Temperature-Controlled Properties of DNA Complexes with Poly(ethylenimine)-*graft*-poly(*N*-isopropylacrylamide)

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End-functionalized poly(*N*-isopropylacrylamide) (PNIPA) was synthesized by living free radical polymerization and conventional free radical polymerization and was used to prepare graft copolymers with poly(ethylenimine) (PEI). The copolymers exhibited lower critical solution temperature (LCST) behavior between 30 and 32 °C and formed complexes with plasmid DNA. The LCST of the copolymers in the DNA complexes increased slightly to ∼34−35 °C. Cytotoxicity of the copolymers was evaluated by measuring lactate dehydrogenase (LDH) release from cells. The copolymers exhibited temperature-dependent toxicity, with higher levels of LDH release observed at temperatures above the LCST. Cellular uptake and transfection activity of the DNA complexes with the PEI-*g*-PNIPA copolymers were lower than those of the control PEI/DNA complexes at temperature below the LCST but increased to the PEI/DNA levels at temperatures above the LCST.

Introduction

Gene therapy shows a potential to treat and prevent a wide variety of genetic and acquired diseases. To fully utilize this potential, safer and more efficient vectors for delivery of genes are required. Vectors based on polyelectrolyte complexes of nucleic acids and synthetic cationic polymers (polyplexes) represent one of the major alternatives to viral vectors that usually do not raise the safety issues associated with viruses. Despite enormous progress in recent years, the efficiency of polyplexes has yet to achieve competitive levels.[1,2]

One of the promising approaches to overcome the difficulties with the low efficiency of polyplexes relies on exploiting specific stimuli to improve the efficiency of polyplex-mediated delivery by temporal and spatial control of their properties.[3] An emerging class of such polyplexes relies on polymers capable to undergo phase transition in response to changes in temperature that can be achieved clinically by localized hyperthermia.[4] This approach is, despite the limited progress so far, very promising not only due to the fast, highly nonlinear, and reversible nature of the responses to small changes in the environment but also due to the number of other stimuli that can be exploited.[5−9] Temperature-responsive vectors described thus far use synthetic acryl-based copolymers or genetically engineered elastin-like polypeptides as the temperature-responsive component.[10−12] One of the most widely used polymers, poly(*N*-isopropylacrylamide) (PNIPA), is a water-soluble polymer that undergoes a coil-to-globule phase transition at 32 °C (lower critical solution temperature, LCST).[13,14] In the earliest studies, temperature-responsive statistical copolymers of NIPA with cationic comonomers were used in gene delivery.[15−18] For example, terpolymers of NIPA with 2-(dimethylamino)ethyl methacrylate and butyl methacrylate were used to prepared complexes with plasmid DNA. The affinity of the copolymers to DNA and transfection activity of the DNA complexes could be controlled by temperature. In addition, the authors established that a temperature regime can be identified that enables control of the disassembly of the vector inside the cells and achievement of elevated transfection levels.[16,17] A carboxyl-terminated NIPA/vinyl laurate copolymer was prepared and coupled with chitosan by Sun et al.[19] The copolymer displayed thermoresponsive behavior with an LCST of about 26 °C. Light scattering analysis revealed that the size of the complex particles was dependent on temperature. The dissociation of the copolymer/DNA complexes could be tuned by varying the temperature. At 37 °C, the collapse of PNIPA was favorable for the formation of compact complexes. On the other hand, the hydrated and extended PNIPA chains facilitated unpacking of DNA from the complexes at 20 °C. In another recent study, physicochemical properties of complexes of DNA with graft copolymers of poly(L-lysine) (PLL) and PNIPA were investigated.[20] It was shown that the phase transition temperature of PNIPA grafts was not significantly affected by grafting onto PLL chains. Increasing the temperature from below to above the LCST caused marked changes in the properties of NIPA-containing polyplexes. The authors demonstrated that the phase transition of PNIPA chains caused an increase of the surface charge and structural density of polyplexes, observations compatible with the coil-to-globule collapse.

Several reports were published recently describing the use of graft copolymers of poly(ethylenimine) (PEI) with PNIPA for gene delivery in vitro.[21−23] The copolymer/DNA complexes displayed variations in gel retardation behavior above and below the LCST, showing a higher affinity of the copolymer to DNA above the PNIPA phase transition. Low levels of transfection were also achieved with the copolymer/DNA complexes. The graft copolymers of PEI with PNIPA showed toxicity comparable to that of PEI. Turk and colleagues reported that attachment of PNIPA to PEI significantly increases the LCST of the copolymer.[21] The surface charge of the DNA complexes was found to decrease when PNIPA-containing copolymers were used. The transfection activity was reported in HeLa cells, showing that copolymers of linear PEI with PNIPA had the highest efficiency of the vectors tested.[21]

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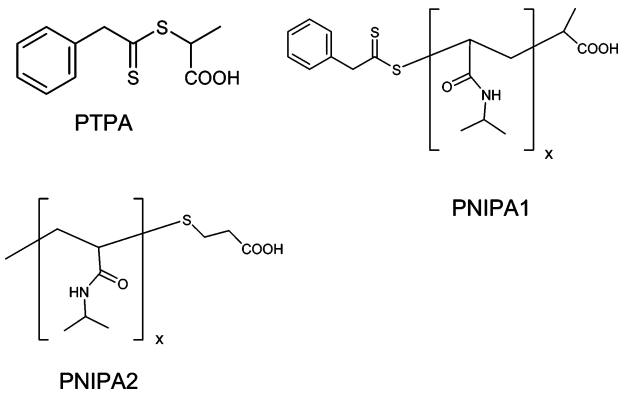

Figure 1. Chemical structures of RAFT chain transfer agent (PTPA), PNIPA1, and PNIPA2.

Our previous studies demonstrated that physicochemical properties of DNA polyplexes can be controlled by the phase state of PNIPA present in their structure.[20] Those studies were performed with complexes of DNA with graft copolymers of PLL and PNIPA. It was hypothesized that the possibility to control physicochemical properties of the polyplexes by changes in temperature could be translated into controlling aspects of their biological activity, such as cellular uptake and transfection efficacy. DNA complexes of PLL-based copolymers, used in our previous studies, are known to exhibit relatively low transfection efficiencies. Here, we extended the previous studies by replacing PLL with one of the most efficient polycations used in DNA delivery, PEI. Our report shows that both the physicochemical properties and to a certain extent the biological activity of PEI-*g*-PNIPA copolymers can be controlled by temperature.

Experimental Section

Materials and Methods. NIPA, azobisisobutyronitrile (AIBN), 3-mercaptopropionic acid (MPA), *N*-hydroxysuccinimide (HSu), (dimethylamino)pyridine (DMAP), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC), and branched PEI (25000 g/mol) were from Aldrich and used as received. Reversible addition−fragmentation chain transfer (RAFT) agent, 2-[(2-phenyl-1-thioxoethyl)-thio]propanoic acid (PTPA) (Figure 1), was synthesized as previously described.[24] DNA plasmids containing luciferase (gWizLuc) and enhanced green fluorescent protein (gWizGFP) reporter genes were purchased from Aldevron (Fargo, ND) as a 5 mg/mL aqueous solution and used without further purification. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen. All other reagents and solvents were purchased from Fisher and used as received.

Synthesis of Heterobifunctional Poly(*N*-isopropylacrylamide) by RAFT Polymerization (PNIPA1). NIPA (1.26 M) and PTPA (9.5 mM) were dissolved in acetone. The solution was transferred into a glass ampule and deoxygenated by a stream of nitrogen. AIBN (1.89 mM) was added after 30 min. The ampule was sealed, and polymerization was carried out in a water bath at 60 ± 1 °C for 24 h. After evaporation of solvent to half the original amount, the reaction mixture was added dropwise to cold diethyl ether. The precipitated polymer was isolated by filtration, dried overnight in a vacuum, and reprecipitated from methanol solution before analysis. The heterobifunctional structure of the polymer was confirmed from ^{1}H NMR analysis with a Varian 400 MHz NMR spectrometer using the phenyl group signal (7.35 ppm) and the methine signal at the carboxylic acid terminus (4.3 ppm).[24] The number-average (M_n) and weight-average (M_w) molecular weights and polydispersity index (M_w/M_n) of the polymer were determined by gel permeation chromatography (GPC) using a Shimadzu LC-10ADVP liquid chromatograph equipped with a CTO-10ASVP Shimadzu column oven and a Polymer Labs PL gel 5 μm mixed C column. The system was equipped with a seven-angle BIMwA static light scattering detector and a BIDNDC differential refractometer (both from Brookhaven Instruments, Inc.). The BIMwA detector was equipped with a 30 mW vertically polarized solid-state laser (660 nm) as a light source. Dimethylformamide was used as an eluent at a flow rate of 1 mL/min at 35 °C. GPC data were analyzed using PSS WinGPC Unity software from Polymer Standards Services. PNIPA1 with M_w = 17040 and M_w/M_n = 1.02 was obtained (Figure 1).

