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The effect of swelling and cationic character on gene transfection by pH-sensitive nanocarriers

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ABSTRACT

We synthesized a series of pH-sensitive vehicles, composed of dimethylaminoethyl methacrylate (DMAEMA) and 2-hydroxyethyl methacrylate (HEMA), to optimize the triggered release of DNA for gene transfection. The purpose of this study was to assess the role of swelling and cationic character independently on transfection; both of which may affect DNA release. Gene transfection was performed by delivering plasmid DNA (pDNA) encoding for luciferase. DNA release was controlled via volumetric swelling by regulating the endosomal pH as a result of inhibiting V ATPases using bafilomycin A1. Increasing the cationic character from 10 to 30 mol% DMAEMA did not increase transfection when swelling was inhibited. Transfection was significantly affected by the rate of pDNA release. pH-sensitive nanocarriers were also compared to vehicles comprised of polyethyleneimine (PEI), dioleoyl trimmonium propane (DOTAP), and poly(lactic-co-glycolic acid) (PLGA, 50:50). pDNA encapsulating DMAEMA/HEMA nanoparticles and PEI/pDNA complexes had reduced transfection when V ATPases were inhibited, whereas pDNA encapsulating PLGA nanoparticles showed no endosomal pH dependence. DMAEMA/HEMA nanoparticles cross-linked with 3 mol% tetraethylene glycol dimethacrylate (TEGDMA) reported equivalent or greater gene transfection relative to the nanocarriers tested at 24 and 48 h.

1. Introduction

Therapeutic gene delivery is based on the concept that recombinant DNA and RNA interference technologies can be used to regulate disease at the molecular level. Nonetheless, treatment of human diseases by genetic material instead of drugs has been limited by effective delivery without cytotoxicity. Nonviral gene delivery vehicles have encapsulated or condensed pDNA with cationic polymers [1–5], degradable polymers [6], or lipids [7], where the pDNA is subsequently released using the low endosomal pH [8]. While it is widely accepted that pH facilitates endosomal delivery, the consequences of vehicle cationic character and the rate of DNA release are poorly understood.

Endocytosis generally occurs by engulfing molecules or therapeutic vehicles by the plasma membrane. Plasma membrane invaginations evolve into endosomes that become lysosomes, acidic compartments responsible for degrading foreign agents. Therapeutic agents (i.e. pDNA) are delivered to the cytoplasm by disrupting endosomes (Fig. 1).

Endosomes undergo acidification by ion pumps. Most primary ion pumps use the energy provided by the hydrolysis of adenosine triphosphate (ATP) to energize ion-transport processes across the cell membrane. V ATPases [9] (i.e. H^+ ATPases), found on endosomal membranes, are responsible for endosome acidification [10,11]. Bafilomycin A1, a proton pump inhibitor, has been used to inhibit endosome acidification. The average pH of the endosome was ~5.5 in the absence and 7.4 in the presence of 200 nM bafilomycin A1 [10]. Bafilomycin A1 has been employed previously to assess endosomal gene delivery [12].

Endocytosed vehicles deliver pDNA by destabilizing the endosomal envelope [13] or buffering against lysosomal degradation by the "proton sponge" effect [2]. Dioleoyl trimethyl ammonium propane (DOTAP) [14] and polyethylenimine (PEI) [15] have been used to condense DNA; however, they both induce cytotoxic effects [15,16]. Peptides [17,18] and fusogenic liposomes [19], activated by low pH, have been designed to induce endosome disruption. pDNA encapsulating poly(lactic-co-glycolic acid) (PLGA, 50:50) nanoparticles have been investigated for gene delivery [20,21], where PLGA degrades by acid hydrolysis. Release of pDNA from the endosome to the cytoplasm is important in facilitating gene transfer.

