Evaluation of copolymers of N-isopropylacrylamide and 2-dimethyl(aminoethyl)methacrylate in non-viral and adenoviral vectors for gene delivery to nasopharyngeal carcinoma

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Abstract: Copolymers of 2-dimethyl(aminoethyl) methacrylate (PDMAEM) with N-isopropylacrylamide (NIPAM) were evaluated for their potential to enhance transgene expression of plasmid DNA (pDNA) and gene delivery by adenovirus vectors. The polymers of varying compositions and molecular weights (MW) were synthesized by free-radical polymerization. Polyelectrolyte complexes (PECs) were prepared with different charge (N:P) ratios of PNIPAM/DMAEM to pDNA. Polymer-modified viral vectors based on non-replicating adenovirus serotype 5 (Ad5), (ΔE1/oriP/*luc*) or (ΔE1/CMV/*luc*) transcriptor/promoter/reporter were constructed by electrostatically coupling PNIPAM/DMAEM (Type I) or PECs (oriP/*luc*, 6.6 kb) (Type II) to the viral capsid. The N:P value at complete condensation was lower for PECs with higher DMAEM content and MW. pDNA binding was enhanced by high MW PNIPAM/DMAEM. Circular dichroism spectroscopy revealed changes to the secondary structure of pDNA and adenovirus capsid proteins in the presence of PNIPAM/DMAEM. The toxicity of PNIPAM/DMAEM to CNE-1 nasopharyngeal cancer (NPC) cells diminished with decreasing DMAEM content and increasing MW. The transfection efficiency of C666-1 NPC cells by PECs increased with DMAEM content and MW. The transduction efficiency of CNE-1 NPC cells by Type I Ad5 vectors improved with DMAEM content, but was independent of MW. The transduction efficiency of Type II Ad5 in C666-1 cells approximated the sum of expression levels of the PECs and Ad5 vectors individually. PDMAEM and PNIPAM/DMAEM demonstrate both transfection and transduction enhancement activity of modified vectors in nasopharyngeal cancer cells in culture.

Keywords: polymers of 2-dimethyl(aminoethyl) methacrylate and N-isopropylacrylamide, polymer-DNA complex, polymer-modified adenovirus, cytotoxicity, transduction efficiency

Introduction

In cancer gene therapy, a major factor impeding the therapeutic efficacy of both non-viral and viral vectors is the inability of the vector to achieve directed expression by localizing at the site of action and effecting gene transfer selectively in the neoplastic cells (Yu and Schaffer 2005; Glasgow et al 2006; Pirollo et al 2000, 2006). Non-viral vectors such as polyelectrolyte complexes (PECs) prepared by condensing plasmid DNA with polycations such as lipids (Ewert et al 2005), poly-L-lysine (PLL) (Ramsay and Gumbleton 2002; Conswell and Huang 2005) and polyethyleneimine (PEI) (Boussif et al 1995; Woodle et al 2001) are hampered by the requirement of a suitable ligand or antibody directed towards a specific cancer cell receptor target. Viral vectors such as adenovirus serotype 2 and serotype 5 (Ad2 and Ad5) have been extensively investigated for cancer gene therapy due to their engineering potential, but are hampered by reticuloendothelial system (RES) clearance, immunogenicity, and

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difficulty controlling the site of viral uptake and expression due to the paucity of expression of the coxsackie adenovirus receptor (CAR) in a variety of malignant cell types (Volpers and Kochanek 2004; Noureddini and Curiel 2005; Shinozaki et al 2006). Attempts to divert adenovirus away from the liver and non-diseased tissues by ablation of the CAR and integrin receptor binding functions of the fiber knob and penton bases, restricts the targeting potential to specific cells and is fraught with technical challenges (Wickham et al 1996; Bilbao et al 1998; Alemany et al 2000; Wickam 2000). Other approaches have sought to exploit adenovirus promiscuity, opting instead to target at the level of expression by restricting viral genome transcription to cells possessing appropriate transcriptional activating markers (Douglas and Curiel 1997; Kirn 2001).

A potentially unifying approach for targeting non-viral and viral vectors involves coupling of polymers that undergo changes in biophysical properties selectively in tumor tissues. By sensing (sensor function) and responding (effector function) to environmental stimuli such as temperature, pH or ionic strength, selective locoregional accumulation of responsive polymer coupled vectors may circumvent the need for specific cellular targets following systemic administration (Kurisawa et al 2000; Takeda et al 2004; Twaites et al 2004, 2005). Surface modification strategies of the adenovirus capsid have been extensively used to target poly(ethylene glycol) (PEG)-coupled so called "stealth" adenovirus vectors bearing target cell specific ligands (Torchilin 1996; O'Riordan et al 1999; Croyle et al 2001). These vectors have shown some improvements in reducing liver clearance and masking capsid protein immunogenicity, while increasing circulation time and improving the potential for perivascular escape (Romanczuk et al 1995; Chillon et al 1998; Woodle et al 2001; Croyle et al 2002; Eto et al 2004; Mok et al 2005). Alternatively, polycation coating of the adenovirus capsid to generate polycation-adenovirus complexes bearing PEI, PLL or various cationic lipid surfaces have demonstrated the potential to enhance transgene expression in CAR deficient cell lines and reduce immunogenic response associated with high vector doses (Fasbender et al 1997; Kaplan et al 1998; Diebold et al 1999; Nicola et al 2000). The principal mechanism by which polycations promote adenovirus transduction is thought to involve electrostatic interaction of cationic polymer segments with the cell membrane. Once in close proximity, cell membrane destabilization by the cationic polymer segments may exacerbate the action of viral proteins used for cell entry, effectively promoting cellular uptake of the vector (Wickam et al 1992).

In this work, we characterize potentially targetable non-viral PEC and polycation-adenovirus complex vectors that incorporate temperature- and pH-responsive polymer components, N-isopropylacrylamide (NIPAM) and 2-dimethyl(aminoethyl) methacrylate (DMAEM) respectively. PolyNIPAM (PNIPAM) is a thermosensitive polymer with a lower critical solution temperature (LCST) near 32 ºC (Heskins and Guillet 1968; Schild 1992). The viability of this polymer in systemic delivery systems was suggested by our previous work that showed low levels of plasma protein adsorption and stimulation of phagocytic activity of human neutrophils by PNIPAM/methacrylic acid (MAA) nanoparticles due to their hydrophilic nature (Moselhy et al 2000). PDMAEM is a cationic polymer at pH 7.4 that has been used extensively in non-viral vectors for gene delivery (Hinrichs et al 1999; Rungsardthong et al 2001; Wakebayashi et al 2004; Funhoff et al 2005). PDMAEM based PECs have been found to transfect a wide variety of cell types due to the ability of the polymer to condense pDNA, buffer lysosomal acidity, and transiently disrupt lipid bilayer membranes (Cheng et al 1999; Wetering et al 2000; Takeda et al 2004). PNIPAM/DMAEM PECs have been shown by Hinrichs et al. to effectively transfect the OVCAR-3 ovarian cancer cell line (Hinrichs et al 1999). Kurisawa et al. have advanced the functional role of NIPAM/DMAEM by utilizing terpolymers with butylmethacrylate (BMA) to effect thermally regulated expression of therapeutic genes (Kurisawa et al 2000). These studies illustrate the versatility of temperature responsive polymers for directed gene expression.

We envisage two classes of adenovirus complexes based on the adenovirus serotype 5 (Ad5) and NIPAM/DMAEM copolymers, Types I and II. These classes are distinguished on the basis of the vector component responsible for gene expression. Type I Ad5 vectors utilize solely viral regulated expression and they are prepared by complexing the polycations with Ad5 directly. Type II complexes acquire plasmid controlled gene expression *via* the complexation of the PECs (bearing transcriptionally active pDNA) electrostatically to the Ad5 capsid. Type II complexes by virtue of their transcriptionally inactive viral genome, may be less likely to induce cell-mediated immune response than Type I vectors in which the viral genome remains intact (Baker et al 1997a, b). Both types of vectors have the potential to reduce immune response due to the shielding of the highly immunogenic fiber knob and penton bases by the polycations, however only the Type I vectors retain the functional genetic elements that may be required for efficient viral entry and gene expression in the cell.

The cancer gene therapy model investigated here is Ad5 gene delivery to nasopharyngeal carcinoma (NPC) cells in culture. NPC is an epithelial malignancy of the head and neck region for which survival outcome after treatment with radial radiation therapy (XRT) needs to be improved (Lee et al 2001). *In vitro* treatment of NPC cells by Ad5 vector expressing wild type p53 has been shown to promote their apoptosis when subjected to hyperthermia, either alone or in combination with XRT (Qi et al 2001). The NPC cells are highly transfectable by Ad5 suggesting that CAR expression is not a limiting factor (Hwang et al 1998). In spite of this, *in vivo* studies in NPC xenograft models have demonstrated poor Ad5 vector performance, suggesting that rapid RES clearance might be an important mitigating factor (Liu 2002). Since modification of nanoparticles by hydrophilic polymer could reduce RES recognition and uptake of the particles (Moselhy et al 2000), we investigated in this work hydrophilic polymer-modified Ad5 vectors to exploit the potential of such vectors to facilitate CAR-independent cell entry and a strategy for targeting leading to the development of improved gene therapies for NPC.

Materials and methods

Materials

N-isopropylacrylamide (NIPAM, 97%, Aldrich) was recrystallized from 70:30 hexane:toluene mixtures and dried in vacuo at room temperature. 2-(dimethylamino) ethyl methacrylate (DMAEM, Aldrich) was distilled under reduced pressure (45 ºC, 5 mmHg). 2,2′-azobisisobuyronitrile (AIBN, Aldrich) was recrystallized from methanol and dried in vacuo. 1,4-dioxane (ACS grade, Fisher), 2-mercaptoethanol (Aldrich) and ethidium bromide (Sigma) were used as received. Distilled deionized (DDI) water was prepared with a Millipore system and used for dilution purposes throughout.

