAMAM dendrimer, the protonation of the primary amino groups occurs between pHs 7 and 10, whereas the protonation of the tertiary amino groups takes place between pHs 3 and 7.[8] Apparently, the pH region, where the tertiary amino groups of the dendrimer interior become protonated, agrees well with the pH region where the increase in the LCST of the IBAM-G5 dendrimer was observed. Thus, it is highly likely that the protonation of the dendrimer interior caused the rise in the LCST of the IBAM-bearing dendrimer. Thomas and co-workers proposed that the charge−charge repulsion induced by the protonation of the tertiary amines of the PAMAM dendrimer could cause molecular expansion, as determined from the polarity change in the dendrimer interior.[9] Therefore, protonation of the interior of the IBAM-G5 dendrimer might induce expansion of the dendrimer and reduce the interaction between the IBAM groups in the periphery of the dendrimer, resulting in an increase in the LCST.

The attachment of IBAM groups to the periphery of the dendrimers gave them a temperature-sensitive property. To know whether this is a general phenomenon or not, we examined the effect of dendritic interior. We prepared PPI dendrimers with 32 and 64 IBAM groups at the periphery by combining an IBAM group to every chain end of DAB-Am-32 and DAB-Am-64 PPI dendrimers, respectively. As can be seen in Figure 3B, the IBAM-terminated DAB-Am-32 and -64 dendrimers changed water solubility at 31 and 27 °C, respectively. Compared with the PAMAM-based dendrimers having the same number of peripheral IBAM groups, the PPI-based dendrimers exhibited LCST at much lower temperature, despite their lower molecular weight. Because the PPI dendrimer is much smaller than the PAMAM dendrimer with the same number of the chain ends,[7b,10] it is likely that the higher density of the peripheral IBAM groups of the PPI dendrimers enhanced interaction between the IBAM groups and caused efficient dehydration at the relatively low-temperature region.

Previously, Kimura et al. prepared the DAB-Am-32 PPI dendrimer having poly(*N*-isopropylacrylamide) arms as a temperature-sensitive host for cobalt phthalocyanins.[11] In that case, the temperature sensitivity was given to the dendrimer by conjugation of the temperature-sensitive poly(*N*-isopropylacrylamide) with the dendrimer. In contrast, for the IBAM-terminated dendrimers, the dendrimer itself could achieve temperature-sensitive water solubility.

In this study, we demonstrated that the attachment of IBAM groups to the chain ends of the PAMAM and PPI dendrimers could give them a temperature-sensitive water solubility. Because the terminal moieties of the dendrimers with high generations are known to be densely packed, it is likely that the terminal IBAM groups form a shell that changes the nature of the dendrimer surface between hydrophilic and hydrophobic in response to the ambient temperature. To the best of our knowledge, the IBAM-terminated dendrimers prepared in the present study are the first dendrimers that exhibit temperature-sensitive characteristics. The IBAM-terminated dendrimers have some remarkable features that the conventional thermosensitive polymers do not have, such as a 3-D structure and a precisely controlled molecular size, though efforts have been made to produce linear polymers with a controlled molecular weight and dispersity.[12] The findings obtained in the present study may generate a new family of thermosensitive polymers.

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**Supporting Information Available:** Experimental procedures and characterization data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

## References

(1) (a) Fréchet, J. M. J. *J. Polym. Sci., Part A: Polym. Chem.* **2003**, *41*, 3713. (b) Hecht, S. *J. Polym. Sci., Part A: Polym. Chem.* **2003**, *41*, 1047. (c) Bosman, A. W.; Meijer, E. W. *Chem. Rev.* **1929**, *29*, 1665. (d) Stiriba, S. E.; Frey, H.; Haag, R. *Angew. Chem., Int. Ed.* **2002**, *41*, 1329.

(2) (a) Jansen, J. F. G. A.; de Brabander-van den Berg, E. M. M.; Meijer, E. W. *Science* **1994**, *266*, 1226. (b) Kojima, C.; Haba, Y.; Fukui, T.; Kono, K.; Takagishi, T. *Macromolecules* **2003**, *36*, 2183. (c) Archut, A.; Azzellini, G. C.; Balzani, V.; DeCola, L.; Vögtle, F. *J. Am. Chem. Soc.* **1998**, *120*, 12187. (d) Zimmerman, S. C.; Wendland, M. S.; Rakow, N. A.; Zharov, I.; Suslick, K. S. *Nature* **2002**, *418*, 399.

(3) Suwa, K.; Morishita, K.; Kishida, A.; Akashi, M.; *J. Polym. Sci., Part A: Polym. Chem.* **1997**, *35*, 3087.

(4) Aoshima, S.; Oda, H.; Kobayashi, E. *J. Polym. Sci., Part A: Polym. Chem.* **1992**, *30*, 2407.

(5) Schild, H. G.; Tirrell, D. A. *J. Phys. Chem.* **1990**, *94*, 4352.

(6) Naylor, A. M.; Goddard, W. A., III; Kiefer, G. E.; Tomalia, D. A. *J. Am. Chem. Soc.* **1989**, *111*, 2339.

(7) (a) Topp, A.; Bauer, B. J.; Klimash, J. W.; Spindler, R.; Tomalia, D. A.; Amis, E. J. *Macromolecules* **1999**, *32*, 7226. (b) Uppuluri, S.; Keinath, S. E.; Tomalia, D. A.; Dvornic, P. R. *Macromolecules* **1998**, *31*, 4498.

(8) (a) Niu, Y.; Sun, L.; Crooks, R. M. *Macromolecules* **2003**, *36*, 5725. (b) Cakara, D.; Kleimann, J.; Borkovec, M. *Macromolecules* **2003**, *36*, 4201.

(9) Chen, W.; Tomalia, D. A.; Thomas, J. L. *Macromolecules* **2000**, *33*, 9169.

(10) Scherrenberg, R.; Coussens, B.; van Vliet, P.; Edouard, G. Brackman, J.; de Brabander, E. *Macromolecules* **1998**, *31*, 456.

(11) Kimura, M.; Kato, M.; Muto, T.; Hanabusa, K.; Shirai, H. *Macromolecules* **2000**, *33*, 1117.

(12) (a) Mao, H.; Li, C.; Zhang, Y.; Furyk, S.; Cremer, P. S.; Bergbreiter, D. E. *Macromolecules* **2004**, *37*, 1031. (b) Yu, S. M.; Tirrell, D. A. *Biomacromolecules* **2000**, *1*, 310.

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# Temperature Sensitization of Liposomes by Use of *N*-Isopropylacrylamide Copolymers with Varying Transition Endotherms

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Three kinds of copolymers of *N*-isopropylacrylamide (NIPAM) with the same conformational transition temperature and varying transition endotherms were synthesized with *N*-acryloylpyrrolidine (APr), *N*,*N*-dimethylacrylamide (DMAM), and *N*-isopropylmethacrylamide (NIPMAM) as the comonomers. Two dodecyl groups were incorporated into the termini of these copolymers as an anchor for the fixation to a liposomal membrane. Egg yolk phosphatidylcholine liposomes having these copolymers were prepared and their temperature-sensitive contents release and association properties were investigated. While these copolymer exhibited a conformational transition at ca. 40 °C, $\Delta H$ for the transition increased in the order of poly(APr-*co*-NIPAM) < poly(DMAM-*co*-NIPAM) < poly(NIPMAM-*co*-NIPAM). The liposomes containing poly(NIPMAM-*co*-NIPAM) showed a drastic release enhancement of entrapped calcein above the transition temperature, whereas the liposomes with poly(DMAM-*co*-NIPAM) and those with poly(APr-*co*-NIPAM) exhibited moderate and slight enhancement of calcein release above that temperature, respectively. On the contrary, the liposomes containing poly(APr-*co*-NIPAM) showed significant aggregation above the transition temperature, but the aggregation was hardly observed for the liposomes having poly(NIPMAM-*co*-NIPAM), indicating that poly(APr-*co*-NIPAM) more efficiently made the liposome surface hydrophobic. Thus, we concluded that the copolymer with a large $\Delta H$ is suitable for obtaining functional liposomes with a temperature-sensitive contents release property, whereas the copolymer with a small $\Delta H$ is appropriate for preparing functional liposomes with a temperature-sensitive surface property.