Synthesis of Poly(*N*-isopropylacrylamide) by Conventional Radical Polymerization in the Presence of Chain Transfer Agent (PNIPA2). A methanol solution of NIPA (1.19 M) and MPA (66 mM) was deoxygenated by a stream of nitrogen. AIBN (2.39 mM) was added after 30 min. The polymerization was carried out in a water bath at 60 ± 1 °C for 24 h. After evaporation of solvent to half the original amount, the reaction mixture was added dropwise to cold diethyl ether. The precipitated polymer was isolated by filtration, dried overnight in a vacuum, and reprecipitated from acetone solution before analysis. Characterization of the polymer was the same as mentioned for PNIPA1. PNIPA2 with M_n = 4060 and M_w/M_n = 2.1 was obtained (Figure 1).

Synthesis of PEI-*graft*-PNIPA Copolymers. PNIPA and DMAP were dissolved in $CHCl_3$ and cooled in an ice bath. HSu in THF was added to the polymer solution slowly. EDC in $CHCl_3$ was added to the above solution and the resulting solution stirred for 30 min. Then the solution was added quickly to ice-cold PEI solution in $CHCl_3$ with vigorous stirring. After 3 h, the solvent was evaporated to one-third the original amount and the polymer was precipitated into cold diethyl ether. The white solid obtained was dried in vacuo, dissolved in water, acidified with 1 M HCl to pH 4, and freeze-dried. The freeze-dried solid was washed extensively with acetone and dialyzed to remove unreacted PNIPA. The graft copolymers were obtained in ~60% yields and were analyzed by ^{1}H NMR. The content of NIPA units was calculated from the ratio of the integral intensities of the methine signal (3.8 ppm) of the isopropyl group of NIPA and the corrected methylene signal of PEI. Two copolymers were prepared with similar weight contents of PNIPA based on PNIPA1 (PEIPNIPA1, 28% PNIPA1) and on PNIPA2 (PEIPNIPA2, 33% PNIPA2). On average, PEIPNIPA1 contained 1.1 PNIPA1 molecules and PEIPNIPA2 contained 5.6 PNIPA2 molecules per PEI molecule.

Formulation of DNA Polyplexes. Polyplexes were prepared at room temperature in 10 mM HEPES buffer (pH 7.4) or HEPES-buffered saline (HBS) (10 mM HEPES (pH 7.4) + 150 mM NaCl) at a final plasmid DNA concentration of 20 μg/mL. PEI/DNA, PEIPNIPA1/DNA, and PEIPNIPA2/DNA complexes were formed by mixing equal volumes of a polycation and plasmid DNA solution to achieve the desired N:P ratio (concentration of amino groups of PEI:concentration of DNA phosphates). A mass of 325 per phosphate group of DNA was assumed in the calculations.

Ethidium Bromide Exclusion Assay. The ability of the synthesized copolymers to condense plasmid DNA was confirmed by measuring the changes in ethidium bromide/DNA fluorescence. DNA (gWIZLuc) solutions at a concentration of 20 μg/mL were mixed with ethidium bromide (1 μg/mL), and the fluorescence was measured using 360 nm excitation and 520 nm emission and set to 100%. The background fluorescence was set to 0% using ethidium bromide (1 μg/mL) solution alone. Fluorescence readings were taken at 25 °C following a stepwise addition of a polycation solution and condensation curves for each polycation constructed.

The effect of temperature on the ethidium bromide/DNA fluorescence at various N:P ratios was determined by measuring the fluorescence intensity between 25 and 41 °C. The complexes with N:P ratios of 1.5, 4.0, and 10 were prepared as described above in 10 mM HEPES (pH 7.4) at a final DNA concentration of 20 μg/mL and an ethidium bromide concentration of 1 μg/mL. The fluorescence intensity was measured using Fluoromax-3 (Horiba Jobin Yvon) (λ_{exc} = 545 nm, λ_{em} = 595 nm) equipped with a programmable digital water bath. The

temperature was increased in 1 °C increments and the fluorescence intensity measured 5 min after each temperature increase. The temperature dependence of the fluorescence intensity of the control samples (DNA + ethidium bromide, ethidium bromide) was determined in the same way. The results are presented as the relative fluorescence intensity using the fluorescence of DNA/ethidium bromide solution at each respective temperature as 100% and the fluorescence of ethidium bromide solution at each respective temperature as 0%.

Determination of the LCST. The phase transition temperatures of the PNIPA and PNIPA-containing copolymers were determined using a ZetaPlus particle size analyzer (Brookhaven Instruments) equipped with a 35 mW solid-state laser (658 nm). Scattered light was detected at a 90° angle. To determine the phase transition temperature, the temperature dependence of the scattering intensity at 90° from 1 mL of a 5 mg/mL solution of a copolymer in a glass cuvette was measured. The temperature was increased in 0.5−1 °C increments in the range 25−40 °C, and the reading was taken 3 min after each temperature increase. For each sample, the determination was performed at least on two separate occasions to confirm the reproducibility.

Hydrodynamic Radius and ζ Potential Determination. The determination of hydrodynamic diameters and ζ potentials of the copolymers and their DNA complexes was performed by dynamic light scattering using the ZetaPlus particle size and ζ potential analyzer. Solutions of copolymers at a 5 mg/mL concentration and complexes of gWIZLuc plasmid (20 μg/mL) with the copolymers prepared at room temperature in 10 mM HEPES or HBS were analyzed. Mean hydrodynamic diameters were calculated from the size distribution by weight, assuming a log-normal distribution using the supplied algorithm, and the results are expressed as a mean of three runs. ζ potential values were calculated from measured velocities using the Smoluchowski equation, and the results are expressed as a mean of 10 runs. The measurements were performed at different temperatures.

Cell Lines. Murine melanoma cell line B16F10 (CRL-6475) was obtained from ATCC. Human endothelial cell line EA.hy926, derived by a fusion of human umbilical vein endothelial cells with a human lung carcinoma A549, was a kind gift from Dr. Edgell (University of North Carolina). Both cell lines were maintained in DMEM supplemented with 4 mM L-alanyl-L-glutamine (Glutamax) and 10% FBS.

Cytotoxicity Assay. The cytotoxicity of the polycations was determined by a CytoTox 96 nonradioactive cytotoxicity assay (Promega), a colorimetric assay that measures lactate dehydrogenase (LDH) released from damaged cells. The cytotoxicity of the polycations was measured in both EA.hy926 and B16F10 cells. Ten thousand EA.hy926 cells were seeded in a 96-well plate. Two days after confluence was reached, the cytotoxicity of the polycations was evaluated by incubating the cells with gradually increasing concentrations of the respective polycations in DMEM/FBS without phenol red. The cells were incubated at two different temperatures: 25 and 37 °C. After 2 h of incubation at the respective temperatures, the medium samples were removed completely and were centrifuged at 250 rpm at 4 °C for 5 min. A 50 μL sample of the supernatant was carefully removed and was diluted with 50 μL of DMEM/FBS without phenol red. The LDH substrate was reconstituted, 50 μL of the reconstituted substrate mixture was then added to each sample, and the plate was immediately protected from direct light. Following incubation for 30 min in the dark at room temperature, 50 μL of the stop solution was added to each sample, and the absorbance was measured at 505 nm. The maximum LDH released was quantified by freeze−thaw lysis of untreated cells followed by medium processing as described above. The results are expressed as the mean percent cytotoxicity relative to untreated cells ± SD. The cytotoxicity of the polymers in B16F10 cells was determined by the same method, except that the experiment was performed 24 h after the cells were seeded.