Endosomal delivery may also depend on the vehicles cationic character. The N/P ratio, the ratio of polycationic polymers or

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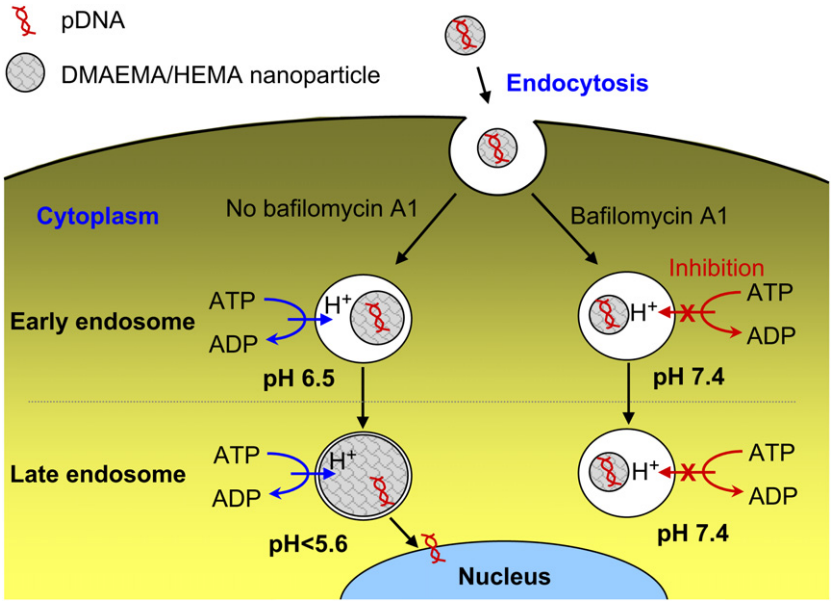

Fig. 1. Schematic representation of pH-sensitive DMAEMA/HEMA nanoparticle-mediated gene transfection with and without bafilomycin A1 as a V ATPase inhibitor.

cationic lipids (with N amine groups) to pDNA (with P phosphate groups), is often used to optimize gene delivery. A high N/P ratio may inhibit pDNA release whereas a low N/P ratio may inefficiently complex the pDNA [22]. In addition, increased cationic character has been linked to increased cell uptake [23]. The contribution of the cationic charge to gene delivery is multifold.

Recently, we synthesized a series of nanoparticles with tunable pH-sensitivity to optimize gene delivery [24]. These particles trigger the release of pDNA in response to changes in pH. Although particles had distinct differences in volumetric swelling as a function of pH, transfection of HeLa cells by pH-sensitive nanoparticles was not dramatically affected by increasing the amount of the pH-sensitive monomer dimethylaminoethyl methacrylate (DMAEMA). Increasing the DMAEMA content simultaneously increased the cationic character and swelling ratio. Therefore, the contribution of pH-induced swelling and cationic character on transfection was unclear.

In this paper, we investigated the role of vehicle swelling and cationicity independently on transfection. Both the extent of swelling and the electrostatic interactions between the cationic DMAEMA and anionic pDNA may affect the rate of pDNA release. We controlled volumetric swelling by altering the endosomal pH as a result of inhibiting V ATPases using bafilomycin A1. Gene transfection was performed by delivering pDNA encoding for luciferase to HeLa, a human cervical cancer cell line, and HEK293, a human embryonic kidney cell line. pDNA encapsulating, pH-sensitive vehicles were benchmarked against naked pDNA, PEI/pDNA complexes, DOTAP/pDNA complexes, and pDNA encapsulating PLGA nanoparticles.

2. Material and methods

2.1. Materials

The monomer dimethylaminoethyl methacrylate (DMAEMA) and comonomer 2-hydroxyethyl methacrylate (HEMA) were purchased from Acros (Morris Plains, NJ, USA). The cross-linker tetraethylene glycol dimethacrylate (TEGDMA) was obtained from Fluka (St. Louis, MO, USA). The bafilomycin A1, polyethyleneimine (PEI, 750 kDa), chloroform, ammonium persulfate, and sodium metabisulphite were purchased from Sigma (St. Louis, MO, USA). 1,2-dioleoyl-3-trimethyl-ammonium-propane (DOTAP) was purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). Pluronic F68 was kindly provided from BASF Corporation (Mount Olive, NJ, USA). Poly(lactide-co-glycolide) (PLGA, 50:50, MW 17000–22000) and polyvinyl alcohol (PVA, MW ~25000) were purchased from Polysciences, Inc. (Warrington, PA, USA) and dichloromethane was purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ, USA). gWiz™ high-expression luciferase vector was purchased from Aldevron (South Fargo, ND, USA) for gene transfection. All cell culture media were purchased from Invitrogen (Carlsbad, CA, USA). All materials were used without further purification. Deionized water (18.2 MΩ) was obtained from a Milli-Q purification system (Millipore Corp., Billerica, MA, USA).

