

Characterization of complexation of poly (N-isopropylacrylamide-co-2-(dimethylamino) ethyl methacrylate) thermoresponsive cationic nanogels with salmon sperm DNA

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Abstract: Thermoresponsive cationic nanogel (TCNG) networks based on N-isopropylacrylamide (NIPAM), 2-(dimethylamino)ethyl methacrylate (DMAEMA), and quaternary alkyl ammonium halide salts of DMAEMA (DMAEMAQ) were synthesized by dispersion polymerization technique. The thermoresponsive properties of TCNGs and TCNG-salmon sperm DNA (sasDNA) polyplexes were characterized in aqueous media of various pH and ionic strength. P[NIPAM] and P[NIPAM/DMAEMA] TCNGs exhibited sharp volume phase transition (VPT) in water at critical temperatures (T_c) of 32 °C and 36 °C, respectively. Quaternized P[NIPAM/DMAEMAQ] TCNGs did not undergo sharp VPT up to 50 °C. The VPT of uncomplexed TCNGs were sensitive to the ionic composition and ionic strength of salts in solution, but were insensitive to pH in the range 5.0 to 7.4. The VPT of P[NIPAM/DMAEMAQ]/sasDNA diminished in magnitude with increasing W_p/W_d suggesting greater compaction of the polyplexes. The distinct phase-transition properties of P[NIPAM/DMAEMA]/sasDNA and P[NIPAM/DMAEMAQ]/sasDNA polyplexes were attributed to the condensing capability of polycations and to differences in the spatial distribution of structural charges in quaternized and nonquaternized networks. The findings demonstrate that stable TCNGs can be prepared with controllable responsive properties determined by the nature of the cationic charge incorporated and may have potential as vehicles for DNA delivery.

Keywords: poly(N-isopropyl acryamide), poly(2-dimethylamino)ethyl methacrylate, polyplex, thermoresponsive cationic nanogels

Introduction

Protonated amines such as poly(2-dimethylamino)ethyl methacrylate, P[DMAEMA], have been extensively studied as transfectants because of their ability to bind and condense DNA into submicron complexes (polyplexes) that protect the DNA from nucleases during transit to the nucleus.¹ P[DMAEMA] polyplexes have demonstrated potential to transfect a wide variety of cell types *in vitro*,^{2,3} but do not attain adequate expression levels *in vivo* to be therapeutically useful.⁴ Improving the *in vivo* performance of P[DMAEMA] polyplexes requires modifications that direct the polyplex to the tissue or region in which the target cells reside and optimize intracellular delivery steps that facilitate gene expression. In particular, the need to reduce the interaction with blood components and the inability of condensed DNA to efficiently dissociate from the polycation during cytoplasmic transport have been identified as major limitations of P[DMAEMA] polyplexes for *in vivo* application.⁵⁻¹⁰

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Most of the approaches that have been used to improve the dissociation properties of P[DMAEMA] polyplexes have utilized hydrolyzable bonds, either within the polymer backbone¹¹ or the on side-chain¹². Veron and colleagues prepared a derivative of P[DMAEMA], bearing hydrolyzable cationic functional groups on pendant side chains¹³. In spite of the ability of the hydrolyzable side-chain to form stable DNA complexes, better transfection results were obtained with P[DMAEMA] polyplexes that dissociated electrostatically rather than by the hydrolysis.

Copolymers of P[DMAEMA] have been synthesized and used as transfectants in which the main purpose of the copolymerized material is to influence the binding or release of the polycation to DNA.¹⁴ If the copolymer added is responsive in nature, ie possesses the ability to sense and respond to external triggers in a controlled manner, an additional functional role may be conferred on these structures, such as regulation of binding or release of DNA.^{15,16} Poly(N-isopropylacrylamide), P[NIPAM], is a thermoresponsive polymer with a lower critical solution temperature (LCST) of ~ 32 °C, ie linear homopolymer chains undergo dehydration and collapse to condensed structures above the LCST. P[DMAEMA] possesses buffering capacity in the low pH environment of the endosome, that may improve transfection efficiency and facilitate cell entry by endocytosis.^{17,18} Hinrichs and colleagues prepared linear copolymers of P[NIPAM/DMAEMA] and evaluated the effect of chain length and copolymer composition on binding of pDNA and transfection efficiency of OVCAR-3 cells (human ovarian cancer).¹⁹ The authors observed a decrease in transfection efficiency of polyplexes with increasing content of NIPAM in the copolymers, though their results did not suggest a cooperative role of P[NIPAM] in the release of pDNA.

Crosslinked cationic nanogels have been suggested as delivery systems for polyphosphates such as pDNA and antisense oligonucleotides in gene delivery or nucleoside analogs in chemotherapy.^{20,21} Most of the cationic nanogels investigated to date have been based on PEI, a polymer associated with considerable *in vitro* toxicity. Although branched PEI and PEG networks have shown reduced toxicity and size reduction,²² they lack responsive characteristics that could potentially mediate processes that may optimize therapeutic response including targeting via temperature and pH-control.

Xu and colleagues first reported on pH- and temperature-responsive hydrogels of PNIPAM, PDMAEMA and poly(hydroxyethyl methacrylate) (HEMA).²³ Subsequent work by the same group led to the development of pentablock

copolymers comprised of PHEMA and PDMAEMA as low toxicity, high transfection efficiency vectors for gene delivery.²⁴

In this work, we investigate the properties of P[NIPAM/DMAEMA] crosslinked thermoresponsive cationic nanogels, TCNGs and complexes of TCNGs with DNA. Our previous studies have shown that the incorporation of P[NIPAM] reduces the cellular toxicity associated with P[DMAEMA] linear copolymers.²⁵ However, the small and tight complexes these polymers form with DNA obfuscate the potentially useful dynamic thermoresponsive characteristics which are not observed in such structures. While the properties of cationic P[NIPAM] nanogels have been previously reported,²⁶ the novel P[NIPAM/DMAEMA] TCNGs described herein, have yet to be investigated.

Experimental

Materials

NIPAM (97%; Sigma-Aldrich, St. Louis MO, USA) was recrystallized from 70:30 hexane:toluene and dried *in vacuo*. 2-dimethyl(aminoethyl)methacrylate (DMAEMA, Sigma-Aldrich) was distilled under vacuum (45 °C, 5 mm Hg) in order to remove inhibitor. N,N'-methylene-bis-acrylamide (MBA), dodecyltrimethylammonium bromide (DTAB), 2,2'-azobis(2-methylpropionamide) dihydrochloride (AIBH), iodomethane, iodopropane, and iodopentane were used as received from the supplier (>98%; Sigma-Aldrich). Tetrahydrofuran (THF; ThermoFisher Scientific, Burlington ON, Canada) was freshly distilled over calcium chloride prior to use. Distilled deionized water (DDIW) was prepared with a Millipore system (Millipore, Billerica, MA, USA) and used for dilution purposes. Salmon sperm DNA (sasDNA) was used as obtained from the supplier, majority of DNA average $\leq 2,000$ bp; 1% TAE agarose gel (Invitrogen, Carlsbad, CA, USA).

