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Enhanced Transfection Efficiency of Poly(*N*,*N*-dimethylaminoethyl methacrylate)-Based Deposition Transfection by Combination with Tris(hydroxymethyl)aminomethane

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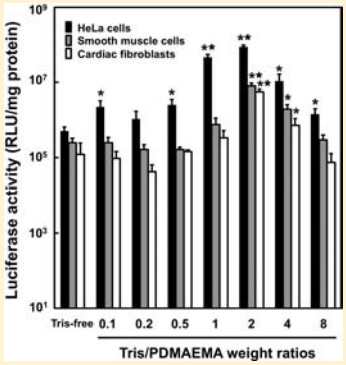
ABSTRACT: We have developed a substrate-mediated transfection method called "deposition transfection technology" using a poly(*N*,*N*-dimethylaminoethylmethacrylate) (PDMAEMA) homopolymer with both thermoresponsive and cationic characteristics. In this study, we enhanced deposition transfection efficiency by using tris(hydroxymethyl)-aminomethane (Tris buffer) as a pH adjuster for transfection solution composed of PDMAEMA and plasmid DNA (pDNA). PDMAEMA with a molecular weight of 9.7×10^4 g mol^{-1} was synthesized by photoinduced radical polymerization. The pH of PDMAEMA solution was increased gradually in the range from 8 to 11 by the addition of Tris, and then the solubility of PDMAEMA was significantly decreased and the dissolution time was extended from 15 to 40 min at Tris/PDMAEMA ratio of 1 and higher. On the other hand, while the polyion complexes (polyplexes) were formed by mixing PDMAEMA with luciferase-encoding plasmid DNA even under an excess amount of Tris at Tris/PDMAEMA ratio of 8, the binding affinity between PDMAEMA and pDNA was decreased with increasing Tris at Tris/PDMAEMA ratio of 2 and higher. When HeLa cells, smooth muscle cells, and cardiac fibroblasts were transfected by the deposition method using polyplex solution containing various amounts of Tris, the transgene expression dramatically increased at a Tris/PDMAEMA ratio of 2 in all cell types, which were more than 150-fold in HeLa cells, 40-fold in smooth muscle cells, and 30-fold in cardiac fibroblasts compared to those in the Tris-free condition. In addition, the enhanced transgene expression by Tris was sustained for over 10 days post-transfection as well as that observed in Tris-free condition. Thus, deposition transfection efficiency can be dramatically enhanced by using Tris buffer as a pH adjuster for polyplex solution.



INTRODUCTION

Controlled gene delivery to specific tissues or cells (targeted transfection) and enhancement of transfection activity are important in fields such as regenerative medicine and gene therapy.[1,2] For the development of controlled transfection technology, nonviral gene carriers such as synthetic cationic polymers and cationic liposomes are considered promising transfection reagents because, in contrast to viral vectors, they are easily able to deliver a diverse set of functional molecules such as signal peptides,[3,4] bioactive molecules,[5,6] thermoresponsive molecules,[7,8] or pH responsive molecules.[9,10]

With this in mind, we developed a novel surface-mediated transfection technology called "deposition transfection" that allowed us to obtain high and sustained transgene expression of plasmid DNA (pDNA) deposited on culture surface, treated by using surfactant polymers for the thermoresponsive surface immobilization of pDNA.[8,11] For the first-generation deposition transfection reagent, we designed a polymer comprising 4 AB-type block branches: a cationic poly(*N*,*N*-dimethylaminopropylacrylamide) (PDMAPAAm) block and a thermoresponsive poly(*N*-isopropylacrylamide) (PNIPAM) block (PDMAPAAm-PNIPAM).[8] This polymer could form polyion complexes (polyplexes) with pDNA and get deposited on a cell culture surface at 37 °C. Transgene expression from this plasmid was observed in cells seeded on this culture surface. Next, we prepared a second-generation reagent by focusing on poly(*N*,*N*-dimethylaminoethylmethacrylate) (PDMAEMA), which is a homopolymer with both cationic and thermoresponsive characteristics. By using PDMAEMA, we significantly reduced the amount of polymer required to produce the polyplexes of polymer with pDNA. In fact, we observed a 25% reduction compared to the amounts required for our previous PDMAPAAm-PNIPAM block copolymer. Compared with the use of commercially available Lipofectamine2000, by using PDMAEMA, we also obtained significantly higher transfection efficiency and cell viability in cell lines and primary cells.[11] Despite this, the transfection efficiencies for primary cells such as cardiac fibroblasts and smooth muscle cells were still lower compared to that obtained using cell lines such as HeLa and Cos-1. Therefore, the application of PDMAEMA deposition transfection technology in many fields such as artificial organ, regenerative medicine, and gene therapy is preferable for the enhancement of transfection efficiency toward primary cells.

The processes of cellular uptake, endosomal escape, cytoplasmic transport, nuclear entry, and disassembly of polyplexes are considered typical barriers for polymer-mediated

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gene delivery.[12] Therefore, many multifunctional intelligent polymers have been synthesized to overcome these barriers.[13−15] On the other hand, Kang et al. focused on the effect of environmental pH, that is, the pH values of polyplex solution, transfection medium, and culture medium on transfection efficiency using cationic polymer, because the environmental pH could affect the characteristics of a polymer and how it polyplexes with pDNA and affects target cell physiology.[16] Further, PDMAEMA is protonated (cationic) at low pH (<7), and deprotonated at high pH (>8) (completely neutral at pH = 10).[17] Therefore, the pH of PDMAEMA solution might affect the particle size, complex formation/deformation potential, and surface charge of its polyplexes with pDNA. Alterations in these parameters can then potentially affect the efficiency of cellular uptake, endosomal escape, cytoplasmic transport, and nuclear entry of polyplexes. However, the effects of pH on the characteristics of polyplexes of PDMAEMA with pDNA and its transfection activity have not been examined. To our knowledge, there is also no report that addresses the effect of the pH of polyplex solutions on substrate-mediated transfection technologies.