2.2. Nanoparticles preparation

pDNA encapsulating, pH-sensitive DMAEMA/HEMA nanoparticles were obtained by adding 100 μL of a DMAEMA/HEMA solution (10/90, 20/80, and 30/70, mol/mol) with 3, 6, and 9 mol% TEGDMA cross-linker, containing 10 μg DNA, to 10 mL of deionized water containing Pluronic F68 (150 mg) and TEGDMA (3 mol%). Aqueous solutions of ammonium persulfate (0.5% w/v) and sodium metabisulphite (0.25% w/v) were added as initiators. The solution was immediately sonicated (200 W, 20 kHz; Digital sonifier 250, Branson Ultrasonics Corp., Danbury, CT, USA) in a laminar flow hood over an ice bath for 10 min (8 s on and 4 s off). The polymerization process was carried out at room temperature for 3 h. DMAEMA/HEMA nanoparticles were collected by high-speed centrifugation at 39000 × g for 20 min (Sorvall RC26 Plus, SA-600 rotor; Thermo Fisher Scientific Inc., Waltham, MA, USA). The particles were washed three times and collected with pH 7.4 phosphate buffer to remove residual surfactant and initiators.

PLGA nanoparticles encapsulating luciferase pDNA were prepared by a water-oil-water (W/O/W) emulsion and then solvent evaporation method. Briefly, 10 μg of pDNA (5 mg/mL in water) was directly added to 20 μL dichloromethane containing 50 mg PLGA and emulsified using a microtip probe sonicator for 10 s. The emulsion was added to 2 mL of 1% (w/v) polyvinyl alcohol solution and sonicated for 30 s at 200 W output in an ice bath to form the W/O/W emulsion. The final double emulsion was agitated using a magnetic stirrer for 3 h to remove dichloromethane completely. PLGA nanoparticles encapsulating pDNA were isolated by high-speed centrifugation at 39,000 × g for 20 min and washed three times with deionized water.

PEI/pDNA and DOTAP/pDNA complexes were prepared as described previously [2,7]. Briefly, 10 μg of pDNA and the desired amount of PEI as indicated by the N/P ratio (MW of PLL = 750 kDa, $N/P = 6$) were diluted into 50 μL of 150 nM NaCl and vortexed gently and spun down briefly. For DOTAP/pDNA complexes, dried DOTAP was rehydrated with distilled water at a final concentration of 1 mM. 10 μg of pDNA and the desired amount of DOTAP (MW of DOTAP = 698.55, $N/P = 2$) were added to deionized water in a final volume of 100 μL. The solutions were incubated for 20 min at room temperature. The N/P ratios were chosen to give high transfection based on previous reports [15,25,26].

2.3. Nanoparticle characterization

A 0.05 wt% aqueous DMAEMA/HEMA nanoparticle suspension was prepared to determine the size and morphology of nanoparticles by transmission electron microscopy (TEM, JEOL 2100; JEOL Ltd., Tokyo, Japan). A 5 μL of the nanoparticle solution was placed on a copper grid (300 mesh, TED PELLA Inc., Redding, CA, USA) supporting a thin film of amorphous carbon. The excess liquid was removed with filter paper and the copper grid was dried in a laminar flow hood. The particle size was analyzed by dynamic light scattering (ZetaPALS; Brookhaven Instrument, Holtsville, NY, USA). The zeta potential of the nanoparticles was measured by electrophoresis (ZetaPALS; Brookhaven Instrument, Holtsville, NY, USA). Nanoparticles were diluted to 0.1 mg/mL in a 10 mM N-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES) buffer at pH 7.4. The size and zeta potential were measured a minimum of three times.