Cells lines

The human nasopharyngeal cancer epithelial cell lines, CNE-1 and C666-1, used in this study have been described elsewhere (Cheung et al 1999; Li et al 2002). The CNE-1 cells were cultured in alpha minimum essential medium (α-MEM, Sigma) and C666-1 in RPMI-1640 (Sigma), respectively at 37 ºC in a humidified incubator containing 5% CO_2. The media were supplemented with L-glutamine (200 mM), penicillin (10,000 units), streptomycin (10 mg in aqueous sodium chloride 0.9%, Sigma) and 10% Fetal Calf Serum (FCS, Wisent Inc.). The cells were passaged by trypsinizing nearly confluent cells in T-75 flasks at 1:6 dilution for CNE-1 cells, and 1:3 dilution for C666-1 cells.

Synthesis and characterization of PNIPAM/DMAEM copolymers

To a Schlenk flask, DMAEM and NIPAM were added in the desired feed proportions to 100 mL of 1,4-dioxane (10%w/v monomers) and heated to 60 °C. For synthesis of polymers of low molecular weight, 2-mercaptoethanol was added as a chain transfer agent (CTA) at a 10:1 monomer/CTA molar ratio. The monomer mixture was allowed to thermally equilibrate and degassed under nitrogen for 10 minutes, followed by rapid injection of 2 mL of free-radical initiator, AIBN, degassed at 60 °C, at a 100:1 monomer/initiator molar ratio. After polymerization for 12 h under nitrogen, the reaction mixture was cooled and the resultant polymers were collected by precipitation into hexane and dried in vacuo. The polymers were dissolved in DDI water and exhaustively dialyzed for 72 h using a SpectraPor 3 membrane with molecular weight cut-off 3500.

The copolymer composition was determined by ^{1}H NMR spectroscopy using Gemini 300 (Varian, Palo Alto, CA) in D_2O at 300 MHz. The relative abundance of NIPAM and DMAEM in the copolymer samples was calculated from the ratio of the integrated area under the peaks corresponding to proton resonances unique to the monomers, that is, δ = 3.9 ppm for NIPAM, 1H, s, $CONHCH(CH_3)_2$, and δ = 4.4 ppm for DMAEM, 2H, t, $COOCH_2CH_2N(CH_3)_2$.

The intrinsic viscosity of polymers was determined from polymer solutions of concentrations ranging from 1 mg/mL to 5 mg/mL in 270 mmol/L KCl using a Cannon Ubbelohde #75 dilution type viscometer. The monovalent electrolyte was added to minimize electrostatic effects on the viscosity of polymer solutions. The average molecular weight of the polymers was determined using the Mark-Houwink (M-H) equation, $\eta = KM_a$, where K and a values for the copolymers were computed from previously published data for NIPAM, $K_{NIPAM} = 5.75 \times 10^{-5}$ and $a_{NIPAM} = 0.78$, and for DMAEM, $K = 9.13 \times 10^{-4}$, respectively (Egoyan 1985; Ganachaud et al 2000), under the assumption of linear additivity of the parameters with respect to the copolymer composition determined by ^{1}H NMR.

The pK_a of the copolymers was determined by potentiometric and conductometric coupled titration of 10 mg/mL polymer solutions with 0.05 N NaOH at 25 °C using a Radiometer Copenhagen ABU-93 triburette autotitrator mated to a Radiometer Copenhagen Model CDM92 conductivity meter. The equivalent points in the titrations

of polymer solutions we found from the inflection points of the titration curves. The pK_a values were derived from the potentiometric data and represented the pH at one half the volume of base required to fully titrate tertiary amino groups on DMAEM. There was good agreement between pK_a values derived potentiometrically and those obtained conductomerically.

Preparation and purification of plasmid PDC312/oriP.luc

Double stranded plasmid DNA (pDNA) PDC312/oriP.*luc* (6 kb) expressing firefly luciferase (*luc*) under control of the oriP promoter was prepared by ligating the SalI/BamHI fragment, containing the oriP.*luc* cassette, isolated from pΔ E1sp1A/oriP.*luc* as described by Li et al (2002) with PDC312 cut with the same enzymes. The plasmid was transformed into *Escherichia coli* DH5-a competent cells and purified using a QIAGEN plasmid Mega kit. The DNA concentration and purity of the pDNA was assessed by measurement of the UV absorption at 260 and 280 nm.

Preparation and purification of Adenovirus

Adenovirus vectors expressing *luc* under the control of a cytomegalovirus (CMV) promoter were amplified in 293 cells using established methods (Graham and Eb 1973), and purified from cell lysates by banding twice on CsCl gradients. Purified virus was then desalted overnight in 1:1000 parts by volume virus solution to Tris-HCl pH 8.0 buffer. Viral concentrations and purity were determined by the absorbance at 260 and 280 nm. The concentration of viral particles was calculated from the optical density at 260 nm (OD_{260}), using the formula 1 $OD_{260} = 1.1 \times 10^{12}$ particles/ml as derived by Maizel et al (1968).

Preparation and characterization of pDNA polyelectrolyte complexes (PECs) and polymer-modified adenovirus

PECs were prepared by the addition of aliquots of polymer stock solution (200 μg/mL in PBS) to pDNA (2 μg) to give the desired molar ratio of nitrogen atoms in the polymers to phosphorous atoms in the DNA (N:P). The N:P ratio was calculated from the equation:

$$N{:}P = \frac{f_{DMAEM} w_{pol} / 157}{w_{DNA} / 325}$$

where f_{DMAEM} is the weight fraction of DMAEM in the copolymer, w_{pol} is the weight of the copolymer, w_{DNA} is the weight of pDNA, and the constants 157 and 325 represent the weight of DMAEM and DNA per nitrogen and phosphorous atom, respectively.

The total volume of solution was adjusted to 400 μl with PBS and vortexed for several seconds. The quantity of reagents was scaled up when necessary. Complex formation was carried out for 30 min at room temperature with gentle mixing using a hematological mixer. PECs used in transfection experiments and for complexation with Ad5 were prepared in the same manner except that either α-MEM or RPMI-1640 was used in place of PBS.

Type I complexes between cationic polymers and Ad5 particles were prepared by mixing stock polymer solutions in either α-MEM (CNE-1 cell infection) or RPMI-1640 (C666-1 cell infection, with Ad5 dilutions at the desired ratio of polymer to virus (typically 100 to 1000 polymer molecules/Ad5 particle) and MOI (see Figure legends). Type II complexes were prepared by mixing PECs as described above with Ad5 dilutions.

Dynamic light scattering and zeta-potential analysis of PECs

The volume–average particle size and size distribution of freshly prepared PECs of different N:P ratios in deionized water at 25 °C was measured using a NICOMP 380ZLS dual zeta/dynamic light scattering instrument equipped with a 10 mW, 632.5 nm laser in particle sizing mode. For zeta-potential measurements, the NICOMP 380ZLS was switched to zeta-mode. All particle size and zeta-measurements were run in triplicate using 5 iterative cycles per sample. Prior to measurements, the NICOMP 380ZLS was calibrated using polystyrene latex particles (Polysicences Inc.) of known hydrodynamic size and surface charge.

Circular dichroism spectroscopy

Complexation of polymers with Ad5 and pDNA was examined by circular dichroism (CD) spectroscopy. PECs and polymer-modified Ad5 were prepared as described earlier. CD spectra of pDNA, Ad5 and PEC and Ad5 complexes were recorded at 20 ºC in a 1 mm path length cuvette using an AVIV model 62A DS spectropolarimeter (Lakewood, NJ). The integration time was 1s and the slit width 2 nm.

Ethidium bromide displacement assay

The ethidium bromide assay was carried out to probe the association of polymers with pDNA. A 200 μL aliquot of

50 μg/mL pDNA in PBS solution was diluted to 2 mL volume in a 1 cm cuvette. A 1 μL aliquot of a 400 μg/mL EtBr solution was introduced to the pDNA and mixed by gentle inversion. The fluorescence emission was recorded (λ_{ex} = 512 nm, λ_{em} = 600 nm, slit width ex/em = 5 nm/5 nm) on a Spex FluoroMax-3 fluorometer. A 5 mg/mL polymer solution was titrated into the pDNA/EtBr solution and the fluorescence emission monitored. The relative fluorescence was calculated from the ratio of the observed fluorescence in presence of polymer relative to that in its absence, correcting for EtBr fluorescence according to the relation:

$$\% F_{rel} = (F_{obs} - F_{EtBr}/F_{DNA\text{-}EtBr} - F_{EtBr}) \times 100\%$$

where F is the fluorescence emission intensity

Gel retardation assay

The gel retardation assay was used to evaluate that binding of the polycation to pDNA was the result of condensation. PECs incorporating 1 μg of pDNA were formed by mixing pDNA stock solution with aliquots of polymer stock solutions (100 μg/ml in PBS). The total volume was adjusted to 200 μl with PBS and complexation was carried out for 1 h at 25 °C. The PEC solution (25 μl) was run on a 0.7 wt.% agarose gel (100 V) in 1 × TAE (40 mM Tris-acetate and 1 mM EDTA, pH 8.3) buffer. DNA bands were visualized by ethidium bromide staining.

In vitro transfection of CNE-1 and C666-1 NPC cells by PECs and polymer-modified Ad5

CNE-1 and C666-1 cells were seeded in a 24-well culture plate at 1×10^5 and 2×10^5 cells/well, respectively. For infection tests, the growth media were removed and the cells rinsed with PBS. A 200 μL aliquot of the Ad5 or polymer-modified Ad5 vector in 2% heat-inactivated FBS media was introduced to the cells, typically at a multiplicity of infection [MOI] = 50 and incubated for 1 h at 37 °C (5% CO_2). The cells were washed 3 times with 200 μL of PBS, followed by addition of a fresh medium containing 10% FBS. The cells were incubated for 24 h to allow for luciferase expression. The medium was then removed and the expressed luciferase was isolated according to the protocol supplied with the Dual-Light® Reporter Gene Assay System (Tropix, Applied Biosystems, Foster City, CA). All samples were run in triplicate using a ThermoLabsystems Luminoskan Ascent luminometer (Thermo Electron Corp., Waltham, MA, USA) for chemiluminescent detection.