## 1. INTRODUCTION

Liposomes have been considered to be a highly attractive material as drug carriers, because of their merits, such as biocompatibility, ability to encapsulate both hydrophilic and hydrophobic drugs, and size controllability (*1*). To increase their usefulness as drug carriers, many efforts have been made to provide liposomes with various functions. pH-Sensitive (*2*−*4*), light-sensitive (*5*−*8*), and temperature-sensitive (*9*−*11*) liposomes have been extensively studied as functional liposomes, which are useful for site-specific and/or cytoplasmic drug delivery.

While several approaches have been employed for the production of functional liposomes, modification of the liposomes with functional polymers is one of the most effective methods (*12*−*14*). For example, Tirrell and coworkers (*3*, *13*) have achieved the sensitization of egg yolk phosphatidylcholine (EYPC) liposomes to pH by the conjugation of a pH-sensitive polymer, poly(2-ethylacrylic acid), which strongly binds to lipid membranes and permeabilizes them. In addition, various synthetic polymers and peptides containing carboxyl groups have been used to render stable liposomes pH-sensitive (*15*−*17*).

Recently, the temperature sensitization of liposomes has been actively attempted by use of thermosensitive polymers, such as poly(*N*-isopropylacrylamide) [poly(NIPAM)] (*14*, *18*−*21*). Poly(NIPAM) is known to exhibit a lower critical solution temperature (LCST) at ca. 32 °C (*22*). This polymer is hydrophilic and soluble in water below the LCST. However, it becomes hydrophobic and forms an insoluble aggregate above this temperature due to hydrophobic interactions and hydrogen bonding. At the molecular level, it undergoes a coil-to-globule transition at the LCST. We have shown that when a homopolymer and copolymers of NIPAM are fixed onto the liposome surface, contents release from the liposome can be triggered by increasing the temperature above their LCST (*18*, *20*, *23*). In addition, it has been demonstrated that surface modification with these thermosensitive polymers provides liposomes with temperature-sensitive surface properties. For example, the hydrophobicity and surface charge density of the thermosensitive polymer-modified liposomes were shown to be controlled by the ambient temperature (*24*, *25*).

The functions of the thermosensitive polymer-modified liposomes are generated through the interaction between the fixed thermosensitive polymer and the liposome membrane. Their performance should be affected by the mechanism and strength of their interaction. The ability of thermosensitive polymers to sensitize liposome membranes should depend on their structures and characteristics. Therefore, for the development of functional liposomes with high temperature sensitivity, it is important to know what characteristics of the thermosensitive polymers will affect their ability to sensitize liposomes.

In this study, three kinds of NIPAM copolymers, which undergo a conformational transition at the same temperature but exhibit different transition endotherms, were synthesized with *N*-acryloylpyrrolidine (APr), *N*,*N*-

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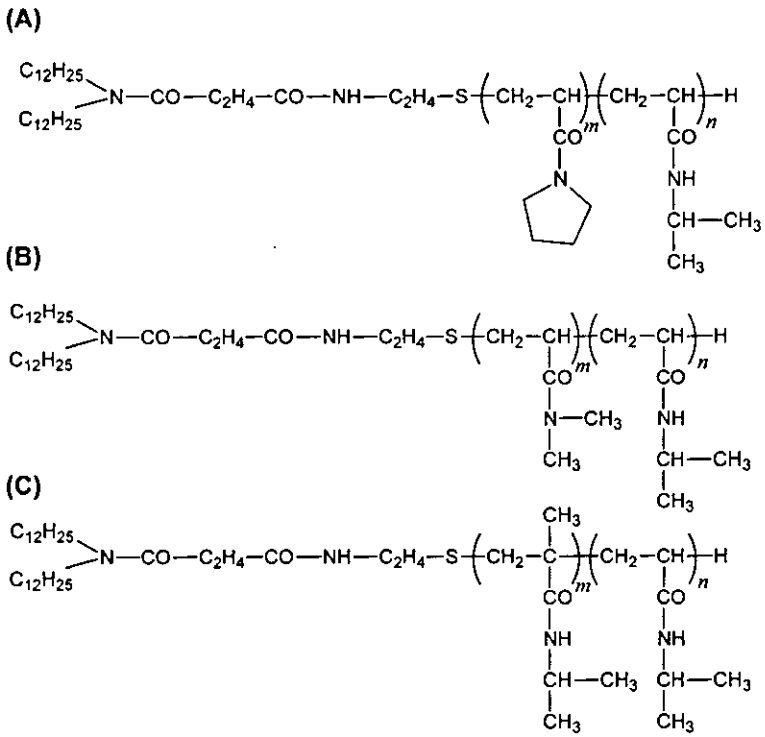


**Figure 1.** Structures of $2C_{12}$-poly(APr-*co*-NIPAM) (A), $2C_{12}$-poly(DMAM-*co*-NIPAM) (B), and $2C_{12}$-poly(NIPMAM-*co*-NIPAM) (C).

dimethylacrylamide (DMAM), and *N*-isopropylmethacrylamide (NIPMAM) as comonomers. Two dodecyl groups were connected to the chain end of these copolymers as an anchor to a liposomal membrane. These anchor-bearing copolymers are termed $2C_{12}$-poly(APr-*co*-NIPAM), $2C_{12}$-poly(DMAM-*co*-NIPAM), and $2C_{12}$-poly(NIPMAM-*co*-NIPAM) (Figure 1). The close correlation between the transition enthalpies of the copolymers and their ability to sensitize liposomes has been described.

## 2. EXPERIMENTAL SECTION

**2.1. Materials.** EYPC was kindly donated by Nippon Oil and Fats Co. (Tokyo, Japan). NIPAM, 2-aminoethanethiol, and Triton X-100 were supplied from Tokyo Kasei (Tokyo, Japan). Calcein, 1,6-diphenyl-1,3,5-hexatriene (DPH), 8-anilino-1-naphthalenesulfonic acid (ANS), and 1-pyrenecarboxaldehyde (PyCHO) were obtained from Sigma−Aldrich Japan (Tokyo, Japan). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was obtained from Wako Pure Chemical Industries (Osaka, Japan). Azobis(isobutyronitrile) (AIBN), tris(hydroxymethyl)aminomethane (Tris), *N*,*N*-dimethylformamide (DMF), and ethylenediamineteraacetic acid disodium salt (EDTA) were from Kishida Chemical (Osaka, Japan). NIPAM and AIBN were purified by crystallization from benzene−*n*-hexane and methanol, respectively. APr was prepared as previously reported (*26*). NIPMAM was synthesized according to the method reported by Jordan et al. (*27*). *N*,*N*-Didodecylsuccinamic acid was synthesized according to the method of Okahata and Seki (*28*).