Synthesis and characterization of quaternary ammonium alkyl halide monomers (DMAEMAQ)

DMAEMAQs were prepared by dissolving 19.1 mmol of DMAEMA in 5 mL of dry THF under stirring, followed by dropwise addition of 22 mmol of CH_3I , $\text{CH}_3(\text{CH}_2)_2\text{I}$ or $\text{CH}_3(\text{CH}_2)_4\text{I}$ dissolved in 5 mL of THF. The reaction was carried out for 12 h at room temperature with magnetic stirring. The crude products were filtered, washed twice with 10 mL aliquots of cold hexane and dried *in vacuo* for 8 h to yield the DMAEMAQ products (white crystalline solid, yield: 81%).

¹H NMR spectra of purified DMAEMAQ were recorded in D₂O on a Gemini 300 NMR Spectrometer (Varian, Palo Alto, CA, USA).

Synthesis of TCNGs

Cationic TCNGs were prepared by dispersion polymerization of NIPAM and DMAEMA or DMAEMAQ monomers using MBA as a crosslinking agent, DTAB as a cationic surfactant and AIBH as a cationic free-radical initiator. The polymerizations were carried out in a 500 mL three-neck flask. Table 1 and Figures 1(a–f) show the reagents and quantities used to prepare the TCNGs used in this study. The specified quantity of reagent was added to the flask and dissolved in 100 mL of DDIW. After complete dissolution of the reagents, 395 mL of deionized water were added to the flask and the solution heated to the desired reaction temperature (50 °C–70 °C) under nitrogen blanket. After 15 minutes, the polymerization was initiated by the addition of a 5 mL aliquot of azo-isobutyro-hydrochloride (AIBH). The polymerization reaction was carried out for 12 h under magnetic stirring at the temperatures indicated in Table 1. The TCNGs were purified by dialysis against DDIW for 72 h using a SpectraPor 3 dialysis membrane (MWCO 3500).

Preparation of TCNG/sasDNA polyplexes

TCNG/sasDNA complexes were prepared by mixing 200 µL of 10 µg/mL sasDNA with aliquots of 100 µg/mL of TCNG to the desired proportion by weight of polymer to DNA (W_p/W_d). The complex suspensions were subsequently diluted to a final volume of 500 µL with either DDIW water or phosphate-buffered saline (PBS) at the desired pH and ionic strength for particle size measurements. For zeta potential measurements, 400 µL of 10 µg/mL sasDNA was mixed with aliquots of 100 µg/mL of TCNG suspension in DDIW to the target W_p/W_d and diluted to a final volume of 2 mL with water.

Characterization of TCNGs and TCNG/sasDNA polyplexes

The particle sizes and zeta-potentials of TCNGs and TCNG/sasDNA complexes were measured by dynamic light scattering (DLS) and electrokinetic mobility measurement, respectively, using a PSS-Nicomp 380ZLS Particle/Zeta Sizer equipped with thermostatic control (PSS-Nicomp, Santa Barbara, CA, USA). TCNGs were diluted with DDIW or PBS for DLS measurements and DDIW for zeta-potential measurements. DLS measurements were run in triplicate using two five-minute cycles for each sample. The intensity-weighted hydrodynamic diameters are reported. Zeta-potential measurements were run using three 30-second cycles for each sample on not less than three independently prepared samples. Ten minutes were allowed for thermal equilibration of samples upon temperature change.

Results

Characterization of TCNGs

The quaternization of DMAEMA was confirmed by ¹H NMR. For the NDQPI6 TCNG, the ¹H NMR chemical shifts of proton resonances were assigned as follows (δ ppm, multiplicity): 0.8, d (CH(CH₃)), 2.0, m (CH₂CH(CH₃)), 3.2, s (N(CH₃)₃⁺I⁻), 3.7, t (CH₂N(CH₃)₃⁺I⁻), and 4.6, t (OCH₂CH₂N(CH₃)₃⁺I⁻) and 5.9, d (CH₂CH(CH₃)). Complete quaternization of the DMAEMA was indicated by the absence of upfield shifted α-CH₂ and β-CH₂ amino proton resonances at 2.5 ppm and 4.1 ppm, respectively.²⁷

Table 2 summarizes the particle size and zeta-potential of P[NIPAM] and P[NIPAM/DMAEMA] TCNGs prepared in this study. The TCNGs N100, ND9-1 and ND9-2 have intensity-averaged hydrodynamic diameters (D_1) at 25 °C in the range of 460–620 nm. NDQMI16, NDQPRI6, and NDQPI6 were significantly smaller ranging from 90–420 nm and have much narrower particle size distributions (data not shown). All of the TCNGs possess positive surface charge, however

Table 1 Recipe for synthesis of TCNGs

Nanogel ^a	Reaction temp (°C)	NIPAM (mM)	DMAEMA (mM)	DMAEMAQ (mM)	MBA (mM)	DTAB (mM)	AIBH (mM)
N100	70	140	–	–	12	32	4
ND9-1	50	120	12	–	12	32	4
ND9-2	70	120	12	–	12	32	4
NDQMI6	70	100	–	6	24	12	8
NDQPRI6	70	100	–	6	24	12	8
NDQPI6	70	100	–	6	36	12	8

Notes: ^aSample nomenclature. Numbers denote percentage of functional (DMAEMA or DMAEMAQ monomer in the feed) relative to the NIPAM content.

Abbreviations: N, NIPAM; D, DMAEMA; DQ, DMAEMAQ; MI, methyl iodide, PRI, propyl iodide, PI, pentyl iodide.