Tris(hydroxymethyl)aminomethane (Tris) is one of the most useful buffering agents that rapidly restores pH and acid−base regulation, and therefore, it has been extensively used not only in basic research fields of biochemistry and molecular biology as the buffer solutions for DNA, RNA, and proteins, but also in clinical practices for diabetic or renal acidosis and metabolic acidosis associated with cardiac bypass surgery or cardiac arrest.[18,19] Tris is a compound with basic property (pH = 11 at 10 mM) having an effective pH range between 7.0 and 9.0. Its pH can be easily controlled by titration with HCl (Tris-HCl acid salt) for use in many experimental purposes including transfection experiments. Here, we suggest that the characteristics of PDMAEMA such as thermoresponsibility, particle size, and complexation/decomplexation of its polyplexes with pDNA could be controlled and optimized for enhancing transfection by changing solution pH by using Tris buffer as a safety pH adjuster. Therefore, in this study, we examined the effects of Tris addition on the thermoresponsive nature of PDAMEMA and the characteristics of its polyplexes with pDNA. We then determined the optimal Tris additive amount for enhancing deposition transfection efficiency of PDMAEMA.

EXPERIMENTAL SECTION

General Methods. ^{1}H NMR spectra were recorded using a 300 MHz NMR spectrometer (Gemini 300; Varian, Palo Alto, CA) with chloroform-d_1 at room temperature. Gel permeation chromatography (GPC) analyses using *N,N*-dimethylformamide as a solvent were carried out using an HPLC-8320 GPC instrument (Tosoh, Tokyo, Japan) in conjunction with Tosoh TSKgel SuperAW-4000 and SuperAW-5000 columns. The columns were calibrated prior to use by using narrow distribution poly(ethylene glycol) standards (Tosoh).

Synthesis and Thermoresponsive Characters of PDMAEMA. Poly(*N,N*-dimethylaminoethylmethacrylate) (PDMAEMA) was synthesized according to the procedure given in our previous report.[11] Briefly, DMAEMA (7.0 g, Tokyo Kasei Co., Tokyo, Japan) was poured into a glass tube (35 × 65 mm, Maruemu Co., Osaka, Japan) under N_2 gas atmosphere. DMAEMA was irradiated for 21 h by using an 18 W fluorescent light (FCL20BL; NEC Co., Tokyo, Japan). After irradiation, reprecipitation was carried out 6 times with chloroform solution in hexane (Kanto Chemical Co., Tokyo, Japan). The final precipitate was dried under reduced pressure, following which, PDMAEMA was obtained (4.3 g, 61.4% conversion). The molecular weight of PDMAEMA was determined to be 9.7 × 10^4 g·mol^{-1} (polydispersity: 4.1) by GPC analysis. ^{1}H NMR: δ 0.8−1.2 ppm (br, $-CH_3$), 1.6−2.0 (br, $-CH_2-CH_3$), 2.2−2.4 (br, $N-CH_3$), 2.5−2.7 (br, CH_2-N), 4.0−4.2 (br, $O-CH_2$).

The lower critical solution temperature (LCST) of aqueous solutions of PDMAEMA (1 mg/mL) mixed with different concentrations of tris(hydroxymethyl)aminomethane (Tris; Wako Pure Chemical Ind. Ltd., Osaka, Japan) (0.5−8.0 mg/mL) was determined using a UV−visible spectrophotometer under a heating rate of 0.5 °C/min (UV-1700; Shimazu, Japan). After complete precipitation of PDMAEMA at 37 °C, the solubility of the PDMAEMA solid in an aqueous Tris solution was observed at 20 °C by optical transmittance change using the UV−visible spectrophotometer.

Surface Analysis. Surface deposition of the PDMAEMA molecules were semiquantitated by surface analysis using attenuated total refraction Fourier transformed infrared spectrometer (FTIR-ATR, IR Pretige-21; Shimadzu, Kyoto, Japan). Briefly, the 100 μL of PDMAEMA solutions (260 μg/mL) containing different amounts of Tris with 0.5- and 2.0-fold weight volume for PDMAEMA were added on the surfaces of cell culture polystyrene plates (Asahi Glass Co., Ltd., Tokyo, Japan). After incubation at 37 °C for 6 h, the surfaces was washed with saline at 20 °C and the absorption spectra from 2600 to 1500 cm^{-1} on these culture surfaces were acquired by FT-IR spectrometry.

Preparation and Characterization of Polyplexes. PDMAEMA was dissolved in saline (100 μg/mL), and then Tris was added to the solution in 0.1−8.0-fold weight volume for PDMAEMA. Aliquots (60 μL) were added to firefly luciferase-encoding plasmid DNA (33 μg/mL, pGL3 control plasmid; Promega, WI) dissolved in 90 μL of DNase-free pure water (Invitrogen, CA) or Tris-HCl-EDTA buffer (pH 8, Wako) to obtain polymer/pDNA ratios, corresponding to cation/anion (C/A) ratio of 8. The solutions (total volume, 150 μL) were mixed using a pipet to prepare polyplexes.