The accumulative pDNA amount in the nanoparticles was quantified by measuring the fluorescence collection of the plasmid combined with PicoGreen reagent (Invitrogen) using a fluorescence microplate reader (SpectraMax Gemini XPS; Molecular Devices Corp., Sunnyvale, CA, USA). pDNA encapsulating DMAEMA/HEMA nanoparticles were put into phosphate buffer (pH 2) titrated by 1 N HCl and vigorously agitated with a magnetic stirrer for 24 h. Also, pDNA encapsulating PLGA nanoparticles were added to chloroform for 5 h, and the amount of encapsulated pDNA was calculated by PicoGreen assay.

2.4. Swelling study

Swelling of DMAEMA/HEMA nanoparticles was performed in buffered solutions of known pH (either pH 5.5, 6.5, and 7.4), composition (10 mM TES, adjusted with 1 N HCl), and temperature (37 ± 0.5 °C). A predetermined amount of freeze-dried DMAEMA/HEMA was put into a scintillation vial containing 10 mL of buffered medium. Samples were placed on a shaker (OS-500 orbital shaker, VWR, West Chester, PA, USA) with a shaking rate of 100 ± 1 rpm in an incubator maintained at 37 °C. The average nanoparticle diameter was measured three times by dynamic light scattering at 0, 1, 2, and 4 h.

2.5. pDNA release from nanoparticles

pDNA release from 30/70 (mol/mol) DMAEMA/HEMA nanoparticles cross-linked with 3 mol% TEGDMA and PLGA nanoparticles was performed in a TE buffer (10 mM Tris–HCl, 1 mM EDTA) at either pH 5.5 and 7.4. DMAEMA/HEMA nanoparticles, encapsulating 2 μg of pDNA, was placed into a 4 mL glass vial containing 2 mL of buffer. The sample was placed on a shaker at a shaking rate of 100 ± 1 rpm in an incubator maintained at 37 °C. At predetermined release time points, samples were collected from the vials and replaced with fresh buffered media. Collected samples were placed into a 96-well plate where the DNA concentration was measured by a fluorescence microplate reader after using the PicoGreen quantification kit. All measurements were performed in triplicate.

2.6. Agarose gel electrophoresis

The integrity of the encapsulated pDNA in DMAEMA/HEMA and PLGA nanoparticles was analyzed by agarose gel electrophoresis in tris-acetate-EDTA buffer (TAE: 40 mM tris-base, 1 mM EDTA, pH 8.5). 20 μL of DMAEMA/HEMA nanoparticles containing 20 μg pDNA was suspended in 500 μL phosphate buffer at pH 2. The suspension was vortexed for 1 d to break down the particles. Supernatant containing the encapsulated pDNA was collected and centrifuged at 15,000 × g for 20 min at 4 °C. Collected pDNA was resuspended in 1 mL of TAE buffer for gel electrophoresis. PLGA nanoparticles containing 20 μg pDNA in 20 μL were added into 1 mL of chloroform and vortexed for 1 d. Subsequently, 1.0 mL of water was added, and the solution was stirred for 10 min. The two-phase solution was separated by centrifugation for 10 min. The aqueous phase was collected and centrifuged at 15,000 × g for 20 min at 4 °C. Obtained pDNA was resuspended in 1 mL TAE buffer and loaded onto a 1.0 w/v% agarose gel containing ethidium bromide (0.5 μg/mL). Gel electrophoresis was performed in TAE buffer at 110 V for 1 h (Wide mini ready sub-cell GT system, Bio-Rad, Hercules, CA, USA). The gel was visualized on a UV transilluminator (Model-20, UVP Inc., Upland, CA, USA) alongside a DNA ladder to indicate the location and size of the DNA.