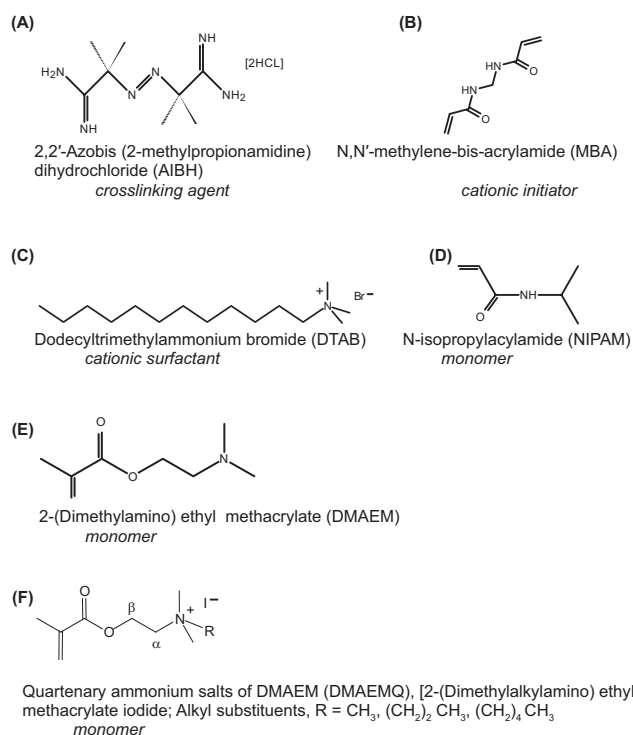


Figure 1 (A–F) Structures and names of substances used in synthesis of cationic polymer TCNGs.

the zeta-potential (ζ) of P[NIPAM/DMAEMA] TCNGs was considerably lower than those of P[NIPAM/DMAEMAQ] TCNGs. In the case of ND9-1 and ND9-2, ζ values were lower than the homopolymer N100 TCNG, in spite of the contribution of positively charged amine group on DMAEMA. For P[NIPAM/DMAEMAQ] TCNGs, ζ values increase with increasing length of the alkyl side chains in the sequence NDQPI6 > NDQPRI6 > NDQMI6. This may suggest that longer positively charged alkyl chains are more exposed at the surface of the TCNG. Collectively, the surface charge data on the TCNGs investigated here point towards dramatic differences in the abundance of charged groups at the surface of TCNGs bearing charged groups of different natures.

Thermoresponsive properties of P[NIPAM/DMAEMA] and P[NIPAM/DMAEMAQ] TCNGs

Effect of synthesis parameters and composition

The synthesis parameters and particle sizes of P[NIPAM/DMAEMA] TCNGs in DDIW are summarized in Tables 1 and 2, respectively. TCNGs containing 8.3%mol DMAEMA were prepared at different temperatures using

various amounts of initiator and cationic surfactant. ND9-1 synthesized at 50 °C has slightly larger diameter and was less responsive than ND9-2 prepared at 70 °C. Synthesis of P[NIPAM/DMAEMA] TCNGs without using DTAB surfactant resulted in broad particle size distributions with mean diameter above 2 μ m (data not shown).

The effect of varying initiator concentration in the feed was investigated on the properties of TCNGs. Presumably, the higher concentration of free radicals generated during the nucleation stage of polymerization due to higher temperature and initiator concentration would generate more foci for particle growth and lead to smaller particles. However, TCNGs prepared at 0.8 mM of AIBH exhibited broad particle size distributions, aggregated on standing and, thus, were excluded from further investigation.

Figure 2 compares the thermal response profiles of the N100 P[NIPAM] TCNG with DMAEMA functionalized TCNGs in DDIW. The N100 TCNG, whose charge originates from AIBH initiator residues and surfactant DTAB, retained its stability through the entire temperature range up to 50 °C. This is in contrast to the TCNGs functionalized with 9%mol DMAEMA, ND9-1 and ND9-2, which lost stability above the LCST.

ND9-2 and N100 exhibited marked responsiveness to temperature, undergoing nearly a 20-fold change in hydrodynamic volume. These transitions are known as volume phase transition (VPT) in which nonlinear collapse of the polymer chains and dehydration of the TCNGs occur through a relatively narrow temperature range. At the pH of the deionized water ~pH 6.5, the tertiary amines of DMAEMA with a pK_a of around 7.0 are expected to be nearly 70% protonated.²⁸ Thus, the diminished thermal response of ND9-1 and ND9-2 TCNGs is likely the result of electrostatic repulsion between charged amine groups that prevents structural collapse of the chains and retains water molecules within the nanogel network.

Effect of quaternization and alkyl side-chain length of TCNGs

The thermal response profiles of P[NIPAM/DMAEMA] TCNGs differ from P[NIPAM/DMAEMAQ] TCNGs as shown Figure 2 and Figure 3, respectively. P[NIPAM/DMAEMA] TCNGs undergo sharp VPT with temperature in the range of 32 °C–36 °C. [NIPAM/DMAEMAQ] TCNGs exhibit near linear shrinkage over a broader temperature range without discernable VPT.

Deswelling ratios D_{20}/D_{50} defined as the ratio of hydrodynamic diameters at temperatures bounding

Table 2 Diameter and zeta-potential of TCNGs

Nanogel	% DMAEMA (% mol) ^a	% DMAEMAQ (% mol) ^a	$D_1 \pm SD$ (nm) ^b (Range)	D_{20}/D_{50} ^c	ζ (mV)
N100	0	–	580 ± 8 (420 to 700)	2.7	+ 5.3 ± 0.8
ND9-1	8.3	–	618 ± 5 (300 to 890)	1.4 ^d	+ 1.1 ± 0.9
ND9-2	8.3	–	580 ± 7 (310 to 820)	2.2 ^d	+ 1.4 ± 1.0
NDQMI6		4.6	90 ± 5 (60 to 120)	1.1	+ 4.1 ± 0.7
NDQPRI6		4.6	160 ± 10 (80 to 250)	1.1	+ 8.4 ± 0.6
NDQPI6		4.2	420 ± 7 (190 to 570)	1.4	+ 12.3 ± 0.7

Notes: ^amol DMAEMA or DMAEMAQ/mol (DMAEMA + NIPAM + MBA) × 100%. ^bMean intensity-averaged hydrodynamic diameter (D_1) in DDIW ± standard deviation of 3 or more independent measurements. The range in a sample represents the average of the minimum and the average of the maximum that bounds 95% of the particle sizes recorded using a Gaussian analysis of the particle size distribution. Results are rounded to two significant figures. ^cRatio of D_1 at 20 °C and 50 °C. ^dRatio of D_1 at 20 °C to D_1 at temperature of loss of particle stability.

the phase transition, 20 °C and 50 °C are considerably smaller for P[NIPAM/DMAEMAQ] TCNGs than those of P[NIPAM/DMAEMA] TCNGs (Table 2). The effect of alkyl side-chain length on the temperature response profiles of P[NIPAM/DMAEMAQ] TCNGs was examined. D_{20}/D_{50} value of 1.1 was obtained for the methyl and propyl quaternary alkyl ammonium salts TCNGs, NDQMI6, and NDQPRI6, respectively. D_{20}/D_{50} value of 1.4 was obtained for the pentyl side chain-bearing NDQPI6 suggesting that the bulkier side chain may reduce the tendency of quaternary alkyl ammonium salts to prevent the collapse of polymer chains within the gel network. The effect of quaternization on deswelling of P[NIPAM/DMAEMAQ] is remarkable given the low quaternized monomer content in these TCNGs (4.2%–4.6%mol). The lack of VPT of P[NIPAM/DMAEMAQ] TCNGs cannot be attributed to crosslinking as the NDQPI6 sample incorporating approximately 36%mol

MBA in the feed showed a modest degree of responsiveness, whereas the NDQMI6 and NDQPRI6 samples containing approximately 24%mol MBA did not undergo deswelling in response to temperature increase. Given the relatively low level of DMAEMAQ in P[NIPAM/DMAEMAQ] TCNGs, the lack of VPT suggests that the distribution of charges and steric effects due to the alkyl side chains impact the collapse of nanogel network. As well, the smaller size P[NIPAM/DMAEMAQ] TCNGs suggests that the nanogel network is considerably less hydrated and, therefore, is less influenced by the dehydration of NIPAM chains around the LCST.