Cellular Uptake of DNA Complexes. The cellular association of the fluorescently labeled polyplexes prepared at various N:P ratios was measured using flow cytometry. B16F10 cells were plated in a 24-well plate at a seeding density of 60000 cells per well, 24 h before the experiment. Plasmid DNA gWiz-Luc (40 μg/mL) in the respective buffer was labeled with YOYO-1, a fluorescent intercalator dye, at a molar ratio of 1 dye molecule per 50 base pairs of the DNA. The labeled DNA was allowed to stand in the dark for 30 min at room temperature. Cells were incubated with the polyplexes in 250 μL of the complete growth medium (DMEM/FBS). The plasmid DNA concentrations in the incubation media were 2.85 and 5.7 μg/mL. The cellular uptake of the above polyplexes was studied by incubating the cells at two different temperatures: 25 and 37 °C. The cells were incubated at the respective temperatures for 3 h, the medium was completely removed, and the cells were washed once with phosphate-buffered saline (PBS), trypsinized, resuspended in PBS, and centrifuged at 850 rpm for 15−20 min. After centrifugation, the supernatant was carefully removed and the cell pellet was resuspended in 150 μL of PBS. The fluorescence associated with the cells was measured using a Becton Dickinson FACSCalibur flow cytometer. YOYO-1 was excited using the 488 nm line of an argon laser and emitted light collected at 520 nm (green fluorescence). Cell debris showing reduced forward and side scatter was excluded from analysis. Histogram plots were constructed using the CellQuest software. The arithmetic mean of the cells incorporating fluorescence from each sample was calculated, and the results are expressed as the mean fluorescence intensity ± SD of triplicate samples. At least 10000 events per sample were counted.

In Vitro Gene Expression. Transfection activity was analyzed using PEI and PEIPNIPA2 polyplexes formulated at various N:P ratios. The plasmid DNA concentration was 20 μg/mL. All transfection studies were performed in 48-well plates with cells plated 24 h before transfection at a seeding density of 30000 cells per well. On a day of transfection, the B16F10 cells were incubated with the complexes in 150 μL of FBS-supplemented DMEM. The plasmid DNA concentrations in the incubation media were 2.85 and 5.7 μg/mL. The transfection activity of the above polyplexes was studied by incubating the cells at two different temperatures: 25 and 37 °C. After 3 h of incubation, the transfection mixture was completely removed and the cells were cultured at 37 °C for an additional 24 h in fresh DMEM/FBS prior to analysis of reporter gene expression.

To determine levels of luciferase expression, the culture medium was discarded and cell lysates were harvested after incubation of cells for 30 min at room temperature in 100 μL of cell lysis reagent buffer (Promega). To measure the luciferase content, 100 μL of luciferase assay buffer (20 mM glycylglycine (pH 8), 1 mM $MgCl_2$, 0.1 mM EDTA, 3.5 mM DTT, 0.5 mM ATP, 0.27 mM coenzyme A) was automatically injected into 20 μL of cell lysate and the luminescence was integrated over 10 s using a single-tube Sirius luminometer (Zylux Corp.). The total cellular protein in the cell lysate was determined by the bicinchoninic acid (BCA) protein assay using a calibration curve constructed with standard bovine serum albumin solutions (Pierce). The luciferase transfection results are expressed as relative light units (RLUs) per milligram of cellular protein. Unless stated otherwise, the results are expressed as the mean RLUs/mg of protein ± SD.

Analysis of GFP expression was carried out using a Becton Dickinson FACSCalibur flow cytometer. After 24 h of incubation, the culture medium was discarded and the cells were washed with PBS, trypsinized, resuspended in PBS, and centrifuged at 850 rpm for 15−20 min. The supernatant was removed, and the cell pellet was resuspended in PBS and fixed in 1.5% paraformaldehyde. GFP was excited using the 488 nm line of an argon laser and emitted light collected at 520 nm (green fluorescence) and 575 nm (red fluorescence) to enable correction of autofluorescence by diagonal gating. Background fluorescence and autofluorescence were determined using untreated cells. Cellular debris showing reduced forward and side scatter was excluded from analysis. The software program CellQuest was used to analyze the data, and the results are expressed as the percentage of GFP-positive cells. GFP expression was also visualized by fluorescence microscopy. The cells were washed once with warm PBS, and 0.5 mL of DMEM/FBS without phenol red was added to each well. The cells were visualized under a Nikon TE2000-U microscope equipped with

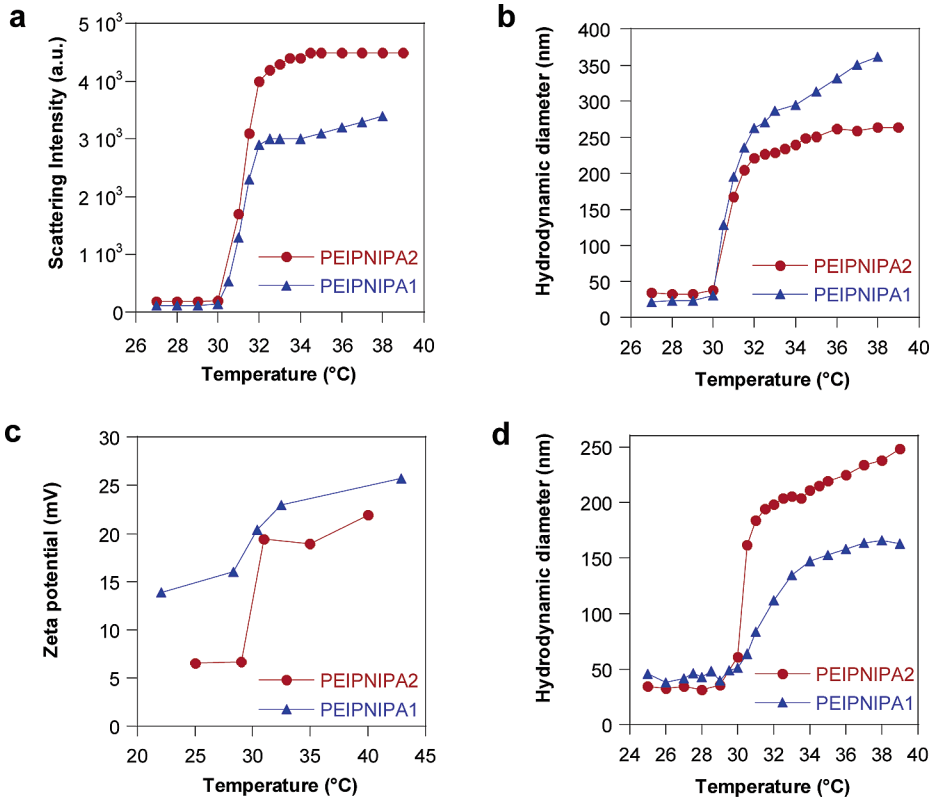

Figure 2. Temperature dependence of physicochemical properties in solutions of PEIPNIPA1 and PEIPNIPA2. The intensity of scattered light (a), hydrodynamic diameter (b), and ζ potential (c) were measured in 5 mg/mL solutions of the copolymers prepared in 10 mM HEPES buffer (pH 7.4). The hydrodynamic diameter was also measured in 5 mg/mL solution in DMEM (d). The solid lines serve only as visual guides.

a heated stage (images were acquired at 37 °C). The GFP expression was imaged using 455−485 nm excitation and 500−545 nm emission filters (DM 495 nm). Images were acquired at 10× magnification to maximize the field of view.