Polyplexes at the same concentration employed for transfection were used for the mean diameter measurement and DNA gel shift assay. The mean diameter of the polyplexes was determined by dynamic light scattering (DLS) on Zetasizer Nano S (Malvern Instruments Ltd., Worcestershire, UK) equipped with a 10 mW He−Ne laser. For the Tris competitive DNA binding assay, 10 μL aliquots of the polyplexes and DNA solutions were incubated for 30 min at 37 °C and loaded into a 1.2% (w/v) agarose gel slab. The gel was electrophoresed at 100 V for 40 min in 1 × TAE buffer (Wako), including 0.05% ethidium bromide (Wako), and imaged using a UV transmitter (Gel Doc XR System; BIO-RAD, CA).

Primary Cell Culture. Beagles, weighing approximately 10 kg, were humanely used in this experiment according to the Principles of Laboratory Animal Care (formulated by the National Institutes of Health, Publication No. 56−23, received in 1985). Cardiac fibroblasts were isolated from beagle heart ventricles. Briefly, the ventricles were separated and minced into small pieces and digested using 0.25% trypsin solution (Invitrogen, CA) at 37 °C for 1 h with gentle agitation. After filtering the digest through a 100 μm nylon mesh (BD Biosciences, NJ) and centrifugation at 1300 rpm for 3 min, the cell pellet was collected. Following resuspension in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Invitrogen Corp.,

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Carlsbad, CA) containing 10% fetal bovine serum (Hyclone Laboratories Inc., Logan, UT), penicillin (200 U/mL; ICN Biomedicals Inc., Aurora, OH), and streptomycin (200 mg/mL; ICN) (growth medium), the cells were placed on a dish (55 cm^2; Asahi Glass Co., Ltd.) with the growth medium and cultured in an atmosphere of 5% CO_2 at 37 °C. Aorta smooth muscle cells were isolated from beagle thoracic aortas by modifying the method described previously by Franzblau et al.[20] Briefly, the endothelial cell layer of an aorta was removed using a cotton swab. De-endothelialized aorta was minced with scissors into small pieces and digested using a solution containing 0.1% collagenase Type I (Wako) and 0.01% elastase (Wako) at 37 °C for 1 h with gentle agitation. After filtering the digest through a 100 μm nylon mesh, the cells were collected and cultured by the method described above. When the cultures were nearly confluent, cells were harvested and subcultivated at 1.0×10^4 cells/cm^2.

Green fluorescence (GFP) expressing-adipose derived stromal cells (ADSCs) were isolated from GFP transgenic Lewis rat by a method described in our previous report.[11] Briefly, approximately 10 g of fat tissue was harvested from the subcutaneous layer of hind limb and digested using 0.1% collagenase type I solution (Wako) at 37 °C for 1 h with gentle agitation. After filtering the digest through a 100 μm nylon mesh and centrifuging it at 1300 rpm for 3 min, the cell pellet was collected and cultured by the method described above. All cells were used for experiments before they reached the fifth passage.

Transfection to Cell Line and Primary Cells. Transfection by the deposition transfection method was performed as reported previously.[11] Briefly, an aqueous solution of the DNA complexes (50 μL; plasmid concentration, 20 μg/mL) was diluted with 150 μL of saline and then added into each well of a 24-well dish (amount of DNA added to each well, 1.0 μg). After incubation at 37 °C for 6 h, HeLa cells, cardiac fibroblasts, smooth muscle cells, ADSCs in 1.0 mL of growth medium were seeded at a density of approximately 5.0×10^4 cells/cm^2 and cultured in an atmosphere of 5% CO_2 at 37 °C for 2−10 days. The luciferase expression of cells transfected with pGL3 control plasmid was analyzed using a luciferase assay as follows. After 2−10 days of cultivation, the cells were lysed with 0.2 mL of cell lysis buffer (Promega). The lysate was centrifuged at 15 000 rpm for 3 min at 4 °C, and 20 μL of the supernatant was analyzed for luciferase activity by using a Luminus CT-9000 luminometer (Dia-Iatron, Tokyo, Japan). Relative light units (RLU) measurements were standardized using the total protein amounts of the cell lysates, which were determined with BioRad protein assays (BIO-RAD, Hercules, CA) using bovine serum albumin as the standard.

Observation of Cellular Uptake of Polyplexes. Cellular uptake of the polyplexes was observed by using fluorescently labeled plasmid DNA (*Label* IT plasmid delivery control-Cy3, Mirus, WI, USA). Polyplexes of Cy3-labeled plasmid DNA and PDMAEMA were deposited followed by cell seeding as described above. After 3 days of cell seeding, the cells were washed twice with PBS (-) and polyplexes were visualized by fluorescent microscope (IX71; OLYMPUS, Tokyo, Japan).

Cell Viability Assays. Cytotoxicity was assessed by performing a cell viability assay using the water-soluble tetrazolium (WST)-8 method (Dojindo, Kumamoto, Japan). Polyplex solutions containing different amounts of Tris at Tris/PDMAEMA ratios from 1 to 8 were added to each well in a 96-well plate (Asahi Glass Co. Ltd.). After incubating at 37 °C for 6 h, HeLa cells, cardiac fibroblasts, or smooth muscle cells in 100 μL of the growth medium were seeded (approximately 1.5×10^4 cells per well) and cultured for 24 h at 37 °C in a 5% CO_2 atm. To each well, 10 μL of WST-8 reagent (5 mmol/L) was added. After 2 h incubation at 37 °C, the absorbance at 450 nm was determined using a Bio-Rad microplate reader (model 680; Bio-Rad laboratories Inc., CA, USA).