Effect of ionic strength

Figures 4 and 5 present the effect of ionic strength and pH on the thermal response profiles of P[NIPAM/DMAEMA] and P[NIPAM/DMAEMAQ], respectively. ND9-2 (Figure 4, main frame) is only slightly sensitive to ionic

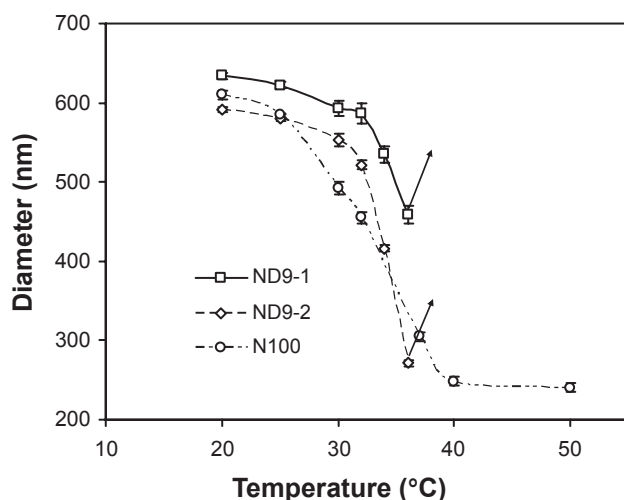


Figure 2 Temperature dependence of particle size of cationic P[NIPAM] and P[NIPAM/DMAEMA] TCNGs in DDIW ($n = 3$). Arrows denote loss of particle stability and aggregation.

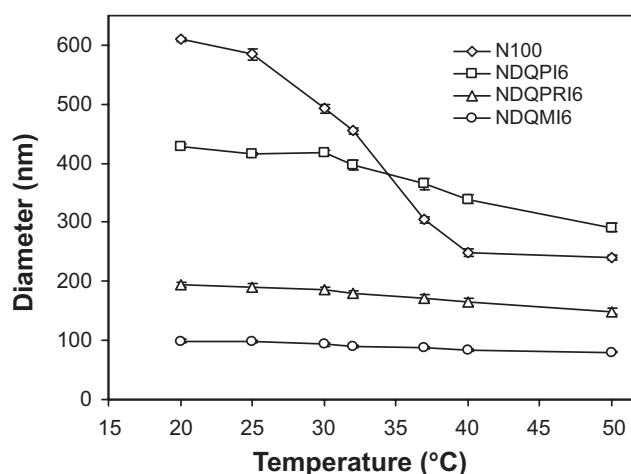


Figure 3 Temperature dependence of particle size of P[NIPAM/DMAEMAQ] TCNGs in DDIW ($n = 3$)/DMAEMA] TCNGs in DDIW ($n = 3$).

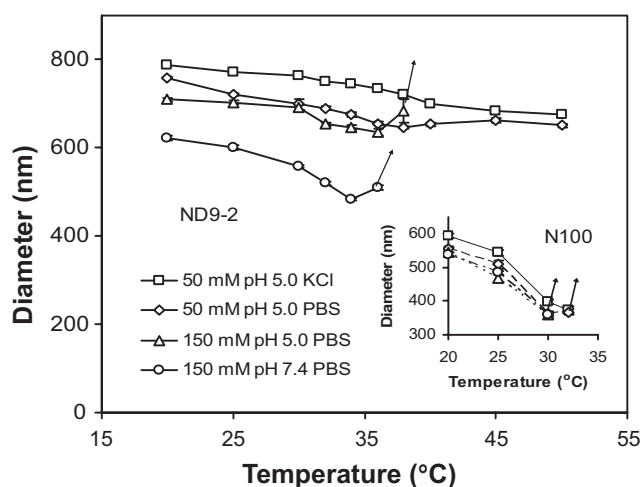


Figure 4 Temperature dependence of particle size of ND9-2 (main frame) and N100 (inset) in media of different pH and ionic strength ($n = 3$).

strength showing a tendency to suppress swelling of gels in high ionic strength media, 150 mM PBS. ND9-2 is sensitive to pH as suggested by the more rapid drop in diameter with temperature at pH 7.4 than at pH 5.0. On the other hand, even with the additional charge present on the ND9-2 due to protonation of the tertiary amine groups of DMAEMA, the TCNG aggregated above 36 °C. Apparently, salt concentration has a considerable influence on the stability of P[NIPAM/DMAEMA] TCNGs. By comparison, N100 (Figure 4, inset) and NDQPI6 (Figure 5) exhibit virtually no sensitivity to pH as evidenced by the invariant nature of the thermal transition profiles with pH. This is anticipated, as neither the initiator residues nor the functional monomer possess ionizable groups.

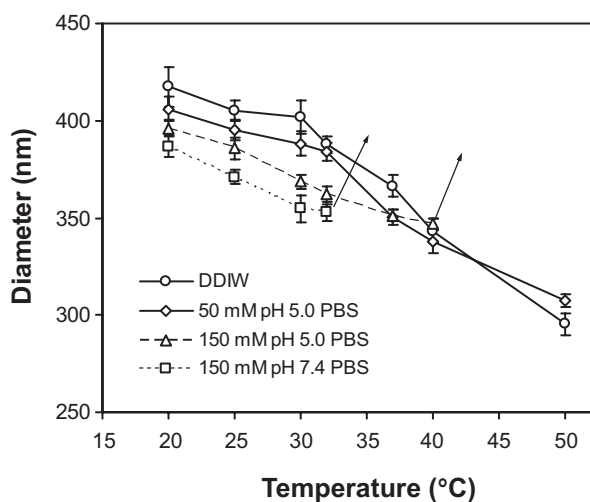


Figure 5 Temperature dependence of particle size of NDQPI6 in media of different pH and ionic strength ($n = 3$).