Results and Discussion

Two types of end-functionalized PNIPA homopolymers were synthesized and used in the preparation of the graft copolymers with branched PEI. Heterobifunctional PNIPA1 (Figure 1) was prepared by the RAFT free radical living polymerization using carboxylic acid-containing chain transfer agent. This allowed synthesis of α-carboxyl-*ω*-phenyldithioacetate-functionalized PNIPA, in which the carboxyl acid end group was utilized for attachment to PEI. The other terminal group of the polymer can be easily converted into a sulfhydryl group by aminolysis and used for attachment of other active ligands in a fashion analogous to the use of heterobifunctional PEG.[25] PNIPA2 was prepared by a traditional free radical polymerization in the presence of mercaptopropionic acid as the chain transfer agent to yield α-carboxy-functionalized PNIPA (Figure 1). Besides the presence of two functional end groups in PNIPA1, the main difference between the two polymers used is the polydispersity index. PNIPA1 has a very narrow distribution of molecular weights, as expected from the living radical polymerization.[26,27] Graft copolymers of PEI and PNIPA were synthesized by a standard carbodiimide coupling between the carboxylic acid end group of PNIPA1 and PNIPA2 and the amino groups of PEI. It was reported previously that the properties of poly(L-lysine)/DNA complexes containing PNIPA were affected by the PNIPA content, and no significant influence of the PNIPA molecular weight was observed.[20] Two PEI-*g*-PNIPA copolymers (PEIPNIPA1 and PEIPNIPA2) were synthesized to contain similar weight contents of PNIPA to confirm the finding also in PEI/DNA polyplexes.

The temperature-responsive behavior of the synthesized graft copolymers was analyzed by light scattering (Figure 2). Phase transition temperatures of NIPA copolymers are usually determined from the temperature dependence of the transmittance of their aqueous solutions under continuously increasing temperature conditions. In this study, the phase transition temperatures were determined from the temperature dependence of the scattering intensity measured at a 90° scattering angle. The temperature was increased stepwise in 0.5−1 °C increments, and the scattering intensity, as well as the mean hydrodynamic diameter, was measured 3 min after each temperature increase. This approach permits sensitive determination of the transition temperature even in copolymer solutions of low concentration exhibiting low levels of molecular association, which may be difficult to capture by the traditional method.[28] The analysis was performed at a copolymer concentration of 5 mg/mL in 10 mM HEPES buffer (pH 7.4). As expected, the scattering intensity increases abruptly between 31 and 32 °C in solutions of both graft copolymers (Figure 2a). This observation is in agreement with published evidence and suggests that the phase transition temperature of the PNIPA grafts is not affected by the conjugation to PEI and is similar to that of PNIPA homopolymers. Block copolymers of PNIPA with hydrophilic polymers and graft copolymers containing PNIPA grafts often exhibit an LCST similar to that of NIPA homopolymers because the cooperative domains in PNIPA blocks and grafts that undergo phase transition are not significantly perturbed by the other component.[20,22,23,29−31] In at least one reported case, however, attachment of PNIPA to PEI resulted in significantly increased LCST values.[21] On the other hand, the LCST of graft copolymers with a PNIPA backbone grafted with hydrophilic

polymers can be increased at high grafting densities, similar to that of random NIPA copolymers with hydrophilic monomers.[8,32] The scattering intensity in the solution of PEIPNIPA2 remains well stabilized above the LCST, suggesting association of the copolymers into relatively stable particles. The scattering intensity of PEIPNIPA1 increases slowly even above the phase transition temperature, indicating further changes in the particle structure and/or size. The control PNIPA homopolymer undergoes uncontrolled aggregation above the LCST that results in polymer precipitation. The control PEI solution exhibits no changes in the scattering intensity with increasing temperature.

The hydrodynamic diameter measured in the copolymer solutions (Figure 2b) exhibits a temperature dependence similar to that of the scattering intensity. With the exception of extremely diluted solutions, the coil-to-globule phase transition of PNIPA homopolymers in aqueous solutions leads to a macroscopic precipitation. Incorporation of hydrophilic components, such as here in the case of graft PNIPA copolymers with PEI, prevents the aggregation and leads to a formation of colloidal associates or micelles.[28−31] Both copolymers form a molecular solution below the LCST as indicated by the hydrodynamic diameters 20−30 nm. The copolymers associate rapidly between 30 and 32 °C and form particles of increasing sizes. The size of the PEIPNIPA1 particles continues to increase slowly above 32 °C, suggesting additional changes in the structure or limited aggregation. The ζ potential measured in the solutions of the copolymers also displays an abrupt change around the LCST (Figure 2c). The ζ potential increases significantly above the LCST. The increase in the ζ potential is most likely the result of the association of the copolymer molecules. The hydrophobic PNIPA molecules form the core and charged PEI molecules form the shell of the associates with a high surface charge concentration. Therefore, the surface charge of micelles is higher than that of polymer coils below the LCST.

The phase transition behavior of NIPA-based polymers is often studied in pure water or under conditions which may not be fully relevant for biomedical applications. Analysis of the phase transition behavior under conditions more relevant for the intended use of the polymers is necessary to translate any promising results into real settings. The transition of the two copolymers was therefore examined in a common cell culture medium (DMEM) (Figure 2d). DMEM is an isotonic solution with pH $\approx$ 7.1−7.4 and contains a mixture of essential amino acids, glucose, vitamins, and inorganic salts. The components of DMEM may affect the structure of hydrating water and therefore in turn also the phase transition of the copolymers. Figure 2d shows changes in the mean hydrodynamic diameter of the copolymers in the temperature interval 25−40 °C. The figure reveals that DMEM has no significant effect on the LCST of PEIPNIPA2. The particles formed, however, are smaller and less stable than those observed in Figure 2b. The phase transition of the copolymer containing PNIPA1 prepared by RAFT polymerization (PEIPNIPA1) is clearly wider than that of PEIPNIPA2 and that observed in 10 mM HEPES buffer (Figure 2b). We hypothesize that a possible reaction of the benzyl end group with amino group-containing components of the DMEM may be responsible for the observed behavior. Indeed, even aminolysis of the terminal phenyldithioacetate groups into sulfhydryl groups with amino groups of PEI within the graft copolymer can be responsible for the observed differences between PEIPNIPA1 and PEIPNIPA2 in this study. These observations strongly suggest that the terminus of PNIPA1 should be modified with a stable functionality before its coupling to PEI.

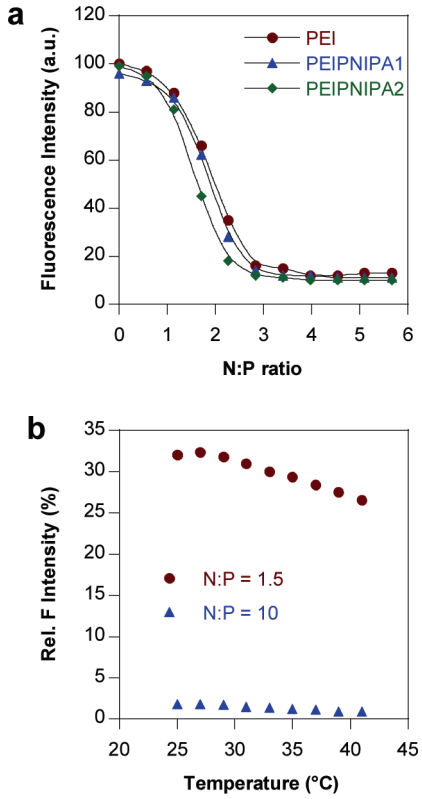

Figure 3. DNA condensation analyzed by ethidium bromide fluorescence. (a) The DNA condensation was determined by ethidium bromide exclusion assay at 25 °C. (b) The temperature dependence of ethidium bromide fluorescence was determined in a solution of PEIPNIPA2/DNA at N:P = 1.5 and 10.

The ability of the copolymers to form complexes with plasmid DNA was evaluated by the ethidium bromide exclusion assay at 25 °C. The assay relies on following the changes in fluorescence intensity of the dye while increasing the N:P ratio (Figure 3a). DNA condensation curves for both copolymers and control PEI show a typical sigmoidal profile with the point of equivalence around a N:P ratio of 2.5. This observation is in full agreement with previous reports and is due to only partial protonation of the PEI amines at the pH used.[33,34] Importantly, there are no significant differences in the DNA-condensing abilities between the copolymers and parent PEI. Because of the similar phase transition and DNA condensation behavior of PEIPNIPA1 and PEIPNIPA2 and because of the apparent chemical instability of the terminal benzyl group in PEIPNIPA1, all the following studies were conducted with PEIPNIPA2.