RESULTS

Thermoresponsive Properties of PDMAEMA. Because PDMAEMA is a slightly cationic polymer, the pH of an aqueous solution of PDMAEMA was approximately 8 (Figure 1). Upon addition of Tris into the PDMAEMA solution, its pH

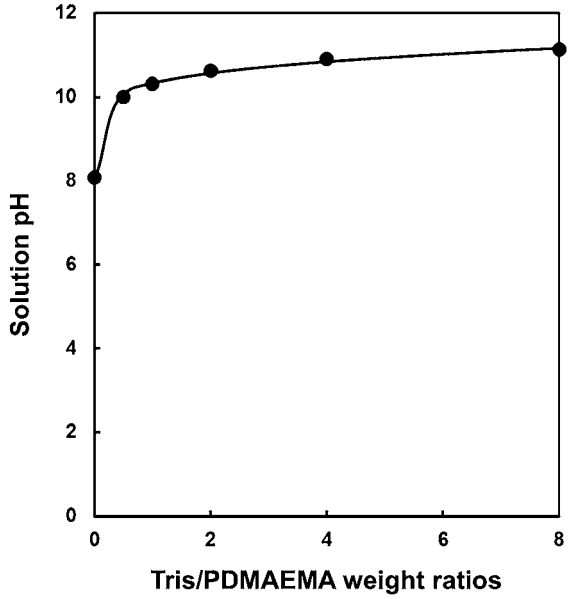

Figure 1. Changes in the PDMAEMA solution pH by the addition of Tris with various amounts. Concentration of PDMAEMA solution: 0.1 mg/mL.

increased gradually, reaching pH 11 in the presence of excess Tris. In addition, we determined that PDMAEMA was thermoresponsive, and its LCST was approximately 26 °C (Figure 2). The LCST was relatively unchanged upon addition of Tris. On the other hand, the solubility of PDMAEMA decreased markedly as the amount of Tris increased (Figure 3). Specifically, PDMAEMA completely dissolved within approximately 15 min, while the dissolution time was extended to 40 min at Tris/PDMAEMA ratios of 2 and above. Thus, the solubility of PDMAEMA in water is inhibited by the presence of Tris.

The PDMAEMA with or without Tris was coated on the culture surface. Upon washing with saline at 20 °C FTIR-ATR absorption signal at 1730 cm^{-1}, originated from C═O stretching vibration of the ester group in PDMAEMA, clearly detected on the PDMAEMA surface coated with Tris, while there was little absorption signal except for 1600 cm^{-1}, originated from C═C bonds of the aromatic hydrocarbons of the polystyrene culture surface, on the Tris-free surface (Figure 4). The adsorption signal level of the PDMAEMA increased with the amount of Tris. Therefore, Tris was effective for the stable deposition of the PDMAEMA on the culture surface.

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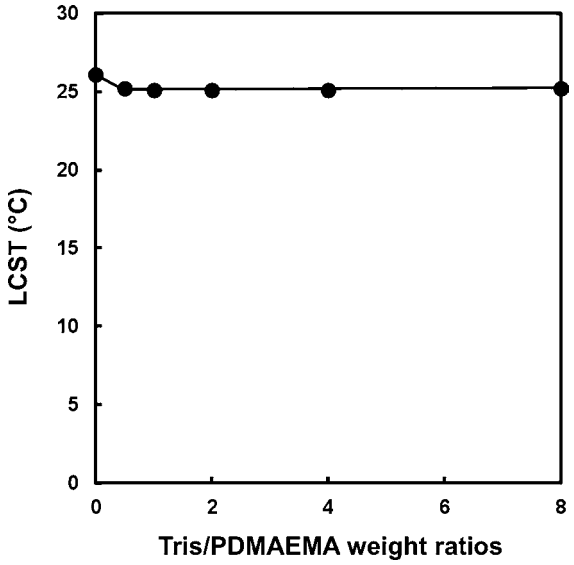

Figure 2. Effect of Tris addition on the LCST of PDMAEMA solution. Concentration of PDMAEMA: 1.0 mg/mL.

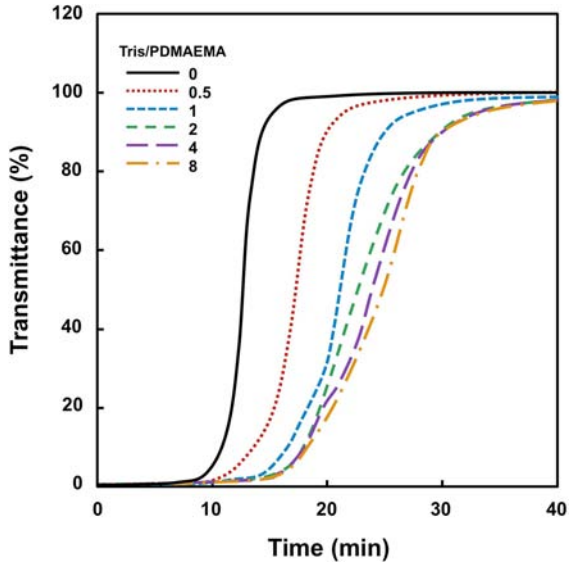

Figure 3. Time course of the transmittance of PDMAEMA solutions containing various amounts of Tris. The PDMAEMA solution was once heated at 37 °C, and then the transmittance was measured at 20 °C. Concentration of PDMAEMA solution: 10 mg/mL.