Both N100 and NDQPI6 exhibit modest sensitivity to ionic strength as evidenced by the contraction of the TCNGs and aggregation in 150 mM PBS. This lack of stability of the TCNGs is consistent with the suppression of the electric double layer surrounding the gels by high-salt environments. A slight shift of the VPT to lower temperature at pH 7.4 may be inferred from the curves. The N100 TCNG did not precipitate up to 50 °C in 50 mM KCl solution or 50 mM PBS. For NDQPI6, lowering the ionic strength of PBS from 150 mM to 50 mM enabled the TCNG to remain stable up to 50 °C. The effect of ionic strength on the thermal response profiles of NDQMI6 and NDQPRI6 mirrored that of NDQPI6, but was less pronounced due to the less responsive nature of those TCNGs (data not shown).

Thermoresponsive properties of TCNG/sasDNA polyplexes

P[NIPAM/DMAEMA]/sasDNA and P[NIPAM/DMAEMAQ]/sasDNA polyplexes were prepared and their physical properties evaluated. Whereas the P[NIPAM] N100 TCNG precipitated upon addition of sasDNA (data not shown), the addition of sasDNA to P[NIPAM/DMAEMA] and P[NIPAM/DMAEMAQ] did not result in the loss of particle stability. As shown in Figure 6, the ND9-2/sasDNA formed stable polyplexes with diameter larger than the ND9-2 alone. The effect of varying the polymer to DNA ratio W_p/W_d on the properties of the polyplexes was investigated. ND9-2/sasDNA particle size decreased with increasing W_p/W_d , however shape of the curves and the temperature at which particle stability was lost (~ 36 °C) did not vary. In the

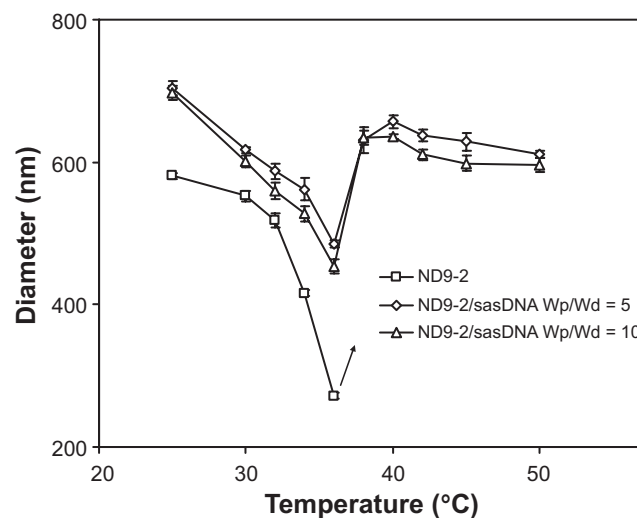


Figure 6 Temperature dependence of particle size of ND9-2 (main frame) with sasDNA in DDIW as a function of polymer:sasDNA weight ratio (W_p/W_d).

absence of sasDNA, ND9-2 exhibited a critical temperature T_c of around 36 °C at which point particle stability was lost. However, complexation of ND9-2 with sasDNA stabilized the structure as evidenced by the leveling of particle size to around 650 nm above this temperature (Figure 6).

The thermal transition profiles of NDQPI6/sasDNA polyplexes in 150 mM pH 7.4 PBS and deionized water are shown in Figure 7 and Figure 8, respectively. The NDQPI6/sasDNA polyplexes aggregate in PBS at a temperature well below that of uncomplexed NDQPI6 ($T_c \sim 36$ °C) and much closer to the LCST of P[NIPAM] ($T_c \sim 32$ °C). This is in contrast to the behavior observed for ND9-2/sasDNA polyplex, which was stabilized by complexation. As with ND9-2/sasDNA, the thermal response profile of NDQPI6/sasDNA profile was insensitive to W_p/W_d . Incubation of the sasDNA with NDQPI6 for a 72 h period did not alter the shape of the transition profile.

The thermal transition profiles of NDQPI6/sasDNA polyplexes prepared in deionized water (Figure 8) differed from those prepared in PBS (Figure 7). Rather than destabilization at T_c , a sharp decrease in the hydrodynamic size of the complexes similar to that of the uncomplexed NDQPI6 was observed. T_c was independent of W_p/W_d and occurred between 25 °C and 30 °C, well below that of ND9-2/sasDNA polyplex.

To further probe the nature of complexation of sasDNA with P[NIPAM/DMAEMAQ] TCNGs, the temperature dependence of surface charge was investigated. As seen in Figure 9, NDQPI6 exhibited a decrease in the value of ζ from +12.3 mV to +5.0 mV through the VPT, with a noticeable decrease near $T_c \sim 36$ °C. However, a decrease in particle volume through the VPT would be expected to increase the

charge density on the surface of TCNGs and, therefore, increase ζ . Noting the relatively small change in the diameter (Figure 3) and decrease in ζ of NDQPI6 with temperature, it appears as though collapse of the polymer chains results in screening of the point charges derived from the quaternized amine functional groups on the gel. NDQPI6/sasDNA polyplexes exhibit highly negative surface charge values (−9.0 mV to −19.8 mV) that decreases with increasing temperature up to 45 °C. At $W_p/W_d = 5$, NDQPI6/sasDNA were slightly more negatively charged than NDQPI6, suggesting that some or all of the additional sasDNA was bound to the surface of the TCNG in the complex. The increase in negative charge of NDQPI6/sasDNA with temperature is likely caused by the 3.5-fold change in hydrodynamic volume of the polyplex through the temperature range. Therefore, it appears that the stabilizing effect of sasDNA is the result of its binding at the surface of the polyplex and that the stabilizing effects are further enhanced as a result of contraction of the gel network during VPT.

Figure 10 shows the change in ζ of NDQPI6/sasDNA and NDQPRI6/sasDNA polyplexes as a function of W_p/W_d . For both of these polyplexes, the slope of the curve decreases substantially at around $W_p/W_d = 2$, suggesting the saturation of DNA-binding capability of the TCNG near this weight ratio. This supports particle size data that showed little change in temperature response of the polyplexes above this weight ratio.

The complexation process of sasDNA with NDQPRI6 was investigated. Figure 11 shows the temperature dependence of the diameter of NDQPRI6/sasDNA polyplexes prepared at different W_p/W_d in water (main frame) and in PBS (inset). NDQPRI6 is relatively insensitive to temperature

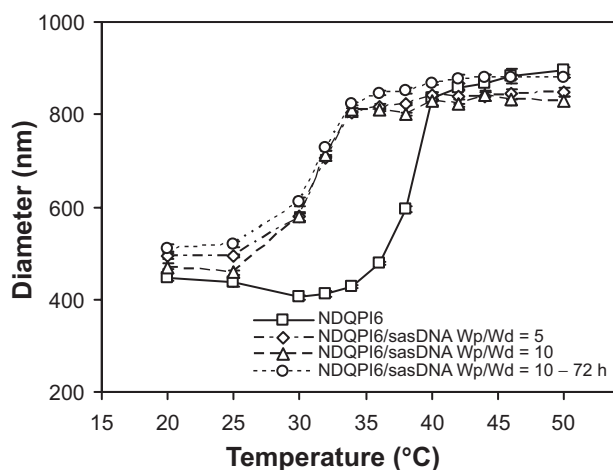


Figure 7 Temperature dependence of particle size of NDQPI6 and NDQPI6/sasDNA in 150 mM pH 7.4 PBS as a function of W_p/W_d and incubation time of 1 h and 72 h.