The ethidium bromide exclusion assay was conducted below the phase transition of the copolymers. The effect of temperature on the ethidium bromide fluorescence in the solution of PEIPNIPA2/DNA complexes was therefore investigated for various N:P ratios (Figure 3b). Continuous decrease of the fluorescence intensity with increased temperature is observed for polyplexes prepared below the stoichiometric ratio (N:P = 1.5) as well as for polyplexes prepared above the stoichiometric ratio (N:P = 10). The absence of a clear transition around the LCST of PEIPNIPA2 and similar temperature dependence observed for control PEI/DNA polyplexes (data not shown) suggest that the phase state of PNIPA within the PEIPNIPA2/DNA polyplexes has no significant effect on the DNA shape that would be reflected in changes of the ethidium bromide fluorescence.[20]

The temperature-controlled behavior of the plasmid DNA complexes with PEIPNIPA2 was first evaluated by measuring

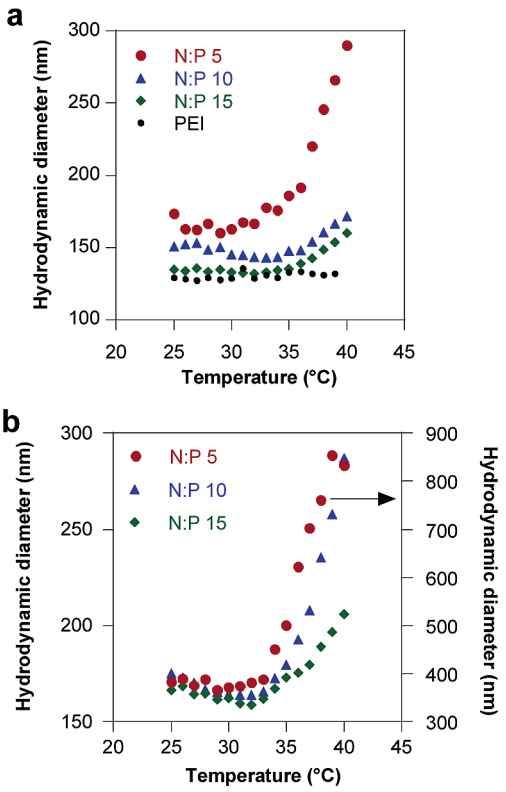

Figure 4. Temperature dependence of the size of DNA complexes of PEIPNIPA2 and PEI. The PEIPNIPA2/DNA complexes were prepared at N:P = 5, 10, and 15 and PEI/DNA at N:P = 5. The DNA concentration was 20 μg/mL. The temperature dependence of the hydrodynamic diameters of the complexes was measured in 10 mM HEPES buffer (pH 7.4) (a) and 10 mM HEPES + 150 mM NaCl (pH 7.4) (b). Secondary *y*-axis: N:P = 5.

the hydrodynamic diameter of the complexes at different temperatures between 25 and 40 °C (Figure 4a). The complexes were prepared at increasing N:P ratios in 10 mM HEPES buffer (pH 7.4). The goal of the initial experiments was to determine whether the LCST of the PNIPA chains is affected by their presence within the DNA complexes. The complexes were formed at 25 °C, and the temperature of the solution was then increased step-by-step with the measurements taken 3 min after each temperature increase. The size of the complexes prepared at N:P = 5 does not change significantly from the original size of ~170 nm between 25 and 32−33 °C. Above 33 °C, the size of the complexes increases significantly, reaching 290 nm at 40 °C. As expected, the complexes prepared at higher N:P ratios are smaller and show a less pronounced increase in size above the LCST. The data in Figure 4a clearly suggest that the temperature-responsive behavior of the copolymers is preserved even within the DNA complexes. In addition, the data suggest that the LCST of the copolymers increases slightly, compared to that of free copolymers, to 33−35 °C. The PNIPA transition is triggered by the loss of hydrating water and is manifested by a collapse of the coil conformation into the more compact globule and by increased polymer hydrophobicity. Previously, it was demonstrated that the phase transition of PNIPA chains increased the structural density of DNA complexes, an observation compatible with the coil-to-globule collapse.[20] It would follow logically that the size of the complexes should decrease above the LCST. Concomitant hydrophobically driven aggregation of the DNA complexes, however, prevents in most cases clear demonstration of the size reduction of individual polyplexes. The increasing size of the PEIPNIPA2/DNA complexes in Figure 4a is a result of the increased hydrophobic character of the complexes above the LCST, which leads to their aggregation. The smaller size of the complexes prepared at higher N:P ratios above the LCST is a result of increased colloidal stability due to the presence of an excess of the copolymer. A note needs to be made about the presence of free PEIPNIPA2 copolymer in the solution of the complexes. Independent of the amount of PEI used for the formation of DNA complexes, purified PEI/DNA polyplexes show almost identical N:P ratios of about 2.5.[35,36] Thus, the complexes prepared at N:P = 5−15 contain between 50% and 80% of the free copolymer. However, the potential for interference of the free copolymer with the light scattering analysis is minimal. First, the concentration of the copolymer is almost 1000-fold lower than the concentration used in Figure 2. Second, the contribution of the large DNA complexes to the total light scattering intensity is much larger than that of the free copolymer. As expected, control PEI/DNA complexes show only small changes in their size in the temperature range studied. This observation is in agreement with the previous finding suggesting that increasing the temperature only results in a small increase in the molecular weight and size of the DNA complexes (from 85 nm at 25 °C to 93 nm at 60 °C).[37] Even though there is no obvious physical reason for significant changes in the size of PEI/DNA complexes in the temperature range studied, Twaites and colleagues reported irreversible aggregation of PEI/DNA complexes upon increasing the temperature from 20 to 40 °C.[22]

The experiment from Figure 4a was repeated under increased ionic strength in HBS (10 mM HEPES (pH 7.4), 150 mM NaCl). The increased ionic strength of the solution leads to a formation of larger particles above the LCST. It also decreases the colloidal stability of DNA complexes only weakly stabilized by electrostatic repulsion and leads to faster aggregation kinetics above the LCST. All these factors are then expected to increase the sensitivity of the LCST determination in solutions of DNA complexes containing PNIPA. The DNA complexes were prepared as above at 25 °C in HBS. As expected, the PEI/DNA complexes (N:P = 5) aggregated rapidly already from the beginning at 25 °C and reached sizes of ~800 nm within 20 min (data not shown). Even though all the PEIPNIPA2/DNA complexes prepared in HBS are larger than those prepared in 10 mM HEPES, their colloidal stability below the LCST is high enough to prevent any significant aggregation with time. Similar to Figure 4a, the size of the complexes is almost constant between 25 and 33 °C. The size of the complexes then increases abruptly above 33 °C. This observation confirms that the LCST of PEIPNIPA2 in the DNA complexes is slightly increased (~33−34 °C) compared to the LCST of the free copolymer (Figure 2). The difference in the LCST is likely to be an effect of the DNA polyplex microenvironment in which PNIPA is present.

The temperature-responsive behavior of PEIPNIPA2/DNA complexes was further confirmed by determining the kinetics of aggregation of the complexes above and below the LCST of the copolymer (Figure 5). The size of DNA complexes is an important determinant of their transfection activity.[38] The aggregation was measured in HBS at two temperatures, 25 and 40 °C. The PEIPNIPA2/DNA complexes are stable below the LCST at 25 °C, showing only a small increase of size over 60 min. This suggests that below its LCST the hydrated PNIPA grafts can provide an effective steric stabilization to the complexes. Above the LCST, the complexes aggregate rapidly over the observed 60 min, most likely due to the increased hydrophobic character of the collapsed PNIPA chains. Control

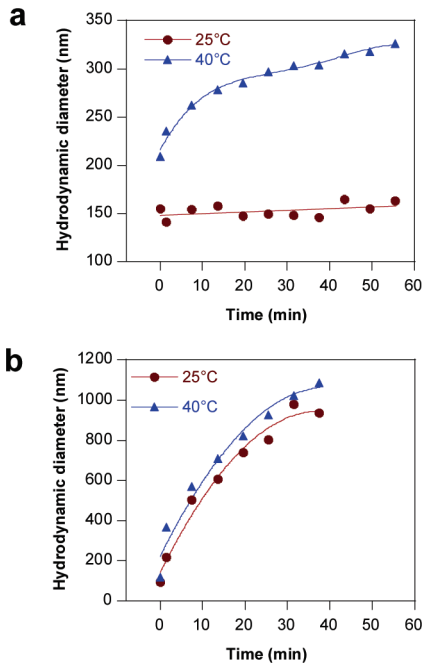

Figure 5. Effect of temperature on the aggregation kinetics of PEIPNIPA2/DNA (a) and PEI/DNA (b) complexes. The complexes were prepared at N:P = 5 and a DNA concentration of 20 μg/mL in 10 mM HEPES buffer (pH 7.4) with 150 mM NaCl.