**2.2. Synthesis of NIPAM Copolymers with Anchor.** Three kinds of NIPAM copolymers having two dodecyl groups at the chain end, $2C_{12}$-poly(APr-*co*-NIPAM), $2C_{12}$-poly(DMAM-*co*-NIPAM), and $2C_{12}$-poly(NIPMAM-*co*-NIPAM) (Figure 1), were prepared according to the method previously reported (*25*, *29*). In brief, for the synthesis of $2C_{12}$-poly(APr-*co*-NIPAM), APr (15.7 mmol), NIPAM (12.8 mmol), 2-aminoethanethiol (0.4 mmol), and AIBN (0.03 mmol) were dissolved in freshly distilled DMF (12 mL) and then the solution was heated at 75 °C for 15 h in $N_2$ atmosphere. The copolymer was recovered by precipitation with diethyl ether and further purified on a Sephadex LH-20 column eluted with methanol. The obtained poly(APr-*co*-NIPAM) having an amino group (1.5 g) at the terminus was reacted with *N*,*N*-didodecylsuccinamic acid ($3.3 \times 10^{-4}$ mol) in DMF (50 mL) with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide ($3.3 \times 10^{-4}$ mol) for 48 h at 4 °C. The copolymer having a didodecyl group, $2C_{12}$-poly(APr-*co*-NIPAM), was purified by an LH-20 column eluting with methanol (yield 1.1 g). The $2C_{12}$-poly(DMAM-*co*-NIPAM) was prepared according to the above method. DMAM (2.4 mmol), NIPAM (9.4 mmol), 2-aminoethanethiol (0.5 mmol), and AIBN (0.13 mmol) were dissolved in DMF (7.5 mL) and then the solution was heated at 75 °C for 15 h in $N_2$ atmosphere. The obtained poly(DMAM-*co*-NIPAM) having an amino group (0.5 g) at the terminal was reacted with *N*,*N*-didodecylsuccinamic acid ($1.5 \times 10^{-4}$ mol) in DMF (15 mL) with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide ($1.5 \times 10^{-4}$ mol) for 48 h at 4 °C. The copolymer having a didodecyl group, $2C_{12}$-poly(DMAM-*co*-NIPAM), was purified as described above (yield 0.25 g). The $2C_{12}$-poly(NIPMAM-*co*-NIPAM) was synthesized by the same method. NIPMAM (15.7 mmol), NIPAM (19 mmol), 2-aminoethanethiol (1.15 mmol), and AIBN (0.3 mmol) were dissolved in DMF (20 mL) and then the solution was heated at 75 °C for 15 h in $N_2$ atmosphere. The obtained poly(NIPMAM-*co*-NIPAM) having an amino group (1.5 g) at the terminus was reacted with *N*,*N*-didodecylsuccinamic acid ($3.3 \times 10^{-3}$ mol) in DMF (50 mL) with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide ($3.3 \times 10^{-3}$ mol) for 48 h at 4 °C. The copolymer having a didodecyl group, $2C_{12}$-poly(NIPMAM-*co*-NIPAM), was purified as described above (yield 1.1 g).

**2.3. Liposome Preparation.** The liposomes were prepared as previously reported (*20*, *29*). EYPC in chloroform solution (10 mg/mL, 1 mL) and the copolymer in chloroform solution [10 mg/mL, 1 mL for $2C_{12}$-poly(NIPMAM-*co*-NIPAM) and $2C_{12}$-poly(APr-*co*-NIPAM) or 4 mL for $2C_{12}$-poly(DMAM-*co*-NIPAM)] were mixed in a flask and the solvent was removed by evaporation. The obtained thin lipid/copolymer mixed membrane was further dried under vacuum overnight and dispersed in 1.5 mL of an aqueous calcein solution (63 mM). The obtained liposome suspension was extruded through a polycarbonate membrane with a pore diameter of 100 nm in an ice-cooled water bath. The free calcein and free copolymer were removed by gel-permeation chromatography (GPC) on a Sepharose 4B column at 4 °C in a 10 mM Tris-HCl-buffered solution containing 140 mM NaCl and 1 mM EDTA solution at pH 7.4. The calcein-loaded liposomes were kept at 5 °C until the measurement.

**2.4. Calcein Release from Liposome.** The release measurements were performed according to the method previously reported (*26*, *29*). An aliquot of dispersion of the calcein-loaded liposomes was added into 2 mL of 10 mM Tris-HCl, 140 mM NaCl, and 1 mM EDTA solution (pH 7.4) in a quartz cell (final concentration of the lipids 10 $\mu$M) at a given temperature, and the fluorescence intensity of the solution was monitored on a spectrofluorometer (Shimadzu RF-5000). The excitation and monitoring wavelengths were 490 and 520 nm, respectively. The percent release of calcein from the liposomes was defined as

$$\% \text{ release} = (F^{t} - F^{i})/(F^{f} - F^{i}) \times 100$$

where $F^{i}$ and $F^{t}$ mean the initial and intermediary fluorescence intensities of the liposome suspension, respectively. $F^{f}$ is the fluorescent intensity of the liposome suspension after the addition of Triton X-100 (final concentration 0.15%).

**2.5. Estimation of the Amount of Copolymer Bound to Liposome.** The amount of the copolymer bound to the liposome was estimated by use of high-performance liquid chromatography as previously reported (*24*, *29*). The liposomes bearing the copolymer were dried under vacuum and then dissolved in methanol/water (7:3 v/v). The solution was filtered through a poly(tetrafluoroethylene) membrane with a pore size of 0.25 $\mu$m. The filtrate (20 $\mu$l) was injected into an SB-803 column (Showa Denko) and the effluent was monitored by the absorbance at 220 nm on a UV detector (Jasco, UV-790). The amount of copolymer was estimated from the absorbance of the separated copolymer. Also, the lipid amount was determined by the method of Bartlett (*30*).

**2.6. Fluorescence Measurements.** Emission spectra of PyCHO were obtained on a spectrofluorometer with excitation at 365.5 nm. Fluorescence polarization ($P$) of DPH and ANS was measured as reported previously (*31*). ANS and DPH were excited at 395 and 360 nm, respectively, and the fluorescence intensities of ANS and DPH were monitored at 467 and 428 nm, respectively. The $P$ value was evaluated by use of

$$P = (I_{p} - I_{v})/(I_{p} + I_{v})$$

where $I_{p}$ and $I_{v}$ represent the intensities of parallel polarized fluorescence and vertically polarized fluorescence, respectively.

**2.7. Other Methods.** The size of liposomes was evaluated by dynamic light scattering on a Nicomp 380 ZLS instrument (Particle Sizing Systems). The weight-average molecular weight ($M_{w}$) and the number-average molecular weight ($M_{n}$) of the polymers were estimated by gel-permeation chromatography on a system equipped with a Shodex KD-803 column (Showa Denko) with differential refractive index detection (Jasco, RI-930) with DMF as an eluent and poly(ethylene glycol) as the standard. Differential scanning calorimetry (DSC) was performed with a DSC 120 microcalorimeter (Seiko Electronics).

## 3. RESULTS AND DISCUSSION

**3.1. Characterization of Copolymer and Copolymer-Modified Liposomes.** It is known that the LCST of poly(NIPAM) can be controlled by copolymerization with comonomers having varying degrees of hydrophilicity/hydrophobicity (*32*). In this study, we used three kinds of comonomers, APr, DMAM, and NIPMAM, to obtain NIPAM copolymers that have different structures but exhibit the same LCST. Because the most attractive application of temperature-sensitive liposomes is the drug delivery application, we chose 40 °C as the LCST of the copolymers. Such temperature-sensitive liposomes can deliver drugs by specifically releasing them at the target site, which is mildly heated above that temperature (*2*). APr and DMAM have already been used to obtain NIPAM copolymers with an LCST around the physiological temperature (*26*, *27*).