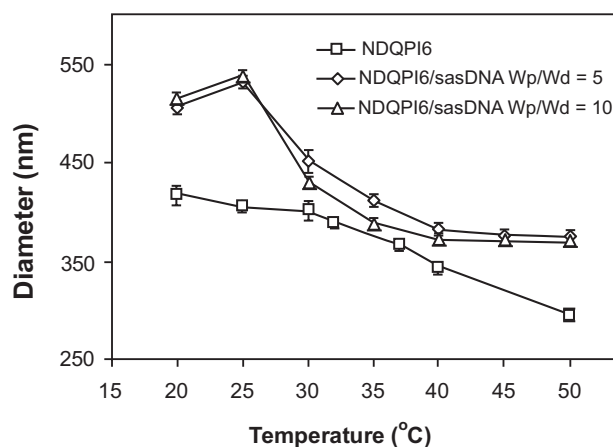


Figure 8 Temperature dependence of particle size of NDQPI6/sasDNA complexes with in DDIW as a function of W_p/W_d .

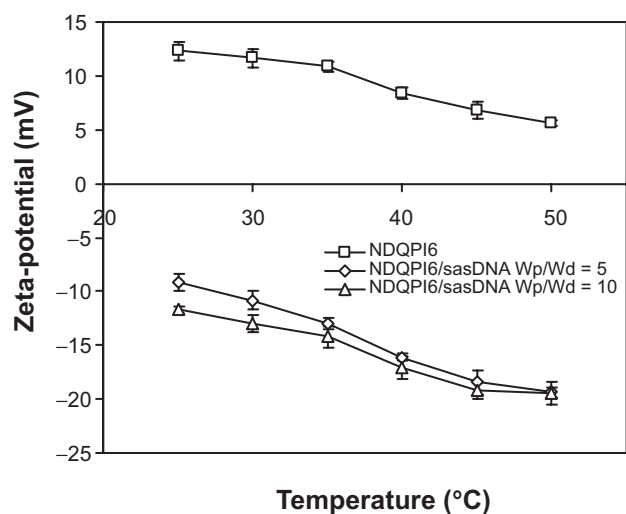


Figure 9 Temperature dependence of zeta-potential of NDQPI6 and NDQPI6/sasDNA in DDIW.

in the absence of sasDNA. Upon addition of sasDNA to the TCNG, a decrease in size at a given temperature occurs as a result of complex formation. At low level of sasDNA addition ($W_p/W_d > 1$), there was little change in the size of the polyplex and the polyplexes were relatively insensitive to temperature similar to the uncomplexed TCNG ($W_p/W_d = 0$). However, at sufficient sasDNA loading, $W_p/W_d \leq 1$, the polyplex increased dramatically in size and exhibited marked temperature dependence characteristic of VPT. At $W_p/W_d = 0.5$ and 1, there is a 4- to 5-fold increase in diameter of the NDQPRI6/sasDNA.

NDQPRI6/sasDNA polyplexes prepared in 150 mM pH7.4 PBS exhibited temperature dependencies similar to

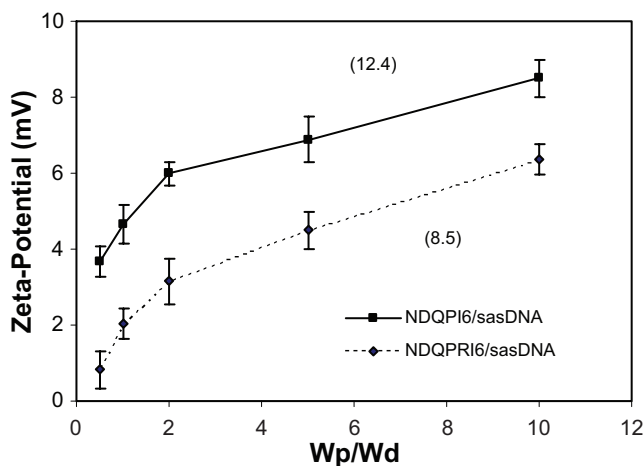


Figure 10 Dependence of zeta-potential of NDQPRI6/sasDNA and NDQPI6/sasDNA on W_p/W_d in DDIW. Numbers in parentheses are zeta-potentials of respective TCNGs in absence of DNA ($W_p/W_d = \infty$).

those prepared in water, suggesting that the presence of buffer salts did not interfere dramatically with the complexation (Figure 11, inset).

To check whether the properties of the NDQPRI6/sasDNA polyplexes were sensitive to the sequence of addition of reagents, the polyplexes were prepared by reverse addition of reagents, ie, DNA to polymer. Full condensation of sasDNA at higher W_p/W_d was not achieved when DNA was added to polymer at a temperature above the VPT (ie, 43 °C) (Figure 12). In contrast, the addition of polymer to DNA resulted in full condensation throughout the temperature range (25 °C to 43 °C) as evidenced by the drop in the diameter to a value near that of the uncomplexed TCNG ($W_d = 0$) above $W_p/W_d = 2$. At temperatures above VPT (ie, >37 °C), the complexation of sasDNA with NDQPRI6 appears to be invariant with the sequence of addition of polycation.

Discussion

We have demonstrated that TCNGs prepared from NIPAM and DMAEMA or DMAEMAQ exhibit distinctive phase transition properties both before and after complexation with sasDNA. The properties of TCNGs and polyplexes were characterized under physiologically relevant conditions of temperature, pH and salt concentration, thereby allowing some insight into the potential of these materials for DNA delivery.

sasDNA has on average 3000 bp and is, therefore, a very large entity approaching the size of typical *Escherichia coli* bacterial plasmid DNA (6000–9000 bp) used in many transfection protocols. Thus, qualitatively at least, sasDNA can serve as a model for characterization of the responsive dynamics of TCNG complexes that may ultimately be prepared from transfectable DNA forms.

For *in vivo* applications, monodisperse polyplexes less than 200 nm in diameter are preferred for systemic circulation in order to efficiently traverse blood capillaries and extravasate to surrounding tissue. However, it has been reported that large-sized, colloiddally unstable lipid/DNA complexes of over 700 nm diameter transfected more efficiently than colloiddally stable 250 nm complexes.²⁹ It is yet to be determined what if any effect particle size of P[NIPAM/DMAEMA] and P[NIPAM/DMAEMAQ] polyplexes has on their DNA delivery potential in either *in vitro* or *in vivo* applications.