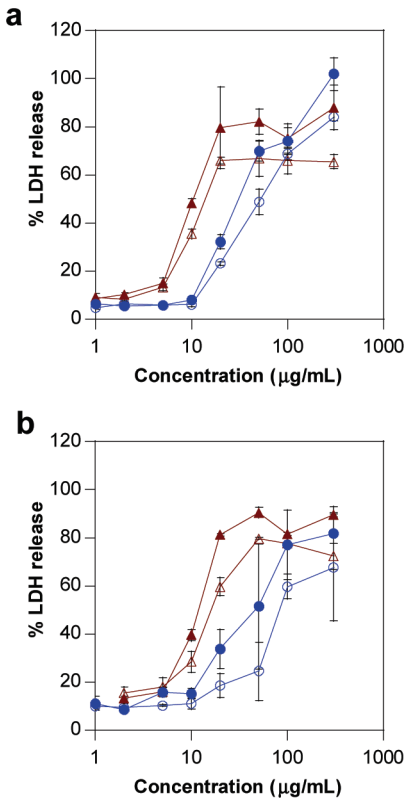

Figure 6. Temperature- and concentration-dependent LDH release from B16F10 (a) and Ea.hy926 (b) cells. LDH release was measured after 2 h of incubation with increasing concentrations of PEI at either 25 °C (△) or 37 °C (▲) and PEIPNIPA2 at either 25 °C (○) or 37 °C (●). Results are shown as the mean and standard deviation from triplicate samples. The solid lines serve only as visual guides.

PEI/DNA complexes show similar rates of aggregation at both temperatures (Figure 5b).

The toxicity of PEI and its complexes with DNA has been reported by a number of investigators in both in vitro and in vivo experiments.[36,39,40] Studies of the cytotoxicity of PEI suggest a multiphase nature of its action.[41,42] The first, relatively rapid process occurs within 0.5−2 h and results from direct plasma membrane destabilization demonstrated by considerable LDH release. The second, slower process that leads to cell death is due to activation of a "mitochondrially mediated apoptotic program" resulting from PEI-induced channel formation in the outer mitochondrial membrane. Structural features believed to most affect the way polycations such as PEI exert their cytotoxic action include the charge density, molecular weight, type of charged center, and molecular flexibility. Rigid molecules have more difficulties attaching to the membranes than flexible molecules. Therefore, polymers with high cationic charge densities and highly flexible polymers, such as PEI, are expected to cause greater cytotoxic effects than those with low cationic charge density and more rigid chains. We have evaluated the cytotoxicity of PEIPNIPA2 by determining the early stages of the toxic action by the measuring release of LDH (Figure 6). Because no data are available on the phase transition behavior in the complex intracellular environment, we hypothesized that the direct plasma destabilization would be most accessible to analysis of the effect of the phase state of the copolymer on its cytotoxicity. Data in Figure 2d show that the phase transition occurs in DMEM. The cells were therefore incubated with PEIPNIPA2 and PEI in serum-free DMEM for 2 h at two temperatures representing conditions below (25 °C) and above (37 °C) the LCST of PEIPNIPA2. The cytotoxicity was determined in a mouse melanoma cell line, B16F10 (Figure 6a), and a human endothelial cell line, EA.hy926 (Figure 6b). As expected, PEI shows higher cytotoxicity than PEIPNIPA2 in both cell lines. The estimated IC_{50} values for PEI at 37 °C are between 10 and 13 μg/mL, while the IC_{50} for PEIPNIPA2 under the same conditions is ∼34 μg/mL in B16F10 and ∼50 μg/mL in EA.hy926 cells. The seemingly large difference in the IC_{50} values is largely determined by the diluting effect of nontoxic PNIPA.[43] When expressed as the number of amino groups per milliliter, the cytotoxicities of PEI and PEIPNIPA2 are more similar at 37 °C, although the PEIPNIPA2 remains less toxic. The cytotoxicity of both polymers decreases at the lower temperature of 25 °C. The IC_{50} values for PEI at 25 °C are between 14 and 17 μg/mL, while the IC_{50} for PEIPNIPA2 under the same conditions is ∼62 μg/mL in B16F10 and ∼86 μg/mL in EA.hy926 cells. PEIPNIPA2 is present in its unimer form at 25 °C and in the form of highly positively charged associates/particles at 37 °C. Although the cytotoxicity of both PEI and PEIPNIPA2 decreases at 25 °C, the cytotoxicity of PEIPNIPA2 decreases significantly more. It is likely that PNIPA chains when present in their hydrated state below the LCST mask the positive charges of PEI and reduce the disrupting interactions of PEI with cell membranes, leading to lower levels of LDH release. Above the LCST, the positive charges of PEI are more exposed and the copolymer forms multimolecular associates, which may allow for an efficient interaction with the membranes. Somewhat surprisingly, both polycations exhibit higher cytotoxicity against the melanoma cells than against the endothelial cells.

The PEIPNIPA2/DNA complexes show temperature-responsive properties (Figure 4). We evaluated whether the thermal responsiveness of physical properties is reflected also in the biological activity of the complexes. It was expected that in particular cellular uptake of the DNA complexes would be affected by the changes in the physical properties due to differences in the surface properties and colloidal stability of PEIPNIPA2/DNA above and below the LCST. The cell uptake was evaluated above (37 °C) and below (25 °C) the LCST in

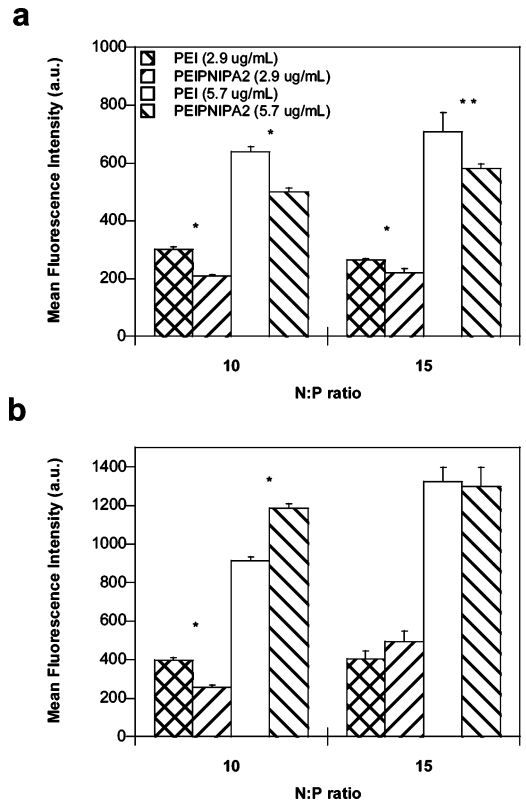

Figure 7. Influence of the incubation temperature and DNA dose on cellular uptake of PEI/DNA and PEIPNIPA2/DNA. Two doses (2.9 and 5.7 μg of DNA/mL) of complexes formulated at N:P = 10 and 15 were incubated with B16F10 cells for 3 h at 25 °C (a) or 37 °C (b) in DMEM supplemented with 10% FBS. Results are shown as the mean fluorescence intensity per cell ± SD of three samples. The same legend applies to both panels. The statistical significance for each PEI/PEIPNIPA2 pair was determined by the Student's *t* test (*, $p < 0.01$; **, $p < 0.05$).