Polyplex Formation and Stability. The diameter of PDMAEMA particles in water was approximately 72 ± 4 nm. Mixing an aqueous PDMAEMA solution and an aqueous DNA (pGL3 control plasmid) solution leads to immediate formation of PDMAEMA/DNA polyplexes, as demonstrated in our previous report. The diameter of the polyplexes formed at 20 °C under C/A ratio of 8 without Tris was 149.1 ± 10.4 nm (Figure 5). Upon warming at 37 °C, the diameter increased slightly to 158.4 ± 0.5 nm. A similar tendency was observed even in the presence of Tris at a Tris/PDMAEMA weight ratio ranging from 1 to 4. The polyplexes formed in Tris−HCl buffer solution (pH 8, Tris/PDMAEMA ratio of 18) were slightly larger than those formed polyplexes. However, a significant increase in particle diameter occurred in the presence of excess Tris (Tris/PDMAEMA ratio of 8) at both 20 and 37 °C.

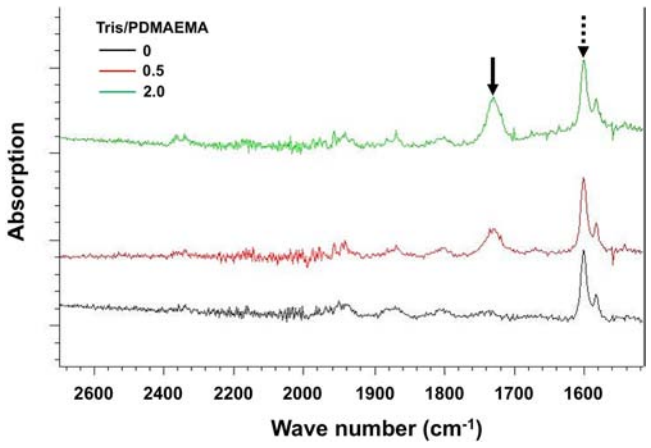

Figure 4. FTIR-ATR spectra of polystyrene culture surfaces coated with PDMAEMA solutions containing different amounts of Tris. Closed and dashed arrows indicate the absorption signals of C═O bonds originated from PDMAEMA and C═C bonds originated from polystyrene, respectively.

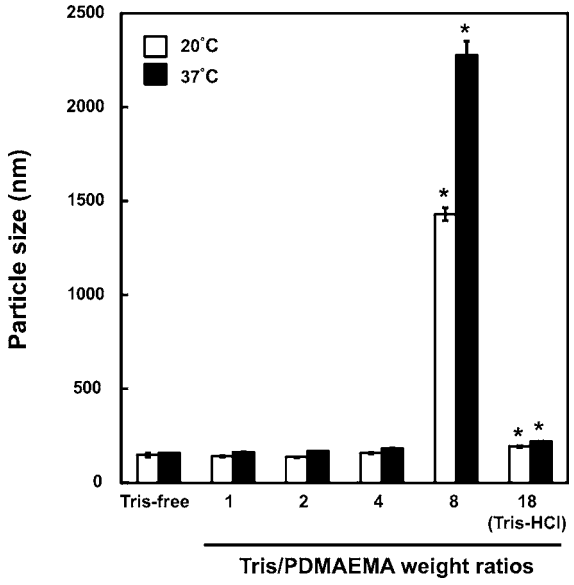

Figure 5. Effect of Tris addition on particle size of the polyplexes prepared by mixing PDMAEMA and pGL3-control plasmid DNA (C/A ratio: 8) at 20 °C (□) and 37 °C (■). The values represent the mean ± SD (n = 3). *P < 0.05 relative to Tris-free conditions (control).

The stability of the polyplexes in the presence of Tris was evaluated using an electrophoretic gel shift assay (Figure 6). No free plasmid DNA bands were observed from polyplex preparations dissolved in Tris-free, Tris/PDMAEMA weight ratio of 1, and Tris-HCl buffer solutions. This indicates that, under these conditions, DNA was fully associated with PDMAEMA. On the other hand, bands of DNA did appear in the presence of Tris in solutions with a Tris/PDMAEMA weight ratio of 2 and higher. The intensity of the band was proportional to the Tris/PDMAEMA weight ratio. This demonstrated that the interaction between DNA and PDMAEMA was inhibited by Tris.

Deposition Transfection Study. HeLa cells or primary cardiac fibroblasts and smooth muscle cells were transfected by deposition transfection (see Methods). In HeLa cells, the luciferase expression at 2 days post transfection increased

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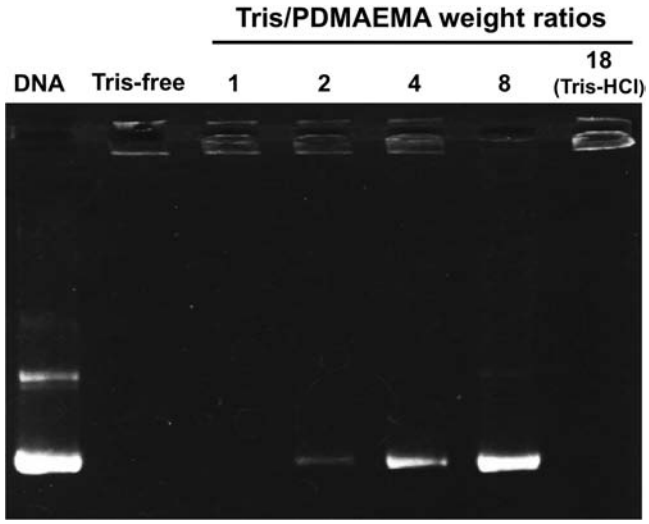

Figure 6. Electrophoretic gel shift assay of the polyplexes of PDMAEMA with pGL3-control plasmid DNA containing various amounts of Tris.

steadily until a Tris/PDMAEMA ratio of 2 was reached, after which it decreased drastically. At the optimal Tris/PDMAEMA ratio of 2, the luciferase activity was more than 150 times that observed in Tris-free conditions. On the other hand, in primary cells, a slight increase in the luciferase expression was observed in the presence of Tris below a Tris/PDMAEMA ratio of 1. However, at a Tris/PDMAEMA ratio of 2, the highest luciferase expression was obtained; compared to Tris-free conditions, there was a 40-fold and 30-fold increase in the activity of the cardiac fibroblasts and smooth muscle cells, respectively.