P[NIPAM/DMAEMA] TCNGs of low DMAEMA content (8.3%mol) undergo extremely rapid dehydration and deswelling in water that results in loss of particle stability and aggregation. At pH 7.4, the dynamics of thermally induced phase transition are little altered by the presence of ionic

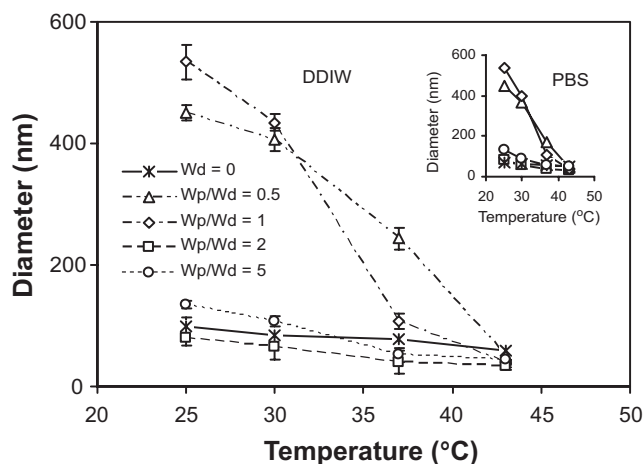


Figure 11 Temperature dependence of diameter of NDQPRI6-sasDNA complexes prepared by adding polymer to DNA in DDIW (main frame) and in 150 mM pH 7.4 PBS (inset) ($n = 3$).

cosolutes and aggregation occurs at a temperature near T_c of TCNGs in DDIW. However, at pH 5.0, tertiary amino groups are highly protonated resulting in highly positively charged TCNGs that resist phase separation at ionic strengths of up to 50 mM. Lopez-Leon and colleagues investigated the effects of salt concentration on cationic P[NIPAM] TCNGs functionalized with 4%mol aminoethyl methacrylate (AEMH).³⁰ As with the DMAEMA functionalized NIPAM TCNGs presented here, the presence of the AEMH as a comonomer with NIPAM necessitated higher electrolyte concentrations to induce chain collapse of the TCNG. Clearly, suppression of the electric double layer will occur in the presence of electrolytes as evidenced by the reduction of particle size

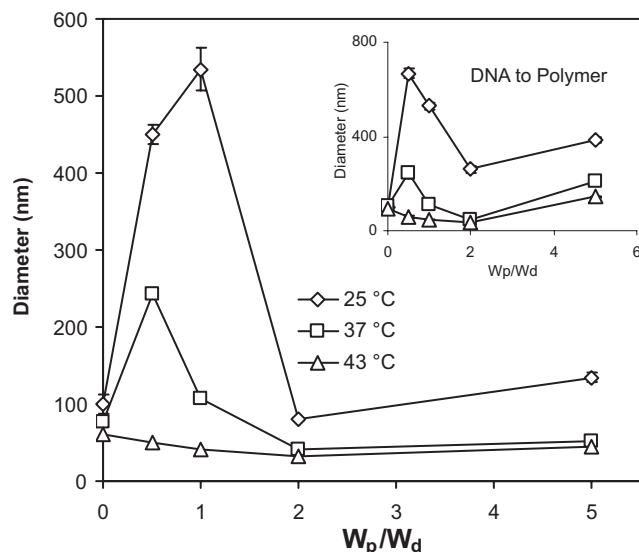


Figure 12 Dependence of the diameter of NDQPRI6-sasDNA on W_p/W_d at different temperatures. Complexes prepared by adding polymer to DNA (main frame) and DNA to polymer (inset).

in presence of electrolytes. Electrolytes also generate a competition for water molecules between ionic species and isopropyl groups on the NIPAM chain. Indeed, “salting-out” of the isopropyl groups in NIPAM has been correlated with the increase in surface tension caused by aqueous salt solutions.³¹ An additional driving force for salt-induced dehydration of P[NIPAM] TCNGs is the increase in osmotic pressure gradient between the polymer phase and bulk solution phase with added electrolyte. However, as pointed out by Lopez-Leon and colleagues, cationic P[NIPAM]-based TCNGs undergo salt-induced dehydration at a rate far in excess of the rate of collapse predicted solely on the basis of osmotic effects ($D_h \propto C_s^n$, where $n = 0.2$ for a purely osmotic-driven mechanism, whereas values of $n = 1$ were reported).

Studies on linear quaternary ammonium ion polymers have shown that they are effective pH-independent condensation agents of DNA, but are beset with high toxicity towards cultured cells.³² The simplest way to reduce the toxicity of quaternary macromolecular condensing agents is to reduce their cation content.³³ In our studies we have used low levels of quaternary cation content of 4%mol. Furthermore, the incorporation of quaternary cations in a nanogel structure may provide a steric barrier against intercalation of quaternary cations into lipid bilayers, thereby reducing induction of cytotoxic mechanisms. McAllister and colleagues investigated the toxicity of 2-hydroxyethylacrylate incorporating the quaternary ammonium cation 2-acryloxyethyltrimethylammonium chloride (AETMAC).³⁴ The authors found that TCNGs prepared with 25 wt % AETMAC had enough surface charge to complex DNA at physiologic ionic strength, but did not induce cell death when incubated with cultured HeLa cells for 40 h. The assessment of toxicity and cellular uptake of P[NIPAM/DMAEMAQ] in various human cell lines is the subject of future work.

A notable finding in this study is the stabilization of P[NIPAM/DMAEMA] and P[NIPAM/DMAEMAQ] TCNGs upon complexation with sasDNA. In the case of P[NIPAM/DMAEMA], complexation resulted in discontinuous phase transition response, whereupon chain relaxation above the volume transition results. Accordingly, the TCNGs recover their original size rather than aggregate as they would in the absence of sasDNA. A plausible explanation for this phenomenon is that of incomplete neutralization of sasDNA charge by tertiary amino groups on the TCNG. In DDIW, slightly more than half of the tertiary amine groups of DMAEMA are positively charged. Furthermore, some fraction of protonated tertiary amines may be sterically inaccessible to phosphate charges on DNA due to shielding effects of NIPAM chains