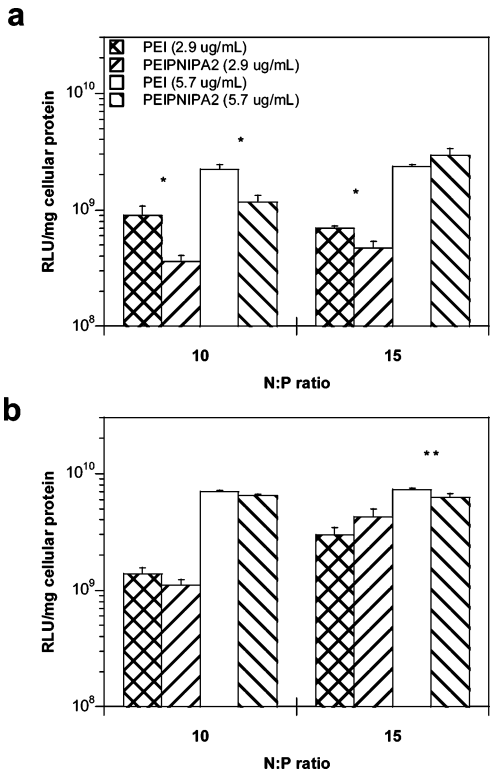

Figure 8. Influence of the incubation temperature and DNA dose on the transfection efficiency of PEI/DNA and PEIPNIPA2/DNA. Two doses (2.9 and 5.7 μg of DNA/ mL) of complexes formulated at N:P = 10 and 15 were incubated with B16F10 cells for 3 h at 25 °C (a) and 37 °C (b) in DMEM supplemented with 10% FBS. Luciferase expression was measured after 24 h at 37 °C. Results are shown as the mean RLUs expressed per milligram of cellular protein ± SD of three replicate assays. The same legend applies to both panels. The statistical significance for each PEI/PEIPNIPA2 pair was determined by the Student's *t* test (*, $p < 0.01$; **, $p < 0.05$).

B16F10 cells at two concentrations of DNA complexes prepared at two N:P ratios (Figure 7). PEI/DNA complexes exhibit higher cell uptake than the PNIPA-containing complexes under all four different conditions below the LCST (Figure 7a). Since PNIPA is present in its extended hydrophilic coil conformation, this observation is consistent with the known effect of nonionic polymers on cellular uptake of DNA complexes.[44] The cellular uptake of both PEI and PEIPNIPA2 complexes increases with increasing temperature to 37 °C (Figure 7b). The observation that the uptake of PEIPNIPA2/DNA increases more significantly than the uptake of PEI/DNA suggests that the phase state of PNIPA can indeed control the biological activity of the DNA complexes. The cell uptake of PEIPNIPA2/DNA prepared at N:P = 15 increases to levels statistically equivalent to those of PEI/DNA. The increase of the cell uptake of PEIPNIPA2/DNA is most significant at the higher dose tested (5.7 μg of DNA/ mL). This observation is consistent with increased surface charge density and reduced colloidal stability of the NIPA-containing complexes above the LCST, which then results in properties very similar to those of PEI/DNA. The data in Figure 7 confirm our hypothesis that the phase state of PNIPA within the PEIPNIPA2/DNA complexes can, to a certain extent, control the cell uptake. Overall, however, the concentration is a more important factor in controlling the levels of cell uptake than temperature.

The transfection activity of PEIPNIPA2/DNA complexes was first studied by measuring the levels of luciferase reporter gene expression in B16F10 cells (Figure 8). Preliminary experiments suggested that N:P ratios between 10 and 15 provide the highest levels of luciferase expression, and the experiments were therefore conducted at N:P = 10 and 15. The transfection activity exhibits trends similar to those of cellular uptake. Generally lower levels of luciferase expression of PEIPNIPA2/ DNA than PEI/DNA at 25 °C are consistent with the lower cell uptake and with the lower activity of DNA complexes containing hydrophilic nonionic polymers (Figure 8a). Increasing the incubation temperature to 37 °C results in a more significant increase of luciferase expression mediated by the PEIPNIPA2 complexes than control PEI complexes. While the luciferase expression at 25 °C is significantly higher in the case of PEI/ DNA, the levels of luciferase expression at 37 °C are essentially identical for both types (PEI and PEIPNIPA2) of DNA complexes studied (Figure 8b). As expected, the transfection efficiency increases with increasing DNA dose and in the case of the lower DNA dose also with increasing N:P ratio. The comparison of the data in parts a and b of Figure 8 suggests that the phase state of PNIPA can indeed control the biological activity of the DNA complexes, although the effect is relatively subtle.

Further transfection studies were performed using the GFP reporter gene to obtain data complementary to the results with the luciferase reporter gene. The percentage of B16F10 cells expressing GFP after transfection with two doses of PEI/DNA and PEIPNIPA2/DNA (N:P = 10 and 15) was analyzed by flow cytometry and fluorescence microscopy. The GFP expression experiments show a temperature pattern similar to that of

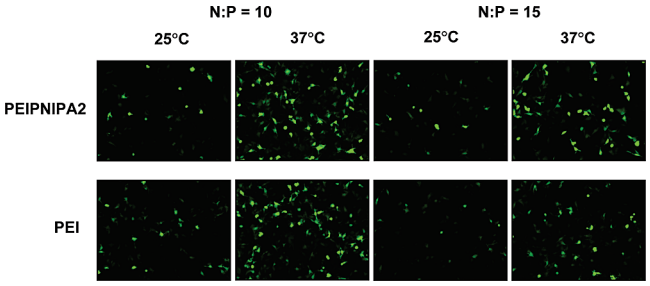

Figure 9. Influence of the incubation temperature on GFP expression. PEI/DNA and PEIPNIPA2/DNA complexes were formulated at N:P = 10 and 15 and incubated with B16F10 cells for 3 h at 25 or 37 °C in DMEM supplemented with 10% FBS (DNA dose 5.7 μg/mL). GFP expression was analyzed after 24 h at 37 °C.

experiments using the luciferase reporter gene. The highest GFP expression for PEINIPA2/DNA (5.7 μg of DNA/mL) is observed at N:P = 10, where the percent of GFP-positive cells increases from 25.0% (SD = 1.3) at 25 °C to 78.0% (SD = 1.1) at 37 °C. PEI/DNA exhibits a similar temperature dependence documented by an increase of GFP-positive cells from 37.4% (SD = 1.1) at 25 °C to 79.5% (SD = 1.9) at 37 °C. Importantly, however, increasing the temperature has again a more pronounced effect on the NIPA-containing DNA complexes than on the control PEI/DNA. The percent of GFP-positive cells increases by a factor of more than 3 upon increasing the temperature from 25 to 37 °C in the case of PEIPNIPA2/DNA but only by a factor of about 2 in the case of PEI/DNA. Increasing the N:P ratio to 15 at the higher DNA dose used results in a decrease of the GFP-positive cells to 53%. The results obtained at the DNA dose of 5.7 μg/mL are illustrated by fluorescence micrographs in Figure 9. It should be noted that flow cytometry is a more sensitive method for detection of GFP expression than fluorescence microscopy. In experiments with the low DNA dose (2.85 μg/mL), the optimum N:P ratio for both PEIPNIPA2 and PEI complexes increased to 15. The NIPA-containing complexes exhibit an increase in the GFP-positive cells from 15.0% (SD = 0.3) at 25 °C to 41.2% (SD = 3.4) at 37 °C, while PEI/DNA complexes show an increase from 22.4% (SD = 1.0) at 25 °C to 42.4% (SD = 1.6) at 37 °C.

Conclusion

These studies demonstrate that temperature-responsive copolymers of PEI and PNIPA can be used to form self-assembly complexes with DNA that exhibit temperature-controlled physical properties and to some extent also biological activity controlled by temperature. In particular, the cytotoxicity of the copolymers is clearly affected by the phase state of PNIPA in the copolymers, as documented by the higher levels of released LDH at temperatures above the LCST of the copolymer than at temperatures below the LCST. The temperature effect on the biological activity of the complexes of the copolymers with DNA was observed in cell uptake and transfection studies. Despite the clear and well-pronounced dependence of the physiochemical properties of the DNA complexes on temperature, changes of the biological activity of the complexes associated with the phase transition of PNIPA are more subtle, yet statistically significant. These results suggest that the physicochemical changes of the DNA complexes associated with the phase transition of PNIPA in the complexes may not represent a sufficiently strong stimulus for controlling the biological activity of the complexes. We hypothesize that incorporation of ligands with a biological activity at the free termini of PNIPA grafts in the copolymers will allow temperature-controlled presentation of the ligands.[45] The ability to control the surface presentation of the ligands can then offer a better possibility to control the biological activity of the DNA complexes than a simple reliance on physical changes of the complexes.