Assay of cellular toxicity of NIPAM/DMAEM polymers

CNE-1 cells were seeded in 96-well culture plates at 2×10^4 cells/well in α-MEM. The medium was removed from plated cells and replaced with various concentrations of polymer (0.25–10 mg/ml) in serum-free α-MEM for 1 h at 37 °C under 5% CO_2. The polymer solutions were then removed and replaced with α-MEM plus 10% FBS. The cells were allowed to proliferate for various time points (ie, 8–48 h) prior to the addition of 100 μL of MTT reagent (α-MEM, 2% FBS). After 3 h incubation in presence of MTT reagent, 175 μL of 0.1% HCl in isopropyl alcohol was added to each well and pipette tip aspirated. The absorbance of the solution was read at 570 nm using a BioRad 3550 microplate reader (BioRad, Hercules, CA). The cytotoxicity was calculated as the percentage of viable cells.

The experiment was repeated in triplicate using three different sets of cultured cells. Within each experiment, the values of six independent measurements (6 wells) at each concentration were obtained. The results are expressed as the mean ± S.D. from data obtained from the three separate measurements.

Transmission electron microscopy

A 50 μL aliquot containing 9×10^9 Ad5 particles in α-MEM was combined with a 50 μL aliquot of polymer solution at a polymer/Ad5 ratio of 100 polymer molecules/particle. The solution was gently aspirated and incubated at 37 °C for 15 min. 10 μL aliquots of polymer-modified Ad5 fixed with OsO_4 and dispersed onto a Formvar-coated TEM grid, stained with lead citrate, and counterstained with 1% aqueous uranyl acetate for 30s. The solution was then removed and the grids dried in air. TEM images were captured using a Hitachi H-7000 (Tokyo, Japan) transmission electron microscope.

Results and discussion

Properties of NIPAM/DMAEM polymers

The chemical composition determined by feed and by ^{1}H NMR, molecular weight and pK_a of the polymers are summarized in Table 1. It is seen that the NIPAM content determined by ^{1}H NMR was slightly lower than the feed for most of the samples, probably because NIPAM is less reactive in copolymerization than DMAEM (Brazel and Peppas 1995; Lee et al 2003). Additionally the copolymers of higher

Table 1 Properties of NIPAM/DMAEM copolymers

Polymer[1,2]	% mol NIPAM (^{1}H NMR)	% mol NIPAM (feed)	M_v (Da x 10^{-4})	pK_a
ND0/100LMW	0	0	1.73	7.5
ND0/100HMW	0	0	289	7.0
ND15/85LMW	10.9	15.0	3.26	7.5
ND15/85HMW	13.1	15.0	197	7.0
ND30/70LMW	22.8	30.0	2.82	7.5
ND30/70HMW	25.8	30.0	193	7.0
ND50/50LMW	52.0	50.0	1.89	7.6
ND50/50HMW	56.6	50.0	87.4	7.1

[1]Copolymer nomenclature: N – NIPAM, D – DMAEM, %mol NIPAM/%mol DMAEM in polymerization feed, LMW – low molecular weight, HMW – high molecular weight.
[2]LMW samples incorporate 10% mol 2-mercaptoethanol relative to total monomer content in polymerization feed.

molecular weight, ie, ND15/85HMW, ND30/70HMW, and ND50/50HMW, consist of more NIPAM than the polymers of low molecular weight, ie, ND15/85LMW, ND30/70LMW, and ND50/50LMW. Since the low MW samples were synthesized by addition of chain transfer agent, the higher NIPAM content in these samples may imply higher reactivity of the CTA with DMAEM monomer. The addition of a chain transfer agent lowers the molecular weight of the polymers dramatically from $87 \times 10^4 - 289 \times 10^4$ dalton to $1.73 \times 10^4 - 2.82 \times 10^4$ dalton. The degree of MW reduction by use of CTA seems to depend on the NIPAM content in the feed. When the molar ratio of NIPAM to DMAEM is 0:100, the MW in the absence of CTA is 167-fold of that with the CTA. As the NIPAM/DMAEM ratio is increased to 50:50, the difference in the MW with or without the CTA is only 46-fold. This result may also suggest the effectiveness of CTA in the termination of DMAEM radicals.

The average pK_a of the tertiary amine groups of DMAEM in the polymers was determined by titration with base. The titration curves of polymers (data not shown) were characterized by broad neutralization profiles suggesting a distribution of pK_as within the polymer. Seemingly the pK_a values were not dependent on the NIPAM content of the copolymers. The pK_as of low molecular weight NIPAM/DMAEM polymers were slightly higher than the high MW analogs. It is possible that the amine groups in low MW samples are buried within the polymer chains and are, therefore, less accessible to the base during titration.

Properties of polymer-pDNA complexes

Figure 1 shows the intensity-average hydrodynamic diameter of PECs at different charge neutralization ratios for low (A) and high (B) MW polymers, respectively. The condensation process is characterized by a sharp rise in the diameter of the PECs followed by a dramatic decrease in diameter that reaches a minimum as the fully condensed state is approached. The diameter of condensed PECs ranged between 115 and 187 nm for all polymers.

The ζ-potential of PECs was measured and plotted in Figure 2 as a function of N:P ratio for low MW (A) and high MW polymers (B). It is shown that the initially negative charges of PECs increase rapidly with increasing N:P ratio and above the point of zero charge (PZC) the PECs become positively charged. The N:P ratio at the PZC, listed in Table 2, increases with increasing NIPAM content and decreasing molecular weight, suggesting that large macromolecular chains and NIPAM units may screen the exposure of the cationic charges to DNA, and thus higher N:P ratios are required for neutralizing the negative charges. The final ζ-potential of the PECs, ζ_{final}, summarized in Table 2 which were found from the plateau of the curves in Figure 2 and, increases with decreasing NIPAM content and increasing MW of the polymers. The composition dependence of ζ_{final} seems to be more marked in high MW samples (Figure 2B) than in low MW samples (Figure 2A). As the positive charges stem from the cationic polymers, the higher the DMAEM content, the higher the positive charges.

Notably, ζ_{max} decreased with increasing NIPAM content and decreasing molecular weight, suggesting that the number of incorporated polymer molecules in the PEC structure does not vary appreciably. These findings are consistent with the DLS data showing that higher molecular weight, DMAEM-enriched polymers are more effective as condensing agent than their lower molecular weight copolymer analogs incorporating NIPAM.

The N:P ratio required for condensation (N:P final, Table 2) increased with decreasing DMAEM content of the polymers This suggests that the primary mechanism for condensation is the binding of negative charge on the pDNA by positively charged DMAEM. Hydrophilic polymers such as PEG are known to imbibe water molecules and promote pDNA condensation by an osmotic mechanism (Kombrabail et al 2005). However, in the NIPAM/DMAEM system, no such effect was observed.

To further probe the nature of NIPAM/DMAEM binding of pDNA, EtBr intercalation and agarose gel electrophoresis assays were performed. These methods complement the light scattering and electrophoresis techniques by providing a measure of the strength of polycation binding to pDNA and may be important indicators of the likelihood of endosomal

escape and, hence, transfection efficiency. In the absence of NIPAM/DMAEM, EtBr intercalates into pDNA yielding a fluorescent complex that emits at ~600 nm. Using this value as the reference, the relative fluorescence intensity of the PECs at various N:P ratios are plotted in Figures 3A and 3B for low and high MW samples respectively. The N:P values required to reduce the fluorescence intensity by 50%, $N:P_{50}$, were found from the plot and are listed in Table 2. There is a sharp decrease in fluorescence intensity as the pDNA/EtBr complex is titrated. As shown in Table 2, $N:P_{50}$ values were markedly lower than the N:P values for neutralizing the negative charges as determined by ζ-potential analysis. PDMAEM readily displaced EtBr with nearly the same efficiency on a per charge basis as PEI. NIPAM/DMAEM copolymers also