Copolymerization of NIPAM and these comonomers was performed in the presence of the chain transfer reagent 2-aminoethanethiol to introduce an amino group at one terminus of the copolymer chain (*34*), which can be used for the attachment of a hydrophobic moiety for the fixation of the copolymer chain to the liposome membrane. The compositions of the copolymers were estimated from the integral ratios of signals derived from NIPAM and the comonomer units in the $^{1}$H NMR spectra (Figure 2). Also, the number-average molecular weight ($M_{n}$), the weight-average molecular weight ($M_{w}$), and polydispersity index ($M_{w}/M_{n}$) of the copolymers were evaluated by gel-permeation chromatography with poly(ethylene glycol) as the standard. The results for the copolymers used in this study are listed in Table 1.

The cloud point measurement was performed to determine the LCST of the copolymer before the conjugation of the anchor. Figure 3 shows the transmittance of the aqueous solutions of these copolymers. The solutions were transparent below 38 °C, indicating that these copolymers were soluble in water below 38 °C. However, the solution became turbid around 40 °C, showing that these copolymers became water-insoluble around this temperature. The LCSTs of poly(APr-*co*-NIPAM), poly(DMAM-*co*-NIPAM), and poly(NIPMAM-*co*-NIPAM) were estimated from Figure 3 and are listed in Table 2.

It is known that the LCST of poly(NIPAM) can be determined by DSC (*22*, *35*). Thus, we examined the calorimetric detection of LCST for these copolymer solutions. Figure 4 depicts the typical DSC curves of these copolymer solutions. These curves exhibited an endotherm centered at approximately the same temperature, 40 °C, which agrees well with their LCST determined from the cloud point measurement. However, the amplitude of the endotherm significantly varies among these copolymers. As shown in Table 2, $\Delta H$ for the phase separation increases in the order poly(APr-*co*-NIPAM) < poly(DMAM-*co*-NIPAM) < poly(NIPMAM-*co*-NIPAM). Because the endotherm is mainly related to the destruction of water around the hydrophobic groups (*32*), this result suggests that the poly(NIPMAM-*co*-NIPAM) forms

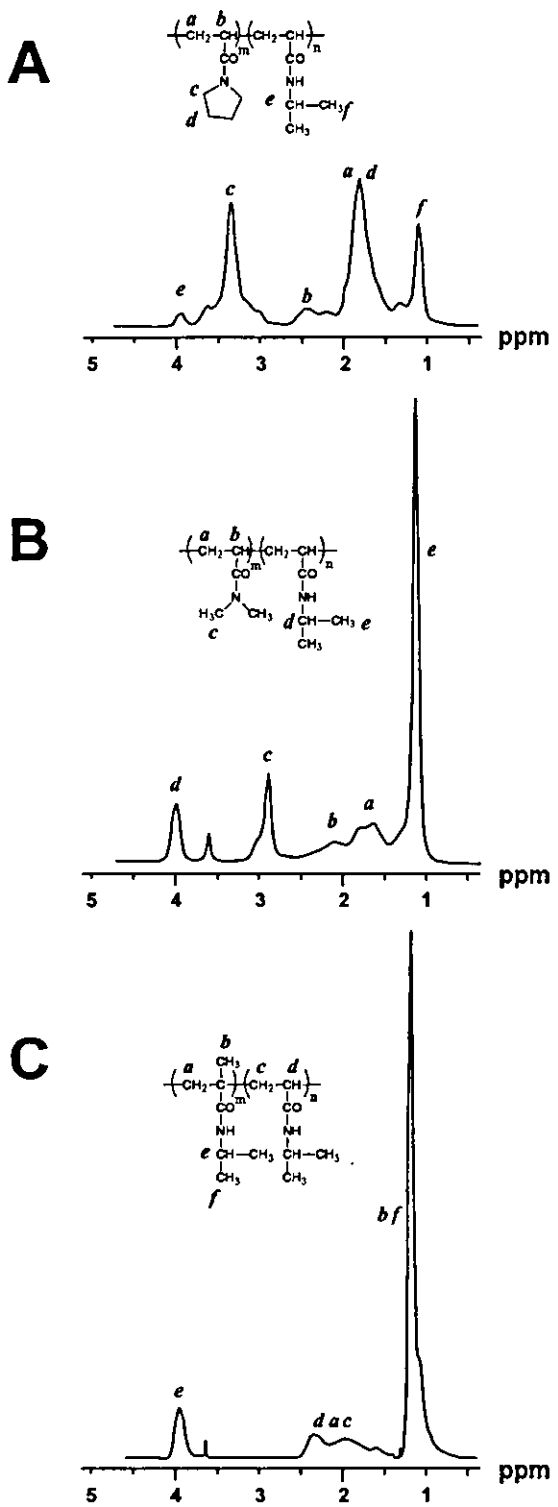


**Figure 2.** $^1$H NMR spectra for poly(APr-*co*-NIPAM) (A), poly(DMAM-*co*-NIPAM) (B), and poly(NIPMAM-*co*-NIPAM) (C) in $CDCl_3$.

the most hydrophobic domain above the LCST, whereas poly(APr-*co*-NIPAM) forms the least hydrophobic domain among these copolymers.

Many studies have shown that a variety of hydrophilic polymers with hydrophobic anchor moieties can be fixed

**Table 1. Characterization of the NIPAM Copolymers**

| copolymer | composition[a] comonomer/ NIPAM (mol/mol) | $M_n$[b] | $M_w$[b] | $M_w/M_n$[b] |
|---|---|---|---|---|
| poly(APr-*co*-NIPAM) | 81/19 | 4100 | 9200 | 2.2 |
| poly(DMAM-*co*-NIPAM) | 28/72 | 4600 | 12800 | 2.8 |
| poly(NIPMAM-*co*-NIPAM) | 46/54 | 4850 | 7550 | 1.6 |

[a] Estimated by $^1$H NMR. [b] Estimated by GPC.

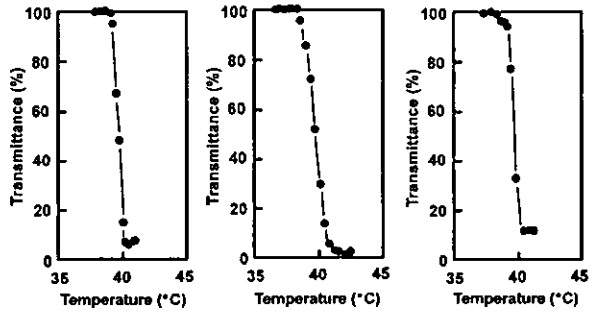


**Figure 3.** Cloud point curves for aqueous solutions of copoly(NIPAM-APr) (A), poly(DMAM-*co*-NIPAM) (B), and $2C_{12}$-poly(NIPMAM-*co*-NIPAM) (C).

**Table 2. LCST of Copolymers**

| copolymer | cloud point (°C) | $T_{max}$[a] (°C) | $\Delta H$ (J/g) |
|---|---|---|---|
| poly(APr-*co*-NIPAM) | 38.6 | 39.0 | 1.4 |
| poly(DMAM-*co*-NIPAM) | 39.8 | 41.1 | 11.8 |
| poly(NIPMAM-*co*-NIPAM) | 39.4 | 39.6 | 26.8 |

[a] Temperature of peak maximum of calorimetric endotherm.