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References and Notes

(1) Pack, D. W.; Hoffman, A. S.; Pun, S.; Stayton, P. S. *Nat. Rev. Drug Discovery* **2005**, *4* (7), 581−93.
(2) Merdan, T.; Kopecek, J.; Kissel, T. *Adv. Drug Delivery Rev.* **2002**, *54* (5), 715−758.
(3) Oupicky, D.; Diwadkar, V. *Curr. Opin. Mol. Ther.* **2003**, *5* (4), 345−50.
(4) Meyer, D. E.; Shin, B. C.; Kong, G. A.; Dewhirst, M. W.; Chilkoti, A. *J. Controlled Release* **2001**, *74* (1−3), 213−24.
(5) Watanabe, M.; Akahoshi, T.; Tabata, Y.; Nakayama, D. *J. Am. Chem. Soc.* **1998**, *120* (22), 5577−5578.
(6) Meewes, M.; Ricka, J.; Desilva, M.; Nyffenegger, R.; Binkert, T. *Macromolecules* **1991**, *24* (21), 5811−5816.
(7) Inomata, H.; Goto, S.; Otake, K.; Saito, S. *Langmuir* **1992**, *8* (2), 687−690.
(8) Bisht, H. S.; Wan, L.; Mao, G.; Oupicky, D. *Polymer* **2005**, *46*, 7945−7952.
(9) Maeda, Y.; Yamamoto, H.; Ikeda, I. *Langmuir* **2001**, *17* (22), 6855−6859.
(10) de Las Heras Alarcon, C.; Pennadam, S.; Alexander, C. *Chem. Soc. Rev.* **2005**, *34* (3), 276−85.
(11) Oupicky, D.; Bisht, H. S.; Manickam, D. S.; Zhou, Q. H. *Expert Opin. Drug Delivery* **2005**, *2* (4), 653−65.
(12) Haider, M.; Hatefi, A.; Ghandehari, H. *J. Controlled Release* **2005**, *109* (1−3), 108−119.
(13) Qiu, Y.; Park, K. *Adv. Drug Delivery Rev.* **2001**, *53* (3), 321−339.
(14) Stayton, P. S.; Shimoboji, T.; Long, C.; Chilkoti, A.; Chen, G. H.; Harris, J. M.; Hoffman, A. S. *Nature* **1995**, *378* (6556), 472−474.
(15) Hinrichs, W. L. J.; Schuurmans-Nieuwenbroek, N. M. E.; van de Wetering, P.; Hennink, W. E. *J. Controlled Release* **1999**, *60*, 249−259.
(16) Kurisawa, M.; Yokoyama, M.; Okano, T. *J. Controlled Release* **2000**, *69* (1), 127−137.
(17) Kurisawa, M.; Yokoyama, M.; Okano, T. *J. Controlled Release* **2000**, *68* (1), 1−8.
(18) Takeda, N.; Nakamura, E.; Yokoyama, M.; Okano, T. *J. Controlled Release* **2004**, *95* (2), 343−55.
(19) Sun, S.; Liu, W.; Cheng, N.; Zhang, B.; Cao, Z.; Yao, K.; Liang, D.; Zuo, A.; Guo, G.; Zhang, J. *Bioconjugate Chem.* **2005**, *16* (4), 972−80.
(20) Oupicky, D.; Reschel, T.; Konak, C.; Oupicka, L. *Macromolecules* **2003**, *36* (18), 6883−6872.
(21) Turk, M.; Dincer, S.; Yulug, I. G.; Piskin, E. *J. Controlled Release* **2004**, *96* (2), 325−40.
(22) Twaites, B. R.; de las Heras Alarcon, C.; Cunliffe, D.; Lavigne, M.; Pennadam, S.; Smith, J. R.; Gorecki, D. C.; Alexander, C. *J. Controlled Release* **2004**, *97* (3), 551−66.
(23) Twaites, B. R.; de Las Heras Alarcon, C.; Lavigne, M.; Saulnier, A.; Pennadam, S. S.; Cunliffe, D.; Gorecki, D. C.; Alexander, C. *J. Controlled Release* **2005**, *108* (2−3), 472−83.
(24) D'Agosto, F.; Hughes, R.; Charreyre, M. T.; Pichot, C.; Gilbert, R. G. *Macromolecules* **2003**, *36* (3), 621−629.
(25) Blessing, T.; Kursa, M.; Holzhauser, R.; Kircheis, R.; Wagner, E. *Bioconjugate Chem.* **2001**, *12* (4), 529−537.
(26) Schilli, C.; Lanzendorfer, M. G.; Muller, A. H. E. *Macromolecules* **2002**, *35* (18), 6819−6827.
(27) Convertine, A. J.; Ayres, N.; Scales, C. W.; Lowe, A. B.; McCormick, C. L. *Biomacromolecules* **2004**, *5* (4), 1177−80.
(28) Konak, C.; Reschel, T.; Oupicky, D.; Ulbrich, K. *Langmuir* **2002**, *18* (21), 8217−8222.
(29) Virtanen, J.; Holappa, S.; Lemmetyinen, H.; Tenhu, H. *Macromolecules* **2002**, *35* (12), 4763−4769.
(30) Lin, H. H.; Cheng, Y. L. *Macromolecules* **2001**, *34* (11), 3710−3715.

(31) Konak, C.; Oupicky, D.; Chytry, V.; Ulbrich, K.; Helmstedt, M. *Macromolecules* **2000**, *33* (15), 5318−5320.
(32) Virtanen, J.; Tenhu, H. *Macromolecules* **2000**, *33* (16), 5970−5975.
(33) Bronich, T. K.; Nguyen, H. K.; Eisenberg, A.; Kabanov, A. V. *J. Am. Chem. Soc.* **2000**, *122* (35), 8339−8343.
(34) Zanta, M. A.; Boussif, O.; Adib, A.; Behr, J. P. *Bioconjugate Chem.* **1997**, *8*, 839−844.
(35) Boeckle, S.; von Gersdorff, K.; van der Piepen, S.; Culmsee, C.; Wagner, E.; Ogris, M. *J. Gene Med.* **2004**, *6* (10), 1102−11.
(36) Zou, S. M.; Erbacher, P.; Remy, J. S.; Behr, J. P. *J. Gene Med.* **2000**, *2* (2), 128−134.
(37) Reschel, T.; Konak, C.; Oupicky, D.; Seymour, L. W.; Ulbrich, K. *J. Controlled Release* **2002**, *81* (1−2), 201−217.
(38) Ogris, M.; Steinlein, P.; Kursa, M.; Mechtler, K.; Kircheis, R.; Wagner, E. *Gene Ther.* **1998**, *5* (10), 1425−1433.
(39) Fischer, D.; Bieber, T.; Li, Y. X.; Elsasser, H. P.; Kissel, T. *Pharm. Res.* **1999**, *16* (8), 1273−1279.
(40) Fischer, D.; Li, Y.; Ahlemeyer, B.; Krieglstein, J.; Kissel, T. *Biomaterials* **2003**, *24* (7), 1121−31.
(41) Moghimi, S. M.; Symonds, P.; Murray, J. C.; Hunter, A. C.; Debska, G.; Szewczyk, A. *Mol. Ther.* **2005**, *11* (6), 990−5.
(42) Godbey, W. T.; Wu, K. K.; Mikos, A. G. *Biomaterials* **2001**, *22* (5), 471−480.
(43) Vihola, H.; Laukkanen, A.; Valtola, L.; Tenhu, H.; Hirvonen, J. *Biomaterials* **2005**, *26* (16), 3055−64.
(44) Oupicky, D.; Carlisle, R. C.; Seymour, L. W. *Gene Ther.* **2001**, *8* (9), 713−724.
(45) Lee, E. S.; Na, K.; Bae, Y. H. *Nano Lett.* **2005**, *5* (2), 325−9.

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