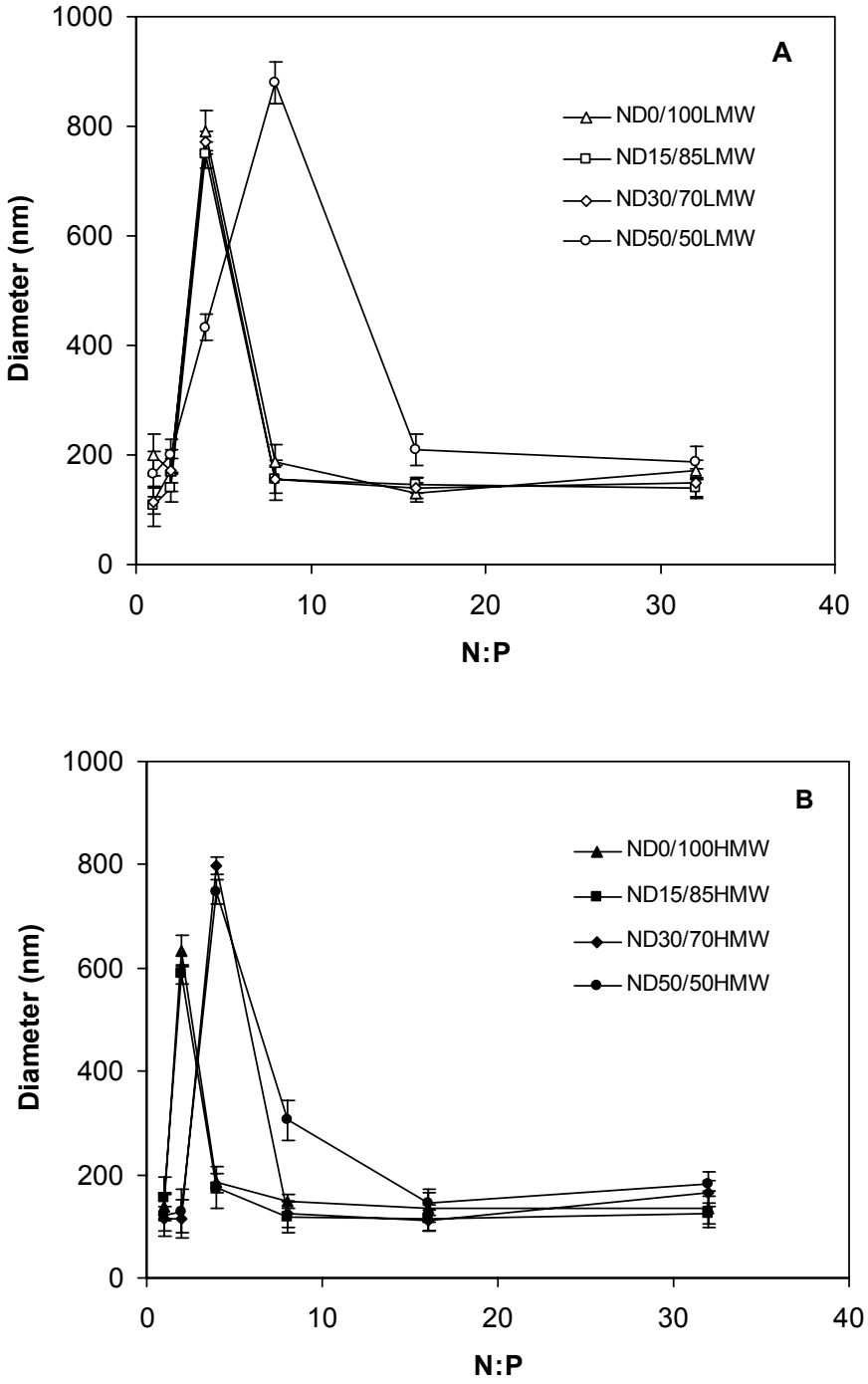

Figure 1 Intensity-averaged hydrodynamic diameter of low molecular weight (**A**), and high molecular weight (**B**) NIPAM/DMAEM PECs at different degrees of charge neutralization of pDNA (n = 3).

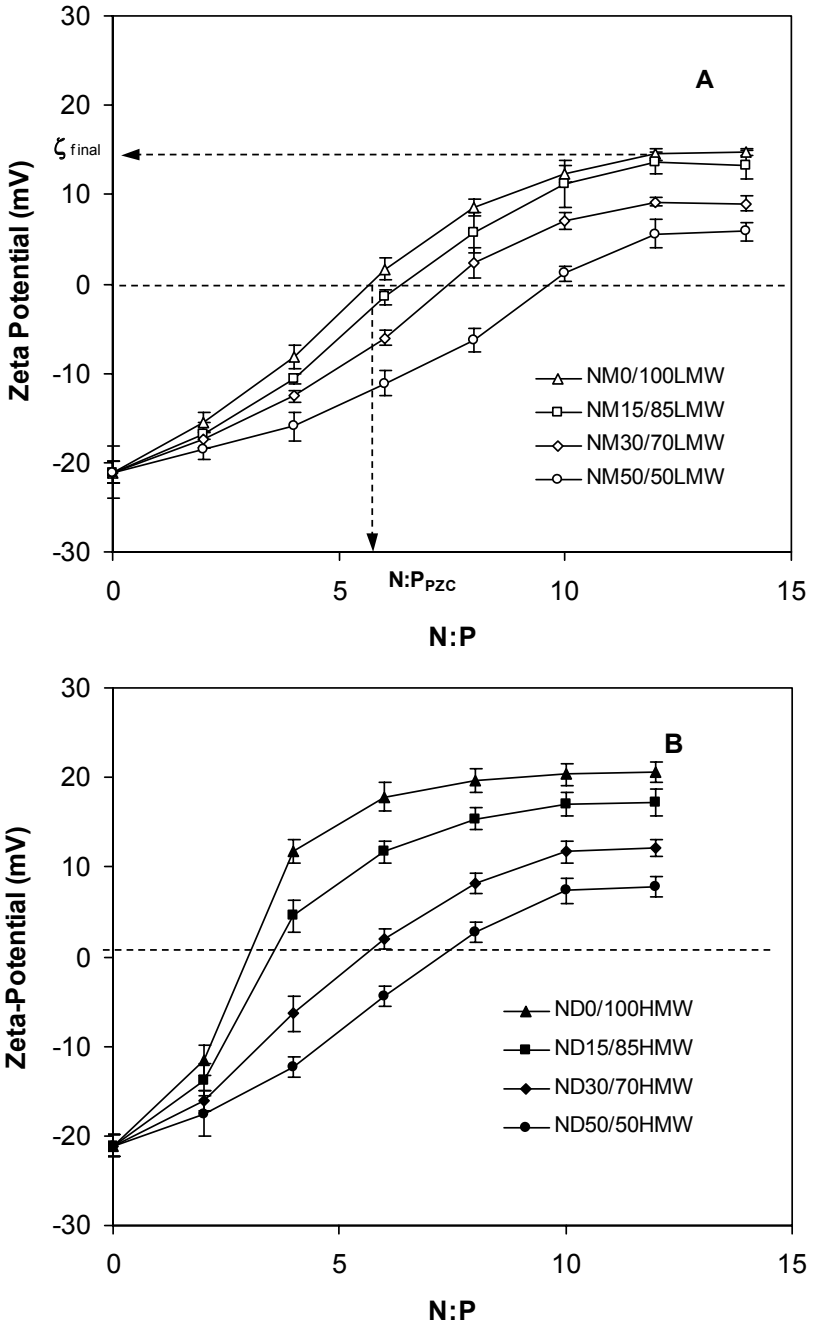

Figure 2 Zeta potential of low molecular weight (**A**) and high molecular weight (**B**) NIPAM/DMAEM PECs at different degrees of charge neutralization of pDNA (n = 3).

readily displaced EtBr in a composition dependent manner. At low levels of NIPAM, displacement occurred nearly as efficiently as with the DMAEM homopolymer. A pronounced decrease in the ability of the polymers to displace EtBr was observed in samples exceeding 50% mol NIPAM content. Notably, the effect of the polymer molecular weight on binding was not as pronounced as would have been expected from the light scattering results. This suggests that low MW NIPAM/DMAEM polymers are able to bind pDNA nearly as efficiently as the high MW analogs, but are not as effective at promoting the structural rearrangement of pDNA that leads to its compaction.

The migration through agarose gels of PEC-complexed DNA prepared from PEI and PLL were compared with the high molecular weight PDMAEM polymer (ND0/100HMW) (Figure 4). In the absence of polycation, two bands attributable to circular (upper band) and supercoiled (lower band) pDNA were observed (lane 2). When pDNA

Table 2 Properties of NIPAM/DMAEM PECs.

Polymer	DLS $D_{I,min}$ (nm)	Zeta-Potential ζ_{final} (mV)	EtBr Intercalation N:P_{PZC}	N:P_{50}
PEI	115 ± 26	25.3 ± 1.0	2.5	0.5
ND0/100LMW	131 ± 30	14.7 ± 0.8	3.9	0.6
ND0/100HMW	133 ± 36	20.6 ± 0.8	3.0	0.6
ND15/85LMW	140 ± 30	13.6 ± 1.2	5.6	0.8
ND15/85HMW	125 ± 17	16.9 ± 1.5	3.5	0.9
ND30/70LMW	140 ± 20	9.2 ± 0.5	7.4	1.7
ND30/70HMW	123 ± 18	12.2 ± 0.8	5.5	1.6
ND50/50LMW	187 ± 30	5.9 ± 1.1	9.6	4.0
ND50/50HMW	146 ± 23	8.8 ± 0.9	7.3	4.2

The diameter, $D_{I,min}$, and the zeta-potential are presented as mean ±SD (n = 3).

was complexed with the polycations at N:P ratios above 2:1, complete retardation of pDNA migration was observed for all of the polymers tested (lanes 3–12). This indicates that PDMAEM binds pDNA as effectively as other more highly charged polycations, when normalized for charge content. Figure 5 shows the effects of NIPAM/DMAEM copolymer composition and N:P ratio on the binding of pDNA. There was clear migration of pDNA in all NIPAM/DMAEM samples complexed at N:P ratios of 0.2:1 and 1:1 (lanes 4–12). However, at N:P of 2:1, the migration of pDNA was retarded in all samples up to 30 mol% NIPAM (lanes 6, 9 and 12). It is interesting to note that only the ND15/85LMW PEC (lane 9) was able to fully retard DNA migration as evidenced by the lack of band in the gel center.

Investigation of pNIPAM/DMAEM and PEC–DNA complexation with adenovirus

CD spectroscopy can be used as a tool to probe changes in secondary and higher order structural features of nucleic acids and proteins in solution. Here, this technique was used to investigate the interaction of NIPAM/DMAEM polymers pDNA and in Type I and Type II modified adenovirus vectors. Figure 6 shows that the CD spectrum of PDMAEM PECs is markedly different from that of the pDNA. The spectrum of ND0/100HMW at N:P = 5:1 exhibited diminished intensity and characteristic red shifts of the 210, 220, 245 and 275 nm bands of native pDNA. PECs formed from the copolymer containing 50% mol NIPAM (ND50/50HMW) at the same N:P ratio (5:1) (Figure 7), exhibited less dramatic changes in spectral bands suggesting reduced levels of interaction between NIPAM-containing polymers with DNA. Increasing the N:P ratio to 10:1 induces a greater change in pDNA structure as evidenced by the reduced band intensity. There is no significant change in the CD spectrum of the ND50/50HMW PECs, when the temperature of the suspending medium was increased to 40 °C (above the LCST of NIPAM). This suggests that the thermo-responsive nature of NIPAM segments do not affect pDNA complexation in the vicinity of physiologically attainable temperatures. Twaites et al. observed a small temperature dependent decrease in the particle size of PECs prepared from NIPAM/DMAEM-co-hexylacrylate terpolymers at 45 °C, that was attributed to local coil-globule NIPAM segment collapse (Twaites 2004). However, our CD data suggests that, at least in the case of NIPAM/DMAEM copolymers, the interaction with pDNA is unaffected by temperature, in the 25–40 °C range. It is possible that changes in the size of the hydration shell of these PECs may account for the small decrease in their particle size at elevated temperatures. The LCST of NIPAM/DMAEM copolymers studied here were in excess of 50 °C at pH 7.4 (data not shown), indicating that positively charged amino groups of DMAEM prevent collapse of NIPAM chains, thereby, further corroborating the CD findings.