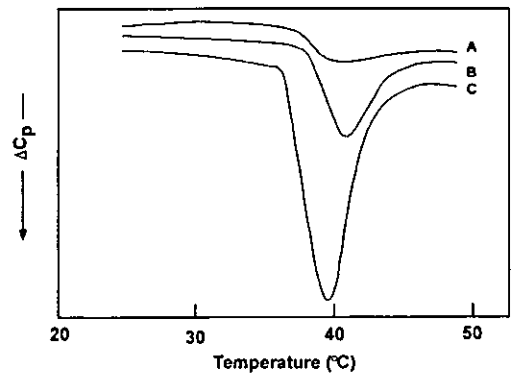


**Figure 4.** Microcalorimetric endotherms for copoly(NIPAM-APr) (A), poly(DMAM-*co*-NIPAM) (B), and poly(NIPMAM-*co*-NIPAM) (C).

to liposome membranes by a hydrophobic interaction between the hydrophobic moieties and liposome membranes (*12*, *15*, *36*). Since these copolymers have an amino group at one terminus of the chain, two dodecyl groups were introduced to the chain end by reacting didodecylsuccinamic acid with the terminal amino group. We have already shown that the didodecyl group can act as an anchor for the fixation of the NIPAM copolymers to the liposome membranes (*26*, *29*, *31*).

Liposomes modified with the copolymers bearing the anchor, $2C_{12}$-poly(APr-*co*-NIPAM), $2C_{12}$-poly(DMAM-*co*-NIPAM), and $2C_{12}$-poly(NIPMAM-*co*-NIPAM), were prepared by hydration of a mixture of EYPC and the copolymer and subsequent extrusion through a polycarbonate membrane with a pore size of 100 nm. The free

copolymer, which was not bound to the liposome membrane, was removed by GPC. The mean diameters and the standard deviations of the EYPC liposomes modified with $2C_{12}$-poly(APr-*co*-NIPAM), $2C_{12}$-poly(DMAM-*co*-NIPAM), and $2C_{12}$-poly(NIPMAM-*co*-NIPAM) were estimated by dynamic light scattering to be 125.7 ± 1.4, 106.6 ± 11.4, and 95.8 ± 3.8 nm, respectively, at 20 °C. It is apparent that these copolymer-modified liposomes possess diameters close to the pore size of the polycarbonate membrane used to prepare the liposomes. The amounts of the fixed copolymer were found to be 0.66, 0.44, and 0.58 mg/mg of liposomal lipid for liposomes modified with $2C_{12}$-poly(APr-*co*-NIPAM), $2C_{12}$-poly(DMAM-*co*-NIPAM), and $2C_{12}$-poly(NIPMAM-*co*-NIPAM), respectively, indicating that these copolymer-modified liposomes contain similar amounts of the copolymers. As indicated in the Experimental Section, the amounts of $2C_{12}$-poly(APr-*co*-NIPAM), $2C_{12}$-poly(DMAM-*co*-NIPAM), and $2C_{12}$-poly(NIPMAM-*co*-NIPAM) mixed with the lipid (10 mg) were 10, 40, and 10 mg, respectively, for the preparation of the liposomes. Thus, their binding efficiencies are calculated to be 66%, 11%, and 58%, respectively. The contents of the didodecylsuccinamic acid residues in these copolymers were estimated to be 0.6, 0.4, and 1.0 mol %, respectively, from $^1$H NMR spectra for these anchor-bearing copolymers. Therefore, the low content of the anchor moiety and relatively high polydispersity (Table 1) of $2C_{12}$-poly(DMAM-*co*-NIPAM) may produce the low fixation efficiency.

**3.2. Calcein Release from Copolymer-Modified Liposomes.** We have already demonstrated that liposomes modified with thermosensitive polymers hardly release their contents below the LCST of the polymer but enhance the contents release above the LCST (*10*, *25*). Figure 5A shows profiles of calcein release from EYPC liposomes modified with these NIPAM copolymers at 25 and 42 °C. As is apparent in Figure 5A, all of the copolymer-modified liposomes did not release calcein at 25 °C. Because these copolymers showed an LCST around 40 °C, this result indicates that well-hydrated chains of these copolymers do not destabilize the liposomal membrane, despite the difference in their molecular structures. In contrast, at 42 °C, an enhancement of the contents release was seen for all of the copolymer-modified liposomes, but the extent of the enhancement was quite different among them. The liposomes modified with $2C_{12}$-poly(APr-*co*-NIPAM) showed only a limited extent of calcein release. However, the liposomes modified with $2C_{12}$-poly(DMAM-*co*-NIPAM) moderately promoted the contents release, and a very rapid and intensive release was observed for the liposomes modified with $2C_{12}$-poly(NIPMAM-*co*-NIPAM).

Figure 5B represents the percent release of calcein from these copolymer-modified liposomes as a function of temperature. For all of the liposomes, the calcein release was enhanced around 40 °C, indicating that the enhancement is triggered by the dehydration of the copolymers above their LCST. For the liposomes modified with $2C_{12}$-poly(APr-*co*-NIPAM), the release gradually increased with increasing temperature above 40 °C. This may be because dehydration of the copolymer chain proceeds with temperature even after its conformational transition. However, even at 50 °C, only 20% of the entrapped calcein molecules were released from the $2C_{12}$-poly(APr-*co*-NIPAM)-modified liposomes. Similarly, for the $2C_{12}$-poly(DMAM-*co*-NIPAM)-modified liposomes, an enhancement in the contents release occurred above 40 °C and the release was promoted by temperature, although the extent of the release was higher than the case

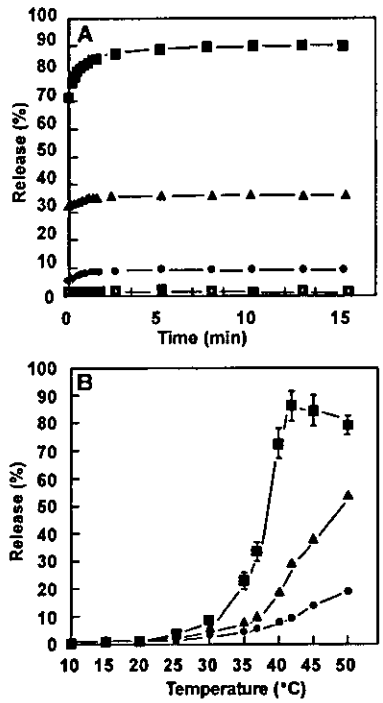


**Figure 5.** Release of calcein from $2C_{12}$-poly(APr-*co*-NIPAM)-modified (○, ●), $2C_{12}$-poly(DMAM-*co*-NIPAM)-modified (△, ▲), and $2C_{12}$-poly(NIPMAM-*co*-NIPAM)-modified EYPC liposomes (□, ■) in 10 mM Tris-HCl and 140 mM NaCl solution (pH 7.4). (A) Time course of calcein release at 25 °C (open symbols) and 42 °C (solid symbols). (B) Percent release after 15-min incubation as a function of temperature.

of the $2C_{12}$-poly(APr-*co*-NIPAM)-modified liposomes. For the $2C_{12}$-poly(NIPMAM-*co*-NIPAM)-modified liposomes, an intensive promotion of the release is seen around 40 °C, indicating that this copolymer chain strongly destabilizes the liposome membrane when dehydrated. A certain degree of contents release was also observed at 35 and 37 °C, although these temperatures are below the LCST. As seen in Figure 4, the DSC curve for the poly(NIPMAM-*co*-NIPAM) solution exhibits a large endotherm between 35 and 45 °C. This fact indicates that partial dehydration occurs even below the LCST. Thus, it is likely that the dehydrated segments of the copolymer chain interact with the liposome membrane and induce the contents release to some extent (*29*).