Consistent with our previous report, elevated luciferase expression was maintained in HeLa cells, smooth muscle cells, and cardiac fibroblasts transfected using the polyplexes for up to 10 days post transfection (Figure 8). The presence of Tris both accelerated and significantly enhanced the luciferase expression—over 50-fold, 55-fold, and 14-fold increases in the luciferase activity of HeLa cells, smooth muscle cells, and cardiac fibroblasts, respectively, compared to those of Tris-free conditions in this observation period.

The cellular uptake of polyplexes was examined by using Cy3-labeled fluorescence plasmid DNA. Figure 9 shows a fluorescence micrograph of the culture surfaces where the GFP-expressing ADSCs were undergoing transfection following the polyplexes of Cy3-labeled plasmid DNA and PDMAEMA were deposited. The weak and blurred red fluorescence signals were observed on the polyplexes-deposited culture surfaces without seeding any cells (Figure 9A), which implies that the red fluorescence derived from Cy3-labeled plasmid DNA were shield by the excess amount of PDMAEMA at C/A ratio of 8. On the other hand, after 72 h of cell seeding, red fluorescence was clearly observed in the cytoplasm of the cells as nanoparticles (Figure 9B), which indicated the uptake of polyplex particles by the cells. When the cells were seeded on the polyplex-deposited surfaces containing Tris at Tris/PDMAEMA weight ratio of 2, which corresponded to the Tris/PDMAEMA ratio that the highest transgene expression was obtained, the numbers of intracellular red nanoparticles were significantly increased compared to that observed in the cells seeded on the surfaces where Tris-free polyplexes were deposited (Figure 9C).

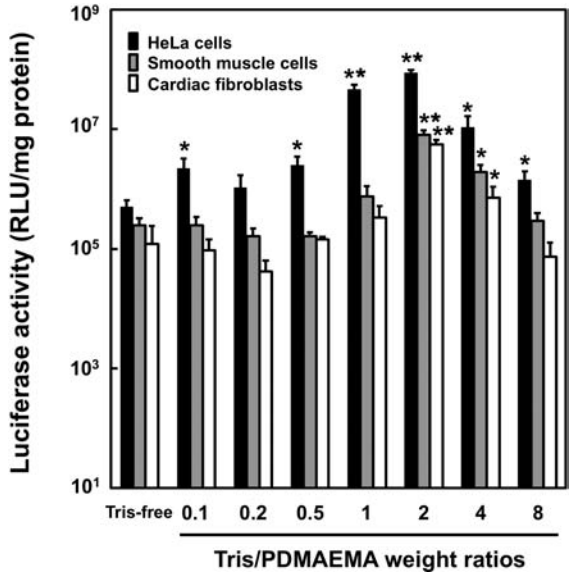

Figure 7. Effect of Tris addition on transgene levels of HeLa cells, smooth muscle cells, and cardiac fibroblasts transfected by deposition method using polyplexes of PDMAEMA with pGL3-control plasmid DNA containing various amounts of Tris. The values represent the mean ± SD (n = 3). *P < 0.05, **P < 0.01 relative to Tris-free conditions (control).

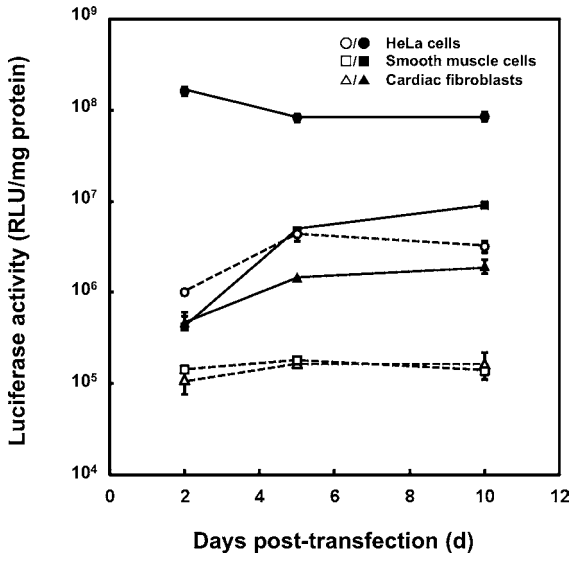

Figure 8. Time course of the luciferase expression levels of HeLa cells (○/●), smooth muscle cells (□/■), and cardiac fibroblasts (△/▲) transfected using polyplexes solution of PDMAEMA with pGL3-control plasmid DNA containing Tris at Tris/PDMAEMA weight ratio of 2 (filled symbol) and without Tris (open symbol and dashed line). The values represent the mean ± SD (n = 3).