CD spectroscopy was used to probe the changes in structural features of Ad5 capsid proteins resulting from complexation with NIPAM/DMAEM polymers and PECs. As illustrated in Figure 8, the CD spectrum of Ad5 reveals bands in the region 210–230 nm, characteristic of an α-helix protein conformation. Abolition of α-helix spectral fine structure of this band was observed when Ad5 was used to form a Type I complex with ND0/100HMW. Even more pronounced changes are observed in the α-helix band of a Type II complex with ND0/100HMW and Ad5. As is seen in Figure 8 by comparing the features of the pDNA and PEC spectra with those of Ad5, it is doubtful that these spectral changes result from a superposition of Ad5 and pDNA spectral features given the constancy of intensity in the region above 240 nm. Therefore, the binding of polymers and PECs onto the Ad5 capsid can be characterized spectrophotometrically by following changes in the spectral characteristics of viral proteins. Indeed, one may speculate as to the nature of the interaction between PDMAEM coatings and viral capsid proteins responsible for cell entry. Mathias et al have reported a conserved Arg-Gly-Asp (RGD) motif in the sequenced penton bases of several adenovirus serotypes that were purported to play a role in viral cell entry via αv integrins (Mathias et al 1994). The predicted secondary structure of the RGD domain of the adenovirus penton base was that of a helix-turn-helix with the RGD domain at the apex of the α-helices. Thus, the CD analysis suggests that PDMAEM and PDMAEM PECs coatings complex

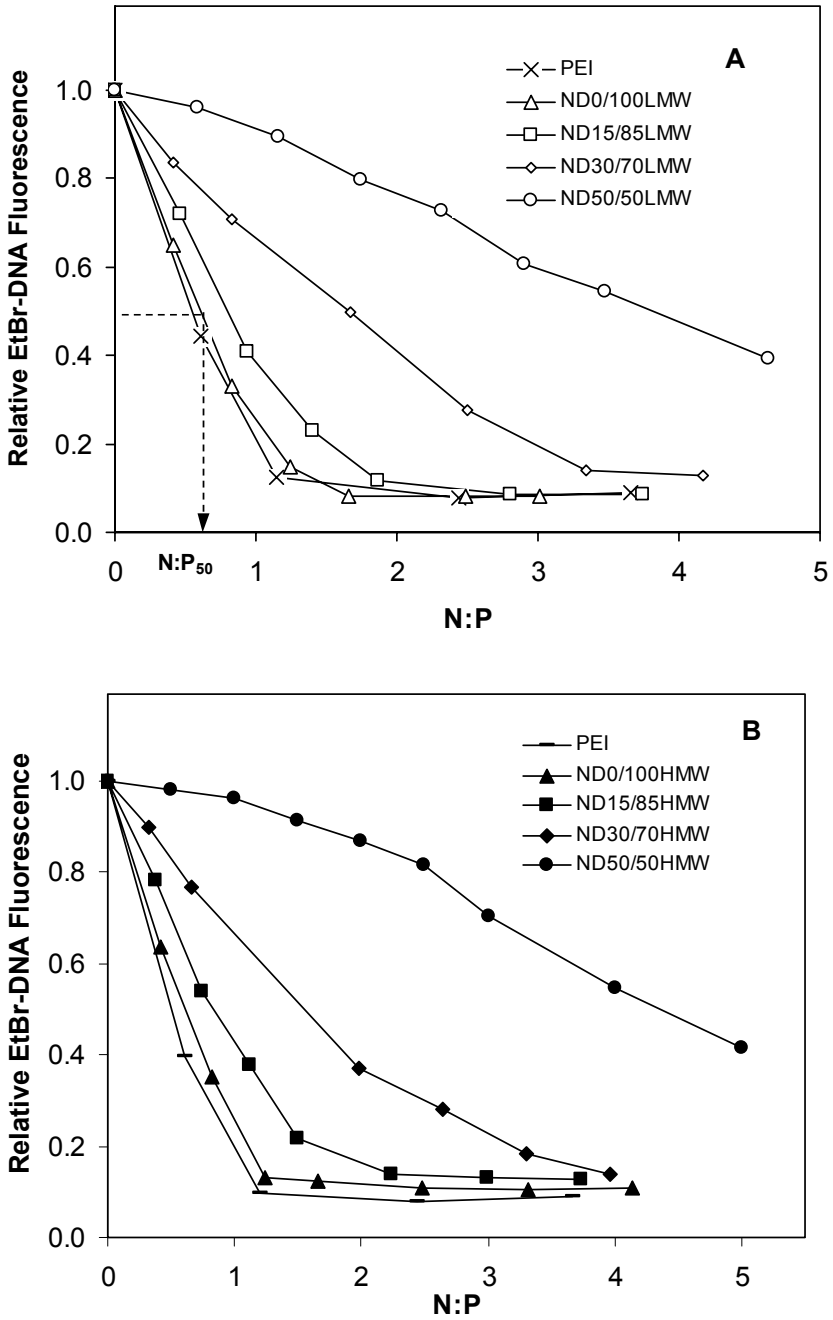

Figure 3 Inhibition of ethidium bromide intercalation complexes with pDNA by low molecular weight (**A**) and high molecular weight (**B**) NIPAM/DMAEM copolymers (n = 3).

with amino acids in the vicinity of Ad5 binding domains. This finding may have implications in terms of the immunologic and internalization role of polymers in Type I and Type II Ad5 vector cell entry.

The binding of NIPAM/DMAEM polymers and PECs to the Ad5 capsid was visualized by TEM. Figure 9 shows low- and high-resolution images of Ad5 and Type II Ad5. The Type II Ad5 had to be diluted extensively to reduce the propensity of viral precipitation. The micrograph shows that binding of PECs does not result in complete coating of the viral capsid. A larger quantity of PEC may be necessary for complete capsid coating.

The results of MTT assay of cytotoxicity of the NIPAM/DMAEM polymers towards CNE-1 cell lines are shown in Figure 10. The toxicity of the polymers increased with molecular weight and the DMAEM content. The PDMAEM homopolymer was fairly toxic with an ID_{50} around 0.2 mg/mL. Most samples were characterized by an abrupt cessation or level-

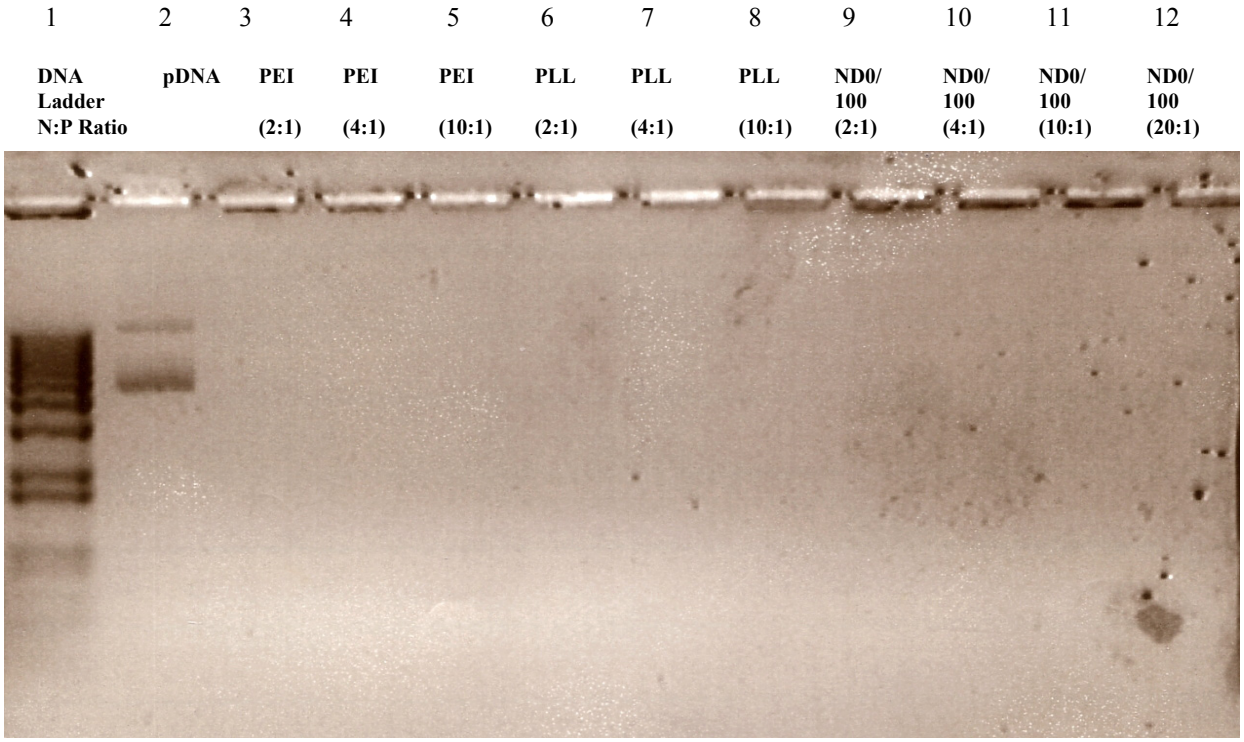

Figure 4 Agarose gel retardation assay of PECs. Lane 1, DNA ladder; lane 2 pDNA; PECs prepared with, lanes 3–5, PEI (N:P); lanes 6–8, PLL, lanes 9–12, ND0/100HMW.