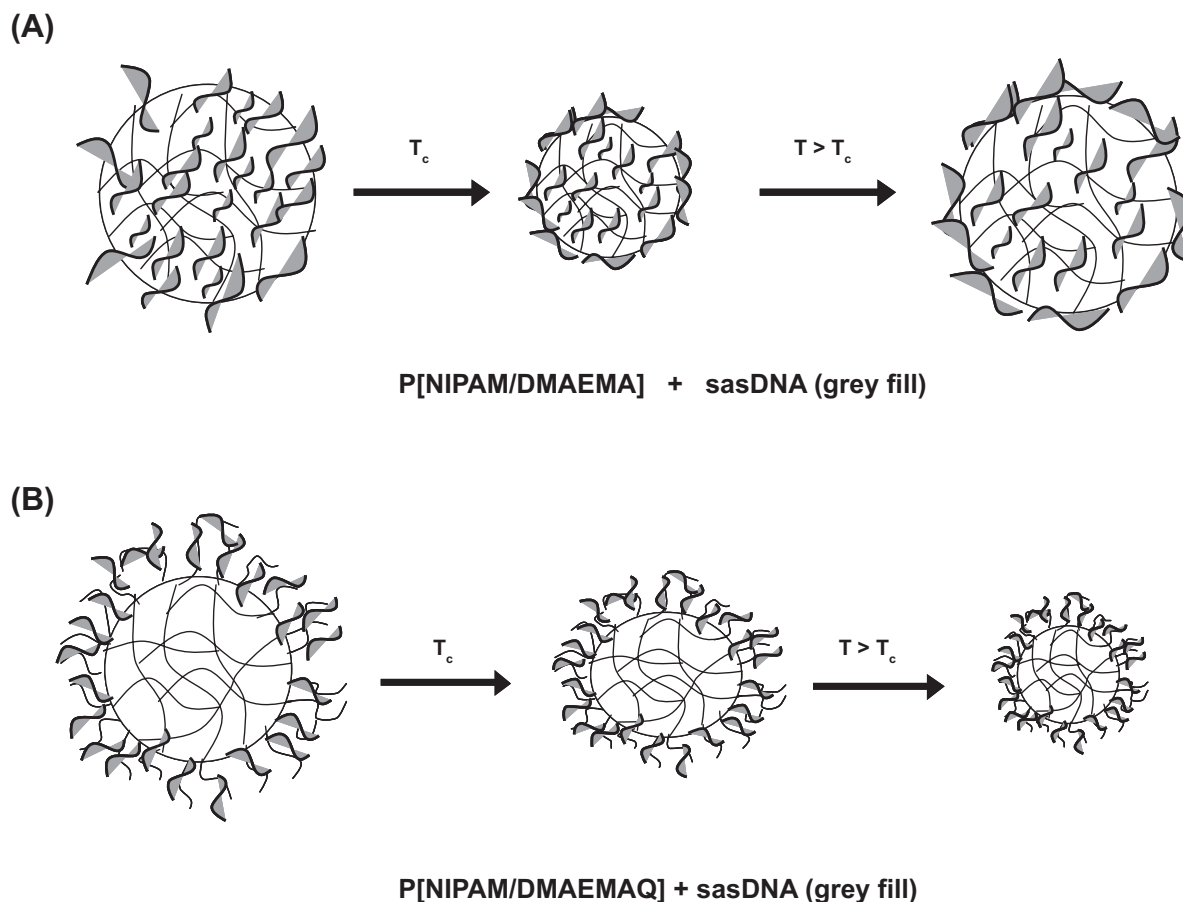


Figure 13 (A,B) Proposed scheme illustrating the effect of temperature on the hydrodynamic size and DNA-binding properties of P[NIPAM/DMAEMA] and P[NIPAM/DMAEMAQ]. **(A)** Redistribution of sasDNA above T_c causing reswelling of polymer chains of P[NIPAM/DMAEMA]. **(B)** Binding of sasDNA to P[NIPAM/DMAEMAQ] results in condensation of DNA and collapse of TCNG above T_c .

and cross-linkages. Presumably, even at very high polymer to DNA ratios such as $W_p/W_d = 10$, full neutralization of the sasDNA does not occur perhaps due to the intercalation of the DNA into the polymer segments within the TCNG. The result is that below the T_c , the polyplex behaves in a manner similar to the uncomplexed TCNG. However, above T_c , some of the sasDNA that is interspersed (but not electrostatically bound) within the TCNG may electrostatically bind to the gel surface upon deswelling during phase transition. As illustrated in Figure 13, the rearrangement of sasDNA at the surface may electrostatically stabilize the TCNG, while hydration and electrostatic effects due to DNA within the TCNG core may contribute to resistance of the polymer core to collapse and restore sufficient hydration to re-swell the gels to their original volume.

The phase transition properties of P[NIPAM/DMAEMAQ]/sasDNA polyplexes differed markedly from nonquaternized P[NIPAM/DMAEMA]/sasDNA polyplexes. Although sasDNA also facilitated stabilization of the TCNGs above T_c , it did so in such a way as to render the otherwise

low-response TCNGs into highly temperature-responsive and aggregation-resistant entities similar to homopolymer P[NIPAM] TCNGs. Titration of the sasDNA with polymer revealed behavior consistent with condensation of sasDNA in a manner similar to that observed with linear P[NIPAM/DMAEMA] copolymers.²⁵ By inference, we propose that in the case of P[NIPAM/DMAEMAQ] TCNGs, there is free and sufficient access of sasDNA to the quaternary ammonium charge and that this enables DNA to be condensed on the periphery of the particle, rendering it positively charged and facilitating a transition similar to P[NIPAM] throughout the temperature range of study. The structural picture that emerges is one that places the quaternary charge on the periphery of the TCNGs extending out into solution with minimal steric hindrance (Figure 13, bottom). This so called “hairy-tether” model of the P[NIPAM/DMAEMAQ] TCNG certainly warrants further study, including electron microscopic imaging and analysis of synthetic factors that would account for the observed polymer and DNA complex dynamics of these TCNGs.

Conclusions

In this study, temperature-responsive P[NIPAM/DMAEMA] and P[NIPAM/DMAEMAQ] TCNGs were synthesized and their temperature-driven deswelling properties characterized under various conditions of pH and ionic strength. The P[NIPAM/DMAEMA] TCNGs exhibited volume transition behavior between 33 °C–36 °C, but aggregated at higher temperatures. The nature of the phase transition and the stability of P[NIPAM/DMAEMA] was sensitive to the ionic strength at low pH where most of tertiary amine groups are protonated. P[NIPAM/DMAEMAQ] TCNGs were much smaller in size than P[NIPAM/DMAEMA] and resisted aggregation up to 50 °C. Complexation of P[NIPAM/DMAEMA] and P[NIPAM/DMAEMAQ] with sasDNA resulted in stabilization of the TCNGs complexes through the volume phase transition.

Future work will investigate the toxicity of TCNGs and DNA-TCNG complexes and explore the transfection potential of nonviral TCNG constructs, including transfection efficiency and the ability to control gene expression by way of temperature and pH modulation.

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