As mentioned above, the extent of partitioning of the copolymers onto the liposome increased in the order of $2C_{12}$-poly(DMAM-*co*-NIPAM) < $2C_{12}$-poly(NIPMAM-*co*-NIPAM) < $2C_{12}$-poly(APr-*co*-NIPAM), although the amounts of the bound copolymer were in the range of 0.44−0.66 mg/mg of lipid and not very different among these copolymer-modified liposomes. Therefore, the observed difference in their degree of contents release behavior cannot be explained from the difference in the extent of the copolymer partitioning. Thus, the ability of the dehydrated copolymer chain to induce the contents release could vary depending on their molecular structures.

**3.3. Association of Copolymer-Modified Liposomes.** Because the surface of the liposomes is covered by the hydrated copolymer chains below the LCST, these copolymer chains would enhance hydration of the liposome surface. However, above the LCST, dehydration of the copolymer chains would cause binding of the dehydrated copolymer chains onto the liposome surface,

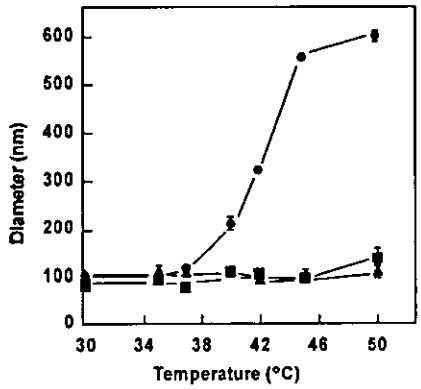


**Figure 6.** Diameters of $2C_{12}$-poly(APr-*co*-NIPAM)-modified (●), $2C_{12}$-poly(NIPMAM-*co*-NIPAM)-modified (■), and unmodified (▲) EYPC liposomes in 10 mM Tris-HCl and 140 mM NaCl solution (pH 7.4) as a function of temperature.

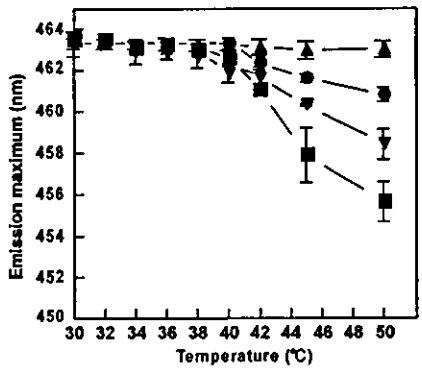


**Figure 7.** Emission maxima for PyCHO in the absence (▲) or presence of poly(APr-*co*-NIPAM) (●), poly(DMAM-*co*-NIPAM) (▼), and poly(NIPMAM-*co*-NIPAM) (■) in water. The concentrations of PyCHO and the copolymers were 1 $\mu$M and 0.4 mg/mL, respectively.

resulting in an increase in hydrophobicity of the liposome surface. Such a temperature-dependent alteration of the characteristic of the copolymer chain on the liposome surface could generate a new type of functional liposome whose surface property is controlled by temperature (*24*). As already shown, from the viewpoint of contents release, $2C_{12}$-poly(NIPMAM-*co*-NIPAM) exhibited the highest ability to sensitize liposomes, whereas $2C_{12}$-poly(APr-*co*-NIPAM) showed the lowest ability among the copolymers prepared in this study. Thus, we examined the temperature-dependent association of the copolymer-modified liposomes for these copolymers.

Figure 6 depicts the apparent diameters of the copolymer-modified and the unmodified EYPC liposomes as a function of temperature. The unmodified liposomes showed essentially the same diameter of ca. 100 nm throughout the experimental temperature range. Both the $2C_{12}$-poly(APr-*co*-NIPAM)-modified and the $2C_{12}$-poly(NIPMAM-*co*-NIPAM)-modified liposomes also exhibited almost the same diameter below the LCST. However, above the LCST, an increase in diameter was observed for both copolymer-modified liposomes, although the extent of the diameter increase is considerably different between them. The $2C_{12}$-poly(APr-*co*-NIPAM)-modified liposomes exhibited a significant increase in the diameter above the LCST, indicating that the dehydrated polymer chains fixed to the liposome made the liposome surface highly hydrophobic and induced aggregation of the liposomes through a hydrophobic interaction. Since the $2C_{12}$-poly(NIPMAM-*co*-NIPAM)-modified liposomes strongly enhanced the contents release above the LCST (Figure 5), we expected that these liposomes would show a more significant association above the LCST. However, the $2C_{12}$-poly(NIPMAM-*co*-NIPAM)-modified liposomes exhibited only a slight increase in the diameter at 50 °C, suggesting that this copolymer does not have the ability to provide a hydrophobic nature to the liposome surface.

**3.4. Interaction of Copolymers with Liposome Membrane.** Since the NIPAM copolymers exhibited an LCST at ca. 40 °C, these copolymers take on a dehydrated globule above this temperature. However, these copolymers induced the contents release from the liposomes and the association of the liposomes in a very different manner. Because these behaviors of the liposomes might be provided by the hydrophobic nature of the copolymers, the above results imply that the hydrophobicity is different among these copolymers taking on a dehydrated state. Thus, we investigated the hydrophobicity of a domain formed by these copolymers using PyCHO. This molecule is known to change the wavelength of the emission maximum ($\lambda_{max}$) of the fluorescence, depending on polarity of the solvent (*37*). By using this property of PyCHO, Schild and Tirrell (*38*) detected collapse of the backbone of the homopolymer and copolymers of NIPAM.

Figure 7 depicts $\lambda_{max}$ of PyCHO dissolved in an aqueous solution with or without the copolymers. In the absence of the copolymers, $\lambda_{max}$ did not change in the experimental temperature range. In contrast, in the presence of the copolymers, a marked blue shift above the LCST was observed. However, the extent of the change in $\lambda_{max}$ was quite different among these copolymers. For poly(APr-*co*-NIPAM), $\lambda_{max}$ was 463 nm below the LCST but slightly decreased above 40 °C, indicating that the collapsed copolymer chain formed a hydrophobic domain. In the case of poly(DMAM-*co*-NIPAM), however, $\lambda_{max}$ decreased more significantly above the LCST. The most remarkable blue shift of $\lambda_{max}$ was observed in the presence of poly(NIPMAM-*co*-NIPAM). Because the $\lambda_{max}$ of PyCHO linearly increases with dielectric constant of the environment (*37*), this result suggests that poly(NIPMAM-*co*-NIPAM) formed a domain with the highest hydrophobicity, whereas poly(APr-*co*-NIPAM) formed a domain with the lowest hydrophobicity.

To obtain information about their interaction with lipid membranes, we next examined their influence on the membrane fluidity of the liposomes using ANS and DPH (Figure 8). The fluorescence probes ANS and DPH have been frequently used to evaluate membrane fluidity and are known to provide information about the surface and the hydrophobic region of the membrane, respectively (*39*). The inverse polarization of fluorescence probes ($1/P$) was taken as an indication of the membrane fluidity (*39*).