The viability of the cells transfected using polyplex solution containing Tris was evaluated for Tris/PDMAEMA ratios from 1 to 8 (Figure 10). In accordance with our previous study,[11] high viabilities greater than 90% were obtained in HeLa cells by PDMAEMA-based deposition transfection, and which were not significantly changed by the addition of Tris for any Tris/PDMAEMA weight ratios (P > 0.05). Similar to HeLa cells, the viabilities of primary cells such as smooth muscle cells and cardiac fibroblasts were not significantly changed by the addition of Tris, even though those of the smooth muscle

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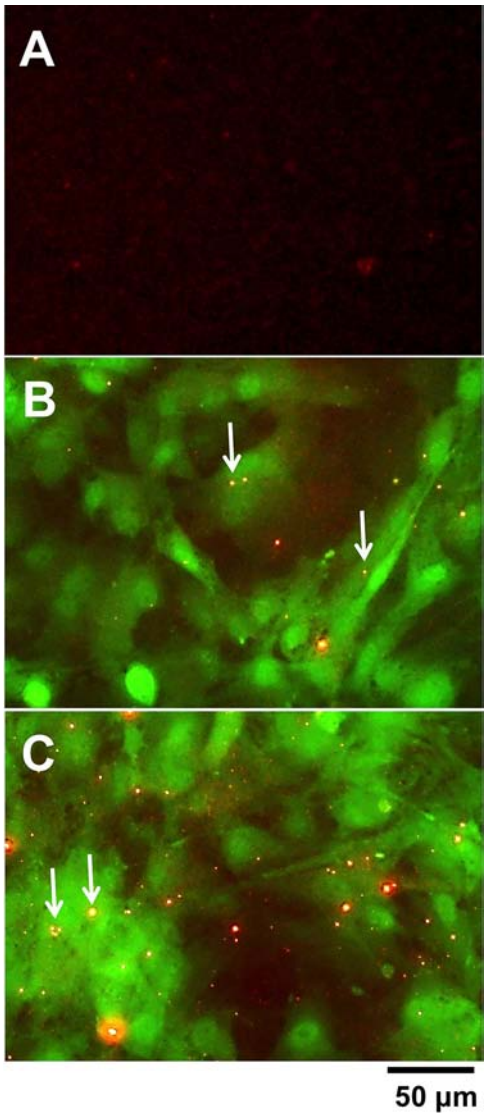

Figure 9. (A) Florescence microscopy images of the culture surfaces where the polyplexes of Cy3-labeled plasmid DNA and PDMAEMA were deposited. Florescence microscopy images of GFP-expressing adipose-derived stromal cells (ADSCs) undergoing the deposition transfection using the polyplex solution without containing Tris (B) and containing Tris at Tris/PDMAEMA ratio of 2 (C). Polyplex solutions (C/A = 8) were incubated on the culture surfaces for 6 h followed by cell seeding. Images were captured 72 h after cell seeding. Green and red indicate the GFP fluorescence of ADSCs and Cy3 fluorescence of plasmid DNA, respectively. Ten regions of interest were analyzed in two independent experiments.

cells and cardiac fibroblasts were a little lower than that of the HeLa cells. These results indicating that the Tris addition has a little effect on the cytotoxicity of PDMAEMA-based deposition transfection.

■ DISCUSSION

In this report, we evaluated the suitability of PDMAEMA, a widely used cationic polymer, for use in delivery of DNA via the substrate-mediated deposition transfection method. Since PDMAEMA is a typical cationic polymer, it is used widely as a nonviral gene delivery carrier. In addition to its cationic properties, PDMAEMA is also a thermoresponsive polymer with a low LCST that ranges from 34 to 37 °C.[21] This unique

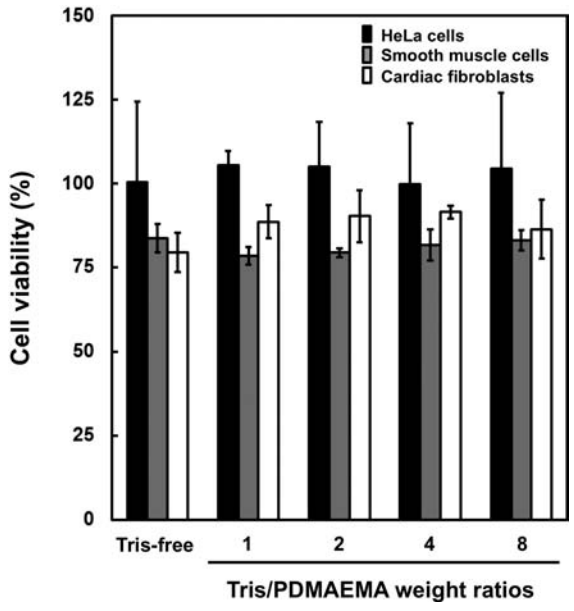

Figure 10. Cytotoxicity of polyplexes prepared by mixing pGL3 control plasmid DNA and PDMAEMA solution containing Tris at different Tris/PDMAEMA weight ratios. The values represent the mean ± SD (n = 3).

combination of properties makes PDMAEMA suitable for bioconjugation with DNA for polyplex formation and amenable to conversion to a water-insoluble form for deposition of polyplexes on a culture dish surface. However, very little biomedical research has been carried out on the dual characteristics of this polymer. In our previous study, we achieved significantly higher transfection efficiency and cell viability by using the deposition transfection method compared with the conventional transfection method using Lipofectamine 2000.[11] Our deposition transfection technique is highly suitable for application in fields such as artificial organ biosynthesis, tissue engineering, regenerative medicine, and gene therapy.