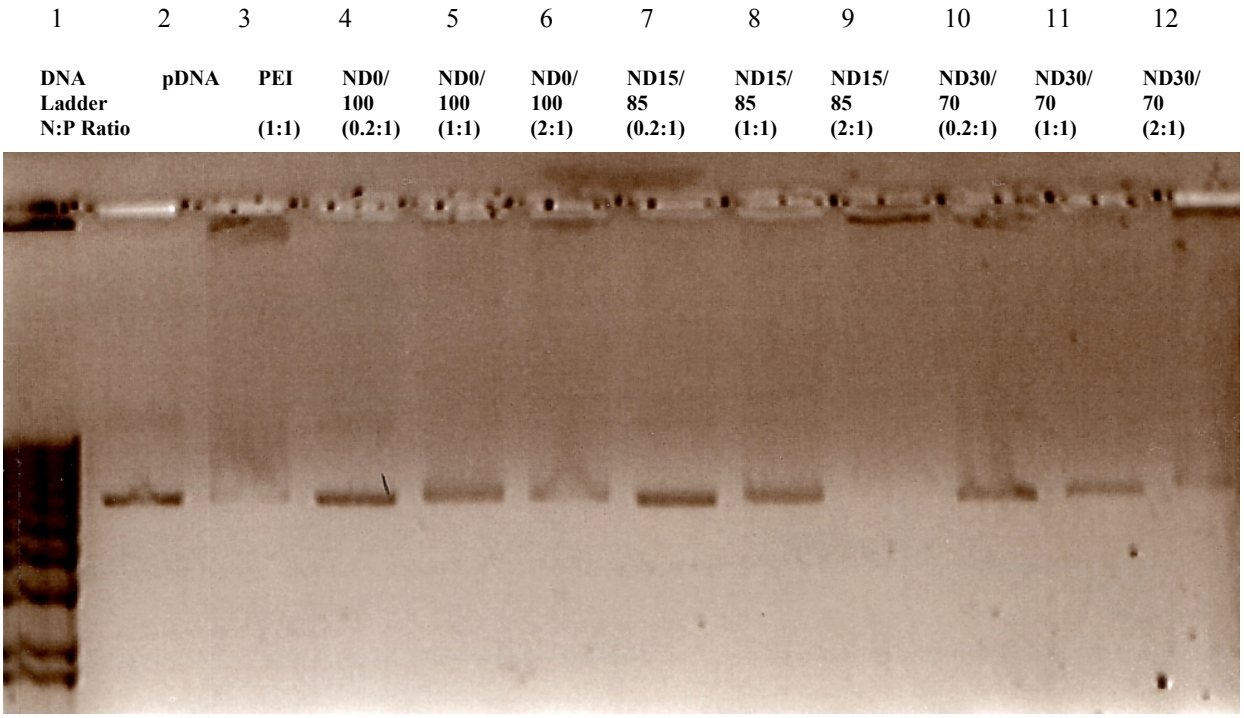

Figure 5 Agarose gel retardation assay of LMW NIPAM/DMAEM PECs. Lane 1, DNA ladder; lane 2 pDNA; PECs prepared with, lanes 3–5, ND0/100LMW; lanes 6–8, ND15/85LMW, lanes 9–12, ND30/70LMW.

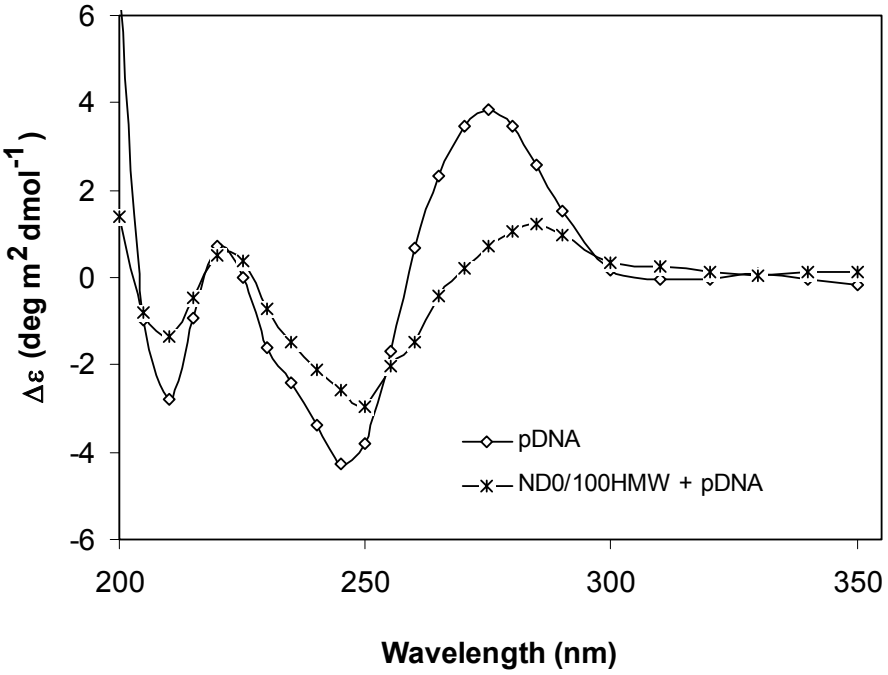

Figure 6 CD spectra of pDNA and ND0/100HMW PECs (N:P = 5:1).

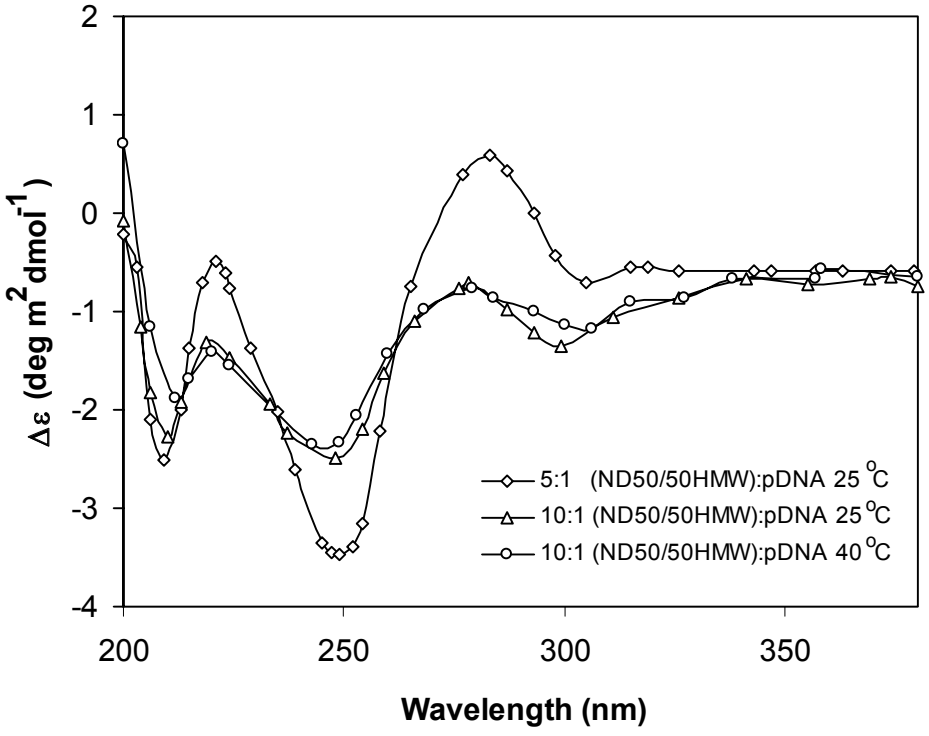

Figure 7 Temperature and N:P ratio dependence of CD spectra of ND50/50HMW PECs.

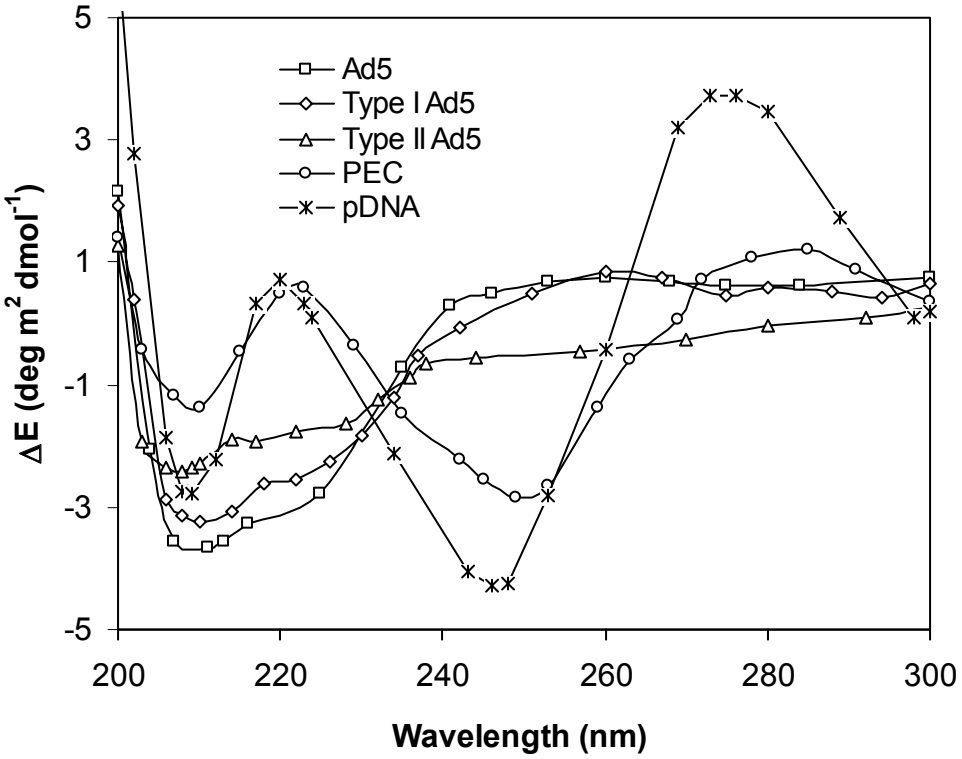

Figure 8 CD Spectra of Ad5; Type I Ad5-ND0/100HMW (1000 molecules/particle); Type II Ad5 coupled with ND0/100HMW:pDNA N:P =10:1; PEC (ND0/100HMW:pDNA N:P =10:1);