Figure 8A–D depicts the temperature dependence of the membrane fluidities of the EYPC liposomes modified with the copolymers and the unmodified EYPC liposomes as monitored by ANS. For the unmodified liposomes (panel A), the membrane fluidity monotonically increased with the increasing temperature. Similarly, the membrane fluidity of the $2C_{12}$-poly(APr-*co*-NIPAM)-modified liposomes (panel B) increased with temperature and no change in the temperature dependence of the membrane fluidity was observed around the LCST. For the $2C_{12}$-poly(DMAM-*co*-NIPAM)-modified liposomes (panel C), a similar increase in the membrane fluidity was seen between 10 and 40 °C. However, the change in the membrane fluidity was slightly suppressed around the LCST. For the $2C_{12}$-poly(NIPMAM-*co*-NIPAM)-modified

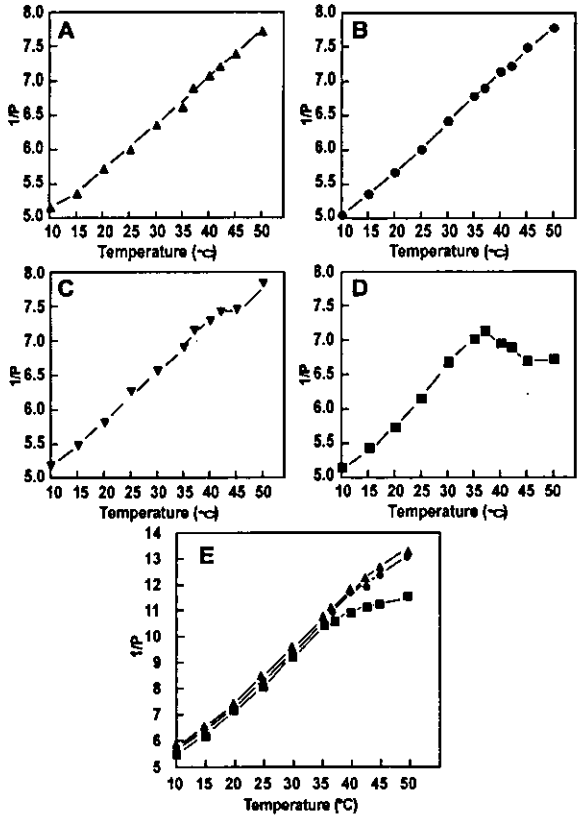


**Figure 8.** Temperature dependence of membrane fluidity ($1/P$) of $2C_{12}$-poly(APr-*co*-NIPAM)-modified (●), $2C_{12}$-poly(DMAM-*co*-NIPAM)-modified (▼), $2C_{12}$-poly(NIPMAM-*co*-NIPAM)-modified (■), and unmodified (▲) EYPC liposomes in 10 mM Tris-HCl and 140 mM NaCl solution (pH 7.4) as monitored by ANS (A–D) and DPH (E). The concentrations of EYPC, ANS, and DPH were 0.4 mM, 4.4 $\mu$M, and 0.44 $\mu$M, respectively.

liposomes (panel D), a marked change in the membrane fluidity took place at the LCST. In this case, the membrane fluidity decreased with temperature above the LCST.

A similar tendency can be seen in the temperature dependence of the membrane fluidity monitored by DPH. As seen in Figure 8E, the membrane fluidity of the $2C_{12}$-poly(APr-*co*-NIPAM)-modified liposomes (●) and that of the unmodified liposomes (▲) increased in almost the same manner. However, for the $2C_{12}$-poly(NIPMAM-*co*-NIPAM)-modified liposomes (■), a remarkable change in the temperature dependence of the membrane fluidity was seen above the LCST. These results indicate that, when hydrated, the chains of these copolymers hardly interact with the lipid membrane. However, when dehydrated, these polymer chains interact with the lipid membrane quite differently. The poly(APr-*co*-NIPAM) chain hardly affected the membrane fluidity even above the LCST, suggesting that their interaction is weak. The poly(DMAM-*co*-NIPAM) chain slightly influenced the membrane fluidity around the LCST, and hence, the copolymer moderately interacts with the membrane. In contrast, poly(NIPMAM-*co*-NIPAM) decreased the membrane fluidity of both the surface and the hydrophobic regions at and above the LCST, indicating that the dehydrated chain of this copolymer strongly interacts with the lipid membrane.

As already described, $\Delta H$ for the conformational transition was quite different among these NIPAM copolymers and increased in the order poly(APr-*co*-NIPAM) < poly(DMAM-*co*-NIPAM) < poly(NIPMAM-*co*-NIPAM) (Figure 4). Because this endotherm is related to the destruction of water around the hydrophobic groups (*32*), it is postulated that the copolymer, which exhibits a large $\Delta H$, forms a domain with a higher hydrophobocity after the transition. In fact, the fluorometric measurement with PyCHO clearly demonstrated that the hydrophobicity of a domain formed by the dehydrated copolymer chain increases in the same order (Figure 7). The copolymer with the higher hydrophobicity, indeed, strongly interacts with the lipid membrane. This is consistent with the result of the membrane fluidity measurement, which showed that poly(NIPMAM-*co*-NIPAM) strongly reduced the fluidities of both the surface and the hydrophobic regions of the lipid membrane (Figure 8). Thus, poly(NIPMAM-*co*-NIPAM) could induce a significant contents release, whereas poly(DMAM-*co*-NIPAM) and poly(APr-*co*-NIPAM) provided the liposomes with moderate and weak contents release properties, respectively.

Since poly(NIPMAM-*co*-NIPAM) strongly enhanced the contents release from the liposomes above the LCST (Figure 5), we expected that these liposomes would show more significant associations above the LCST. However, contrary to our expectation, the liposomes having poly(NIPMAM-*co*-NIPAM) chains exhibited only a slight increase in the diameter at 50 °C (Figure 6). Rather, poly(APr-*co*-NIPAM) enhanced the aggregation of the liposomes much more strongly. As is seen in Figure 8D, the dehydrated chain of poly(NIPMAM-*co*-NIPAM) affected the fluidity of the hydrophobic region of the lipid membrane. This fact implies that this copolymer chain deeply penetrates into the lipid membrane and, hence, is buried in the membrane. In contrast, the poly(APr-*co*-NIPAM) chain did not decrease the membrane fluidity even after the conformational transition, suggesting that the dehydrated copolymer chain still exists on the surface of the liposome. Thus, the dehydrated copolymer chains of poly(APr-*co*-NIPAM) could efficiently cover the liposome surface and make its surface hydrophobic.

## 4. CONCLUSIONS

In this study, we prepared three kinds of NIPAM copolymers with the same conformational transition temperature and varying transition endotherms, and then we investigated their ability to sensitize EYPC liposomes to temperature. We found that their abilities correlated with their $\Delta H$ values for the transition. The copolymer with a large $\Delta H$ is suitable for obtaining functional liposomes that drastically release contents above the LCST, whereas the copolymer with a small $\Delta H$ is appropriate for preparing functional liposomes whose surface becomes hydrophobic above the LCST. These liposomes can release the encapsulated drugs at, or bind to, a target site that is heated above the LCST. Therefore, these liposomes may have potential usefulness for site-specific drug delivery.

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## LITERATURE CITED

(1) Lasic, D. D. (1993) *Liposomes, from physics to applications*, Elsevier, Amsterdam.