In this study, we showed that the Tris base enhanced the efficiency of the deposition transfection method in primary cardiac fibroblasts and smooth muscle cells without Tris dosage-dependent cytotoxicity (Figures 7 and 10). In this method, deposition of the polyplexes from PDMAEMA and DNA onto the culture surface is the most essential step; this in turn is dependent on the thermoresponsive character of PDMAEMA. At low PDMAEMA:DNA ratios, deposition is unstable. On the contrary, high PDMAEMA:DNA ratios result in significant deposition of DNA on the culture dishes, but the surface is coated with a lot of PDMAEMA. In both cases, this can lead to inhibition of transgene expression. Therefore, we set the cation/anion (C/A) ratio to 8, since we found this was optimal from our preliminary study. We reasoned that, if a higher robust deposition could be obtained at the constant C/A ratio of 8, transgene expression would be further increased. As shown in Figure 3, the solubility of PDMAEMA at 20 °C was markedly decreased as the concentration of Tris increased, and the dissolution time was extended from 15 to 40 min at Tris/PDMAEMA ratios of 2 and more. However, the LCST remained constant independently of Tris concentration (Figure 2). Moreover, the increased amount of deposited PDMAEMA on the culture surfaces was confirmed by FTIR-ATR analysis in a Tris dosage-dependent manner (Figure 4). Therefore, we

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concluded that the most robust deposition was obtained at Tris/PDMAEMA ratios of 2 and higher.

The dependency of PDMAEMA dissolution on the amount of Tris was causally related to the ensuing change in the solution pH as shown in Figure 1. This is likely because DMAEMA monomers are protonated at low pH and easily dissoluble in water due to intermolecular electrostatic repulsion. On the other hand, the monomers are deprotonated at high pH and may be difficult to dissolve in water because of intermolecular hydrophobic interactions. Indeed, Xiong et al. reported that the solubility of PDMAEMA-*b*-poly(acrylic acid) was altered by changing the pH of the solution.[22]

We also observed that the interaction between PDMAEMA and pDNA was inhibited by increasing amounts of Tris (Figure 6). The inhibition was clearly observed at Tris/PDMAEMA weight ratios greater than 4:1 and was virtually complete at a ratio of 8:1. In addition, at the 8:1 ratio, the particle size of the polyplexes increased significantly (Figure 5), indicating the degradation of the polyplexes by weak interaction between PDMAEMA and pDNA. As mentioned above, PDMAEMA is protonated (cationic) at low pH (<7) and deprotonated at high pH (>8). Therefore, the loss of DNA binding was due to the disappearance of PDMAEMA ionic properties. The data from the electrophoretic gel shift assay and the above-mentioned thermoresponsive study led us to conclude that a Tris/PDMAEMA weight ratio of 2 is the optimal condition for transgene expression. Indeed, at this ratio, the significantly larger number of endocytosed Cy3-labeled plasmid DNA compared to that transfected using Tris-free PDMAEMA were observed in the primary ADSCs (Figure 9B and C), and the highest level of transgene expression was obtained in HeLa cells, primary cardiac fibroblasts, and smooth muscle cells (Figure 6).

In general, transgene expression occurs after the release of pDNA from cationic polymers (decomplexation) in the cytosol or nucleus.[12] Therefore, the affinity between cationic polymers and pDNA inside the cells is a critical factor that determines efficient transfection. Indeed, Godbey et al. examined the effect of polyplex solution pH on the transfection efficiency by using poly(ethyleneimine) (PEI) as the transfection reagent.[23] However, this group found that the pH of PEI prior to forming a complex with pDNA did not create a significant difference from the transfection efficiency. This was because the pH adjustments made to the PEI/pDNA complex solutions were nullified once they were dipped into the culture medium. By contrast, in this study, we prepared the polyplex solutions at relatively high pH ranges from 8 to 11 by the addition of Tris (effective pH buffering range, 7−9) (Figure 1), and then coated the culture surface with them prior to adding cells suspended in media. In addition, the change in the affinity between PDMAEMA and pDNA that is due to altered solution pH was also observed when analyzed in electrophoresis buffer solution at a fixed pH of 8.3 (Figure 6). Therefore, the pH buffering of the polyplexes by Tris buffer could enhance their deposition in the culture surface microenvironment, after which they would be endocytosed by cells at a relatively higher pH when compared with the pH of the culture medium. This may contribute a more robust decomplexation of polyplexes during the endocytosis and cytosol trafficking inside cells.

After internalization of polyplexes, the first barrier for exit is considered to be endosomal escape. Indeed, it is well-known that the endosomal escape of polyplexes is a crucial rate-limiting step for efficient transfection. Here, polymers such as PEI or PDMAEMA acquire a cationic charge due to protonation of their many amino residues at acidic pH (<7). When they are incorporated into endosomes, where the pH is relatively low (ca. 5−7), their cationic charges increased because of further protonation. This proton consumption causes destabilization and rupture of the endosome and ultimately leads to polyplex release via the so-called "proton sponge effect."[24] Furthermore, Tris is known to bind DNA not only by electrostatic interactions, but also by hydrogen bonds.[25] Therefore, if polyplexes are combined with Tris through hydrogen bonds and endocytosed at relatively high pH, deprotonated PDMAEMA and Tris may consume a lot of protons in the endosome, further accelerating the proton sponge effect and associated polyplex release. In any case, further studies are required to delineate the mechanism by which Tris addition enhances the deposition transfection method.

In our previous study, we obtained sustained transgene expression for 2 weeks by deposition transfection using PDMAEMA.[11] Consistent with this, the enhanced luciferase expression by the addition of Tris at a Tris/PDMAEMA ratio of 2 was maintained for at least 10 days post-transfection in HeLa cells and also in primary cells (Figure 8). This suggests that the enhancement of transfection by the addition of Tris is continuous, rather than transient, and appears to occur without deleterious effects to the cells.

■ CONCLUSION

In this study, we increased the utility of deposition transfection technology by using Tris buffer as a pH adjuster for polyplexes solutions composed of PDMAEMA and pDNA. This leads to a dramatic enhancement of transfection efficiency in cell lines and in primary cells. We believe that our findings confirm that deposition transfection is a valuable method for many research and clinical fields, including regenerative medicine and gene therapy.

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Notes

The authors declare no competing financial interest.

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