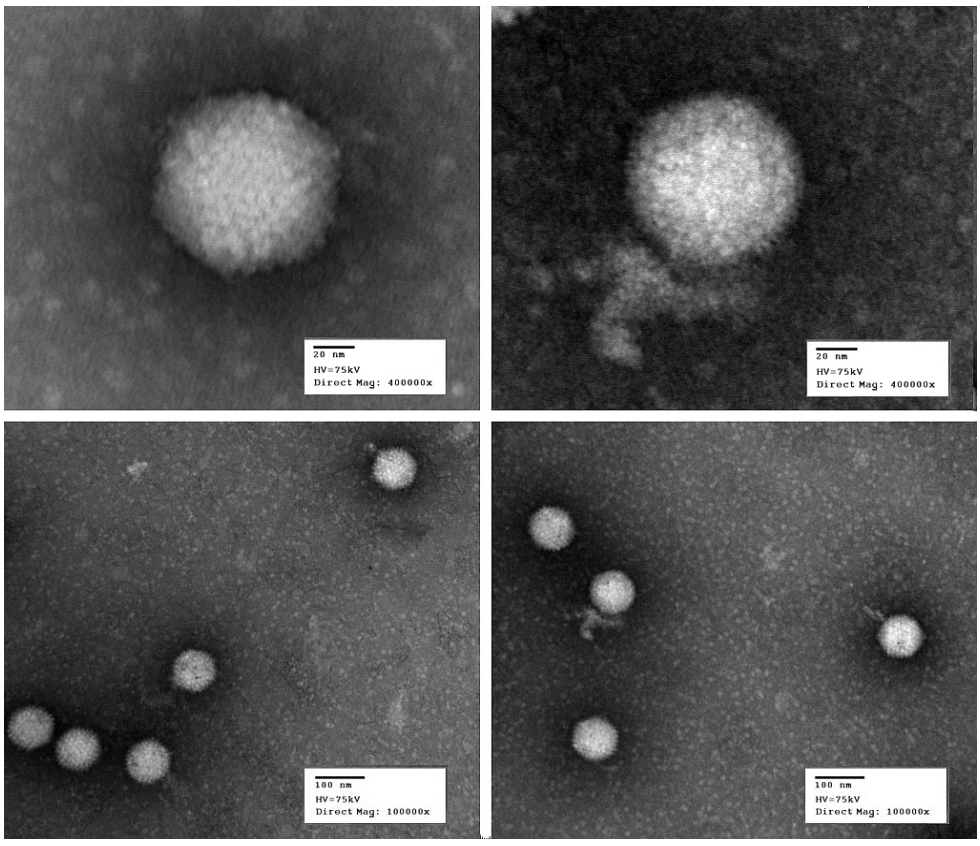

Figure 9 Transmission electron micrographs of adenovirus (top left), ND0/100HMW polymer-modified adenovirus at high and low resolution (top right and bottom plates, respectively).

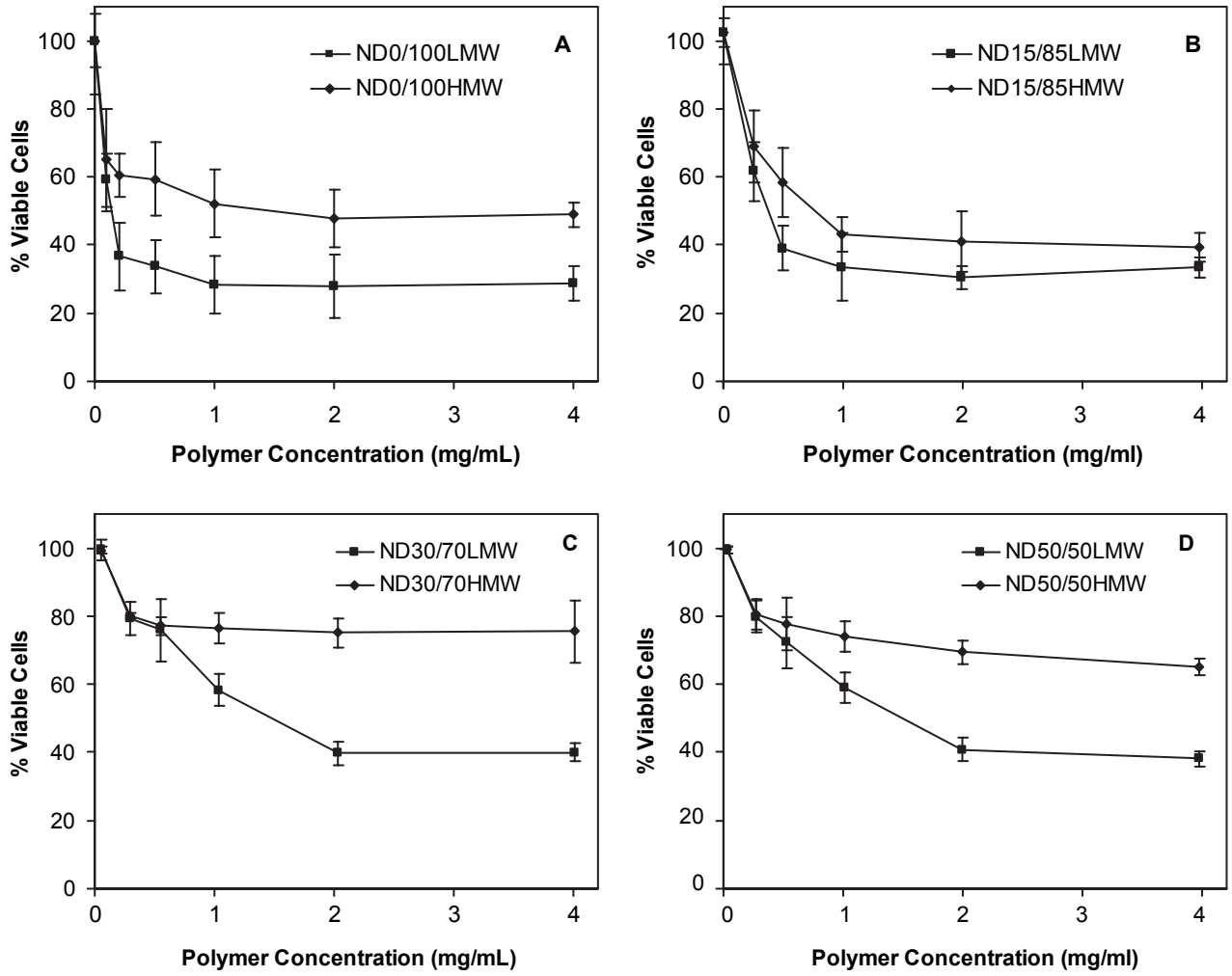

Figure 10 Toxicity of NIPAM/DMAEM copolymers towards CNE-1 nasopharyngeal carcinoma cells; A) ND0/100; B) ND15/85; C) ND30/70; D) ND50/50 (n = 3).

ing off of toxicity above a threshold value which increased with NIPAM content and decreasing molecular weight.

Effect of polymers on the tranduction/ transfection efficiency

Figure 11 shows the results of transfection of C666-1 NPC cells by NIPAM/DMAEM PECs. The level of expression of firefly luciferase provides an indication of the efficiency of transfection by the PECs. In each case, high molecular weight NIPAM/DMAEM polymers were on the order of 5-fold more efficient at expressing the reporter gene than were the low molecular weight analogs. Transfection levels decreased markedly with increasing NIPAM content in the polymer. Notably, the transfection with PDMAEM homopolymer PECs was as effective as or better than PECs prepared from the established transfectant, PEI. The drop off in transfection levels between PDMAEM homopolymers and ND0/50 polymers was in excess of 90%. Since, the NIPAM-bearing polymers were effective at condensing pDNA, elucidation of the origin of this dramatic change in transfection efficiency may require further studies on the compositional dependence of PECs for endosomal escape and protection of pDNA from nucleases.

In Figure 12, the transduction efficiencies of CNE-1 NPC cells by type I polymer-modified Ad5 vectors are compared. The efficiencies of the highly branched and highly charged PEI-modified vectors were compared with those derived from PDMAEM and NIPAM/DMAEM copolymers. PEI-modified Ad5s transduced CNE-1 cells more efficiently than the untreated Ad5s at the same infectivity ratio. Moreover, increasing the quantity of PEI coating the virus by 100% produced a concomitant increase in the expression level. The PDMAEM homopolymers that were used to modify the Ad5, were almost as effective at promoting reporting gene expression as the PEI-modified Ad5 vectors. For the PDMAEM and NIPAM/DMAEM polymers, the

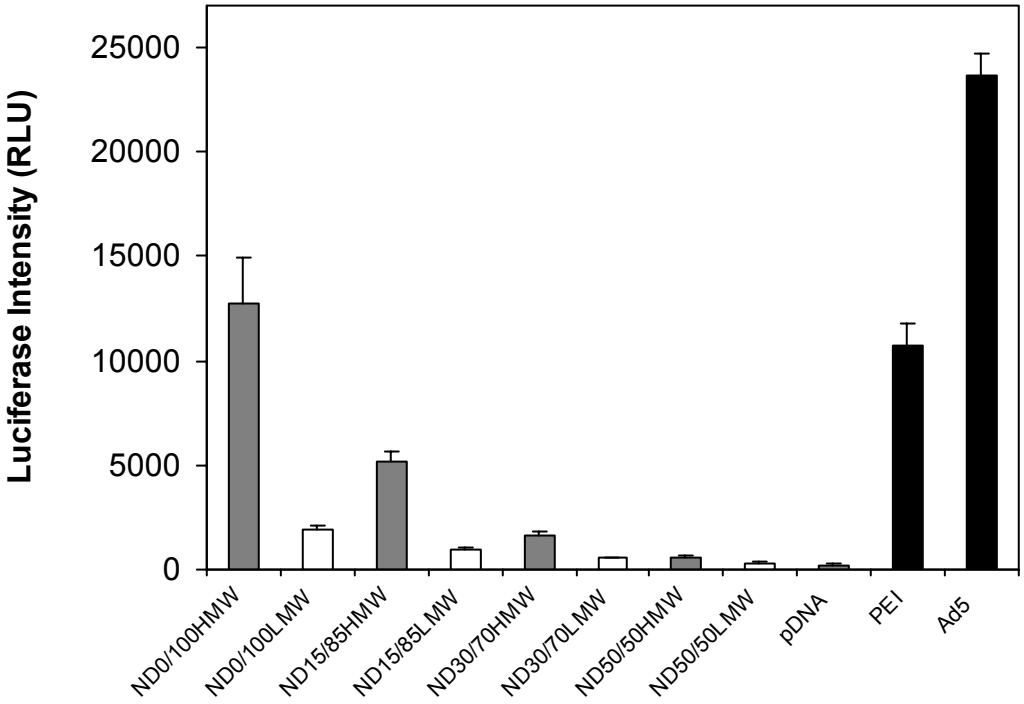

Figure 11 Luciferase expression in C666-1 nasopharyngeal carcinoma cells of NIPAM/DMAEM PECs. Grey shaded samples represent high molecular weight NIPAM/DMAEM PECs; white bars represent low molecular weight NIPAM/DMAEM PECs. Black filled samples are the negative (pDNA) and positive controls, PEI and Ad5 oriP/Luc. For PECs N:P = 10 Ad5 MOI = 50, (n = 3).