(2) Yatvin, M. B., Kretz, W., Horwitz, B. A., and Shinitzky, M. (1980) pH-Sensitive liposomes: possible clinical implications. *Science 210*, 1253−1255.
(3) Maeda, M., Kumano, A., and Tirrell, D. A. (1988) $H^+$-induced release of contents of phosphatidylcholine vesicles bearing surface-bound polyelectrolyte chains. *J. Am. Chem. Soc. 110*, 7455−7459.
(4) Rui, Y., Wang, S., Low, P. S., and Thompson, D. H. (1998) Diplasmenylcholine-folate liposomes: An efficient vehicle for intracellular drug delivery. *J. Am. Chem. Soc. 120*, 11213−11218.
(5) Pidgeon, C., and Hunt, C. A. (1983) Light sensitive liposomes. *Photochem. Photobiol. 37*, 491−494.
(6) Anderson, V. C., and Thompson, D. H. (1992) Triggered release of hydrophilic agents from plasmalogen liposomes using visible light or acid. *Biochim. Biophys. Acta 1109*, 33−42.
(7) Mueller, A., Bondurant, B., and O'Brien, D. F. (2000) Visible-light-stimulated destabilization of PEG-liposomes. *Macromolecules 33*, 4799−4804.
(8) Sato, T., Kijima, M., Shiga, Y., and Yonezawa Y. (1991) Photochemically controlled ion permeability of liposomal membrane containing amphiphilic azobenzene. *Langmuir 7*, 2330−2335.
(9) Yatvin, M. B., Weinstein, J. N., Dennis, W. H., and Blumenthal, R. (1978) Design of liposomes for enhanced local release of drugs by hyperthermia. *Science 202*, 1290−1293.
(10) Kono, K., and Takagishi, T. (2004) Temperature-sensitive liposomes. *Methods Enzymol. 387*, 73−82.
(11) Needham, D., Anyarambhatla, G., Kong, G., and Dewhirst, M. W. (2000) A new temperature-sensitive liposome for use with mild hyperthermia: characterization and testing in a human tumor xenograft model. *Cancer Res. 60*, 1197−1201.
(12) Ringsdorf, H., Schlarb, B., and Venzmer, J. (1988) Molecular architecture and function of polymeric oriented systems: models for the study of organization, surface recognition, and dynamics of biomembranes. *Angew. Chem., Int. Ed. Engl. 27*, 113−158.
(13) Thomas, J. L., and Tirrell, D. A. (1992) Polyelectrolyte-sensitized phospholipid vesicles. *Acc. Chem. Res. 25*, 336−342.
(14) Kono, K. (2001) Thermosensitive polymer-modified liposomes. *Adv. Drug Delivery Rev. 53*, 307−319.
(15) Kono, K., Zenitani, K., and Takagishi, T. (1994) Novel pH-sensitive liposomes: liposomes bearing a poly(ethylene glycol) derivative with carboxyl groups. *Biochim. Biophys. Acta 1193*, 1−9.
(16) Meyer, O., Papahadjopoulos, D., and Leroux, J.-C. (1998) Copolymers of *N*-isopropylacrylamide can trigger pH sensitivity to stable liposomes. *FEBS Lett. 421*, 61−64.
(17) Bailey, A. L., Monck, M. A., and Cullis, P. R. (1997) pH-Induced destabilization of lipid bilayers by a lipopeptide derived from influenza hemagglutinin. *Biochim. Biophys. Acta 1324*, 232−244.
(18) Kono, K., Hayashi, H., and Takagishi, (1994) Temperature-sensitive liposomes: liposomes bearing poly(*N*-isopropylacrylamide). *J. Control. Release 30*, 69−75.
(19) Kitano, H., Maeda, Y., Takeuchi, S., Ieda, K., and Aizu, Y. (1994) Liposomes containing amphiphilic poly-(*N*-isopropylacrylamides) prepared by using a lipophilic radical initiator. *Langmuir 10*, 403−406.
(20) Hayashi, H., Kono, K., and Takagishi, T. (1999) Temperature sensitization of liposomes using copolymers of *N*-isopropylacrylamide. *Bioconjugate Chem. 10*, 412−418.
(21) Chandaroy, P., Sen, A., and Hui, S. W. (2001) Temperature-controlled content release from liposomes encapsulating Pluronic F127. *J. Control. Release 76*, 27−37.
(22) Heskins, M., and Guillet, J. E. (1968) Solution properties of poly(*N*-isopropylacrylamide). *J. Macromol. Sci., Chem. A2*, 1441−1455.
(23) Hayashi, H., Kono, K., and Takagishi, T. (1996) Temperature-controlled release property of phospholipid vesicles bearing a thermo-sensitive polymer. *Biochim. Biophys. Acta 1280*, 127−134.
(24) Hayashi, H., Kono, K., and Takagishi, T. (1998) Temperature-dependent associating property of liposomes modified with a thermosensitive polymer. *Bioconjugate Chem. 9*, 382−389.
(25) Kono, K., Henmi, A., and Takagishi, T. (1999) Temperature-controlled interaction of thermo-sensitive polymer-modified cationic liposomes with negatively charged phospholipid membranes. *Biochim. Biophys. Acta 1421*, 183−197.
(26) Kono, K., Nakai, R., Morimoto, K., and Takagishi, T. (1999) Thermosensitive polymer-modified liposomes that release contents around physiological temperature. *Biochim. Biophys. Acta 1416*, 239−250.
(27) Jordan, E. F., Jr., Riser, G. R., Parker, W. E., and Wrigley, A. N. (1966) Mechanical properties and transition temperatures for copolymers of N-*n*-alkylacrylamides and acrylonitrile. *J. Polym. Sci.: Part A-2 4*, 975−996.
(28) Okahata, Y., and Seki, T. (1984) pH-Sensitive capsule membranes. Reversible permeability control from the dissociative bilayer-coated capsule membrane by an ambient pH change. *J. Am. Chem. Soc. 106*, 8065−8070.
(29) Kono, K., Yoshino, K., and Takagishi, T. (2002) Effect of poly(ethylene glycol) grafts on temperature-sensitivity of thermosensitive polymer-modified liposomes. *J. Control. Release 80*, 321−332.
(30) Bartlett, G. R. (1959) Phosphorus assay in column chromatography. *J. Biol. Chem. 234*, 466−468.
(31) Kono, K., Henmi, A, Yamashita, H., Hayashi, H., and Takagishi, T. (1999) Improvement of temperature-sensitivity of poly(*N*-isopropylacrylamide)-modified liposomes. *J. Control. Release 59*, 63−75.
(32) Feil, H., Bae, Y. H., Feijen, and Kim, S. W. (1993) Effect of comonomer hydrophilicity and ionization on the lower critical solution temperature of *N*-isopropylacrylamide copolymers. *Macromolecules 26*, 2496−2500.
(33) Chung, J. E., Yokoyama, M., Aoyagi, T., Sakurai, Y., and Okano, T. (1998) Effect of molecular architecture of hydrophobically modified poly(*N*-isopropylacrylamide) on the formation of thermoresponsive core−shell micellar drug carriers. *J. Control. Release 53*, 119−130.
(34) Kaneko, Y., Sakai, K., Kikuchi, A., Yoshida, R., Sakurai, Y., and Okano, T. (1995) Influence of freely mobile grafted chain length on dynamic properties of comb-type grafted poly(*N*-isopropylacrylamide) hydrogels. *Macromolecules 28*, 7717−7723.
(35) Schild, H. G., and Tirrell, D. A. (1990) Microcalorimetric detection of lower critical solution temperatures in aqueous polymer solutions. *J. Phys. Chem. 94*, 4352−4356.
(36) Kitano, H., Akatsuka, Y., and Ise, N. (1991) pH-Responsive liposomes which contain amphiphiles prepared by using lipophilic radical initiator. *Macromolecules 24*, 42−46.
(37) Kalyanasundaram, K., and Thomas, J. K. (1977) Solvent-dependent fluorescence of pyrene-3-carboxaldehyde and its applications in the estimation of polarity at micelle−water interfaces. *J. Phys. Chem. 81*, 2176−2180.
(38) Schild, H., and Tirrell, D. A. (1991) Microheterogeneous solutions of amphiphilic copolymers of *N*-isopropylacrylamide. *Langmuir 7*, 1319−1324.
(39) Ohtoyo, T., Shimagaki, M., Otoda, K., Kimura, S., and Imanishi, Y. (1988) Change in membrane fluidity induced by lectin-mediated phase separation of the membrane and agglutination of phospholipids vesicles containing glycopeptides. *Biochemistry 27*, 6458−6463.

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