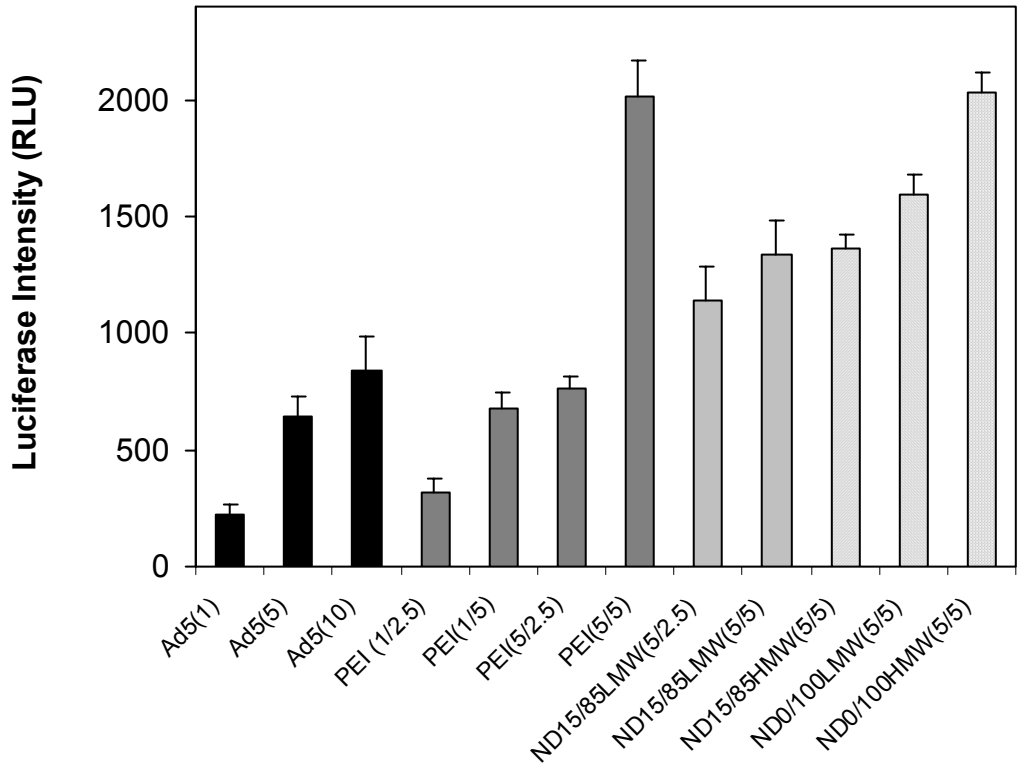

Figure 12 Luciferase expression in CNE-1 transfected with Type I Ad5-CMV/*luc*. Numbers in parentheses denote (MOI/ng polymer) in Ad5 suspension (n = 3).

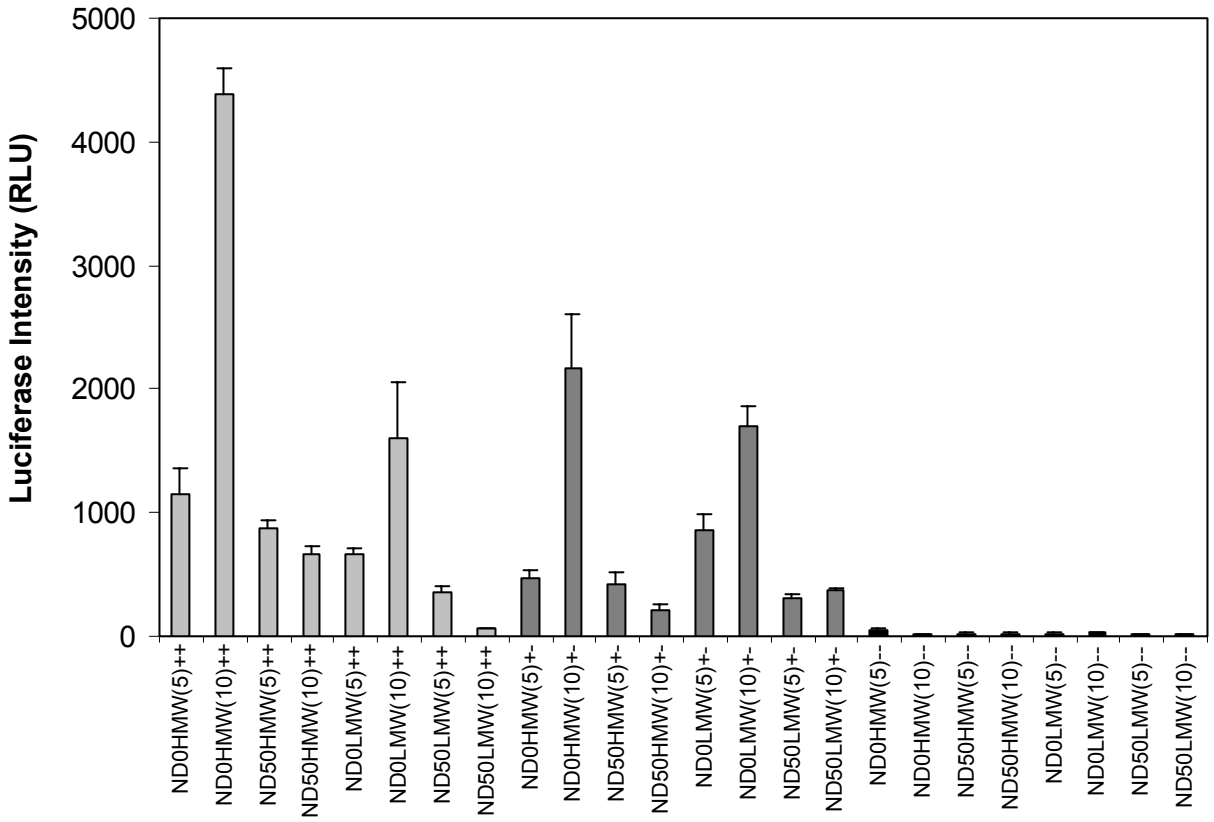

Figure 13 Luciferase expression in C666-1 transfected with Type II Ad5-CMV/Luc complexes at MOI = 10 pfu, N:P ratio of PEC in parentheses. + denotes active expression via plasmid or adenovirus, –denotes UV knockout; the first sign refers to plasmid, the second to Ad5 (n = 3).

HMW samples yielded slightly higher levels of expression than the LMW samples. Interestingly, the expression levels of ND15/85 modified Ad5s were only slightly lower than those of the PDMAEM homopolymers and essentially independent of molecular weight.

Figure 13 shows the results of tests of transfection and transduction efficiency of Type II polymer-modified Ad5 vectors. The Type II vectors utilizing both functionally expressive pDNA and Ad5 (denoted +/+) exhibited the highest transgene expression levels. The vectors that incorporated the PECs that exhibited the highest transfection efficiency (ND0/100HMW) also had the highest expression. Expression levels decreased when the NIPAM content in the PEC of the Type II vector was increased. When the Ad5 was functionally knocked out by irradiation, expression of the Type II vector should be a consequence of the condensed pDNA alone. The dark shaded bars in Figure 13, in most cases indicate that 50% or more of the total transgene expression resulted from the pDNA. It is difficult to ascertain whether this is caused by interference of transduction of Ad5 by PECs or simply due to the greater accessibility of the PECs to the cell surface thereby facilitating more facile cellular entry.

Conclusion

Copolymers of NIPAM/DMAEM were tested as components of non-viral transfectants and as modifiers of adenoviral vectors for gene delivery. As transfectants, NIPAM/DMAEM copolymers were shown to effectively condense pDNA across a broad range of molecular weights and compositions to sizes suitable for gene delivery application. CD spectra revealed small red shifts and diminution of spectral band intensity of pDNA indicating structural changes upon complexation with cationic polymers. PECs prepared from copolymers of higher MW and higher DMAEM content were more efficient transfectants of nasopharyngeal cell lines. This was offset by a modest increase in toxicity of these polymers. NIPAM/DMAEM polymers effectively modulated gene expression observed by adenovirus vectors both in Type I and Type II polymer-modified Ad5 vector systems. This suggests that these materials can be used to enhance gene expression either by mediating infection of adenovirus expressing systems or using pDNA plus the adenovirus as a carrier. Future studies will investigate the potential of these polymers to facilitate hyperthermic localization of the PECs and the polymer-modified Ad5 vectors and assess their biodistribution and RES avoidance.

Acknowledgment

The authors would like to gratefully acknowledge funding from Canadian Institutes of Health Research to F.F. Liu and X.Y. Wu, the Natural Sciences and Engineering Research Council of Canada to X.Y. Wu, the Rosenstadt Fund to S. Sarkar and the Ben Cohen Fund to J. Moselhy. We are also thankful to Dr. T. Chalikian for allowing us to use the CD instrument in his lab.

List of abbreviations

LMW–low molecular weight; HMW:high molecular weight; PMV–polymer-modified virus; Ad5–adenovirus serotype 5; PEC–polyelectrolyte complex; N:P–nitrogen:phosphate mole ratio

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