2.7. Gene transfection

HeLa and HEK293 cells in the exponential growth phase were detached with 1× ethylene-diamine tetra-acetic acid (EDTA)-trypsin from a tissue culture dish (Falcon, Frankin Lakes, NJ, USA). HeLa and HEK293 cells (5×10^4 cells/well) were inoculated in a 96-well plate with 200 μL of high glucose Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 °C in a 5% CO_2, humidified incubator. After an 18 h incubation, cell growth media was removed, and the cells were washed with PBS three times to remove serum. Bafilomycin A1 was added to culture wells to achieve 0, 20, or 300 nM final concentration in 200 μL serum-free growth medium and incubated for 30 min 20 μL of the pDNA complexes and nanoparticle suspensions (containing 2 μg pDNA) were diluted with serum-free media containing the same concentration of bafilomycin A1 and added to culture wells. After a 4 h incubation, culture media were replaced with fresh growth media containing bafilomycin A1, serum, and 1% penicillin-streptomycin. The luminescence of transfected cells was measured at 8, 24, and 48 h after the addition of pDNA complexes and pDNA-loaded nanoparticles relative to non-transfected cells. The transfection efficiency was analyzed by relative light units (RLUs) using a microplate reader with luminescence reading (SpectraMax Gemini XPS). For comparison, transfection of HeLa and HEK293 cells was performed using equivalent amounts of naked DNA. The obtained RLUs were normalized with respect to protein concentration in the cell extract examined using the Bio-Rad DC protein assay kit.

2.8. Endosomal staining

LysoSensor™ dye (LysoSensor Yellow/Blue DND-160, Invitrogen) was used to monitor endosomal pH visually. HeLa cells (5×10^4) were cultured on 96-well glass-bottom tissue culture dishes (MatTek Corp., Ashland, MA, USA). After an 18 h inoculation, HeLa cells were incubated for 30 min at 37 °C in the absence or presence of bafilomycin A1 (0, 20, and 300 nM, respectively). And then, 20 μL of the PEI/pDNA complexes and nanoparticle suspensions (containing 2 μg of plasmid pDNA) were diluted with serum-free media and added in culture dishes. After a 4 h incubation, the culture media was replaced with fresh 2 mL DMEM containing 10% FBS and 5 μM LysoSensor DND-160 for 5 min. Cells were washed with Dulbecco's phosphate buffered saline (D-PBS) three times and examined with confocal microscopy (Zeiss LSM 510 META, Carl Zeiss, Inc., Thornwood, NY, USA).

2.9. Cell viability

The cytotoxicity of PEI/pDNA and DOTAP/pDNA complexes and pDNA encapsulating PLGA and DMAEMA/HEMA nanoparticles was evaluated by a Live/Dead (viability/cytotoxicity) assay for mammalian cells (Molecular Probes, Eugene, OR, USA) with HeLa and HEK293 cells. HeLa and HEK293 cells (10^4 cells/well) were added to a 96-well cell culture dish (Falcon) with 200 μL DMEM supplemented with 5% FBS and 1% penicillin-streptomycin. After inoculating the cells for 18 h, 20 μL of DMAEMA/HEMA and PLGA nanoparticles containing 2 μg pDNA were resuspended with growth medium and added into a cell culture dish. After a 24 h incubation, cells were rinsed twice with 100 μL of D-PBS. Live/Dead assay reagents, which contained 4 mM calcein AM and 2 mM Ethidium homodimer-1, were added to the cell culture dish. After a 30 min incubation at room temperature, cell viability was measured with a fluorescence microplate reader. Also, the Live/Dead assay on PEI/pDNA and DOTAP/pDNA complexes was performed for comparison. All cultures were performed at 37 °C, balanced with 5% CO_2 in air in a humidified incubator.

2.10. Statistical analysis

Unless otherwise mentioned, triplicate data were obtained and presented as mean ± standard deviation. Statistical difference was analyzed using analysis of variance with Student's *t*-test on the significance level of $p < 0.05$.

3. Results

3.1. Nanoparticle synthesis and characterization

pDNA encapsulating, pH-sensitive nanoparticles composed of 10/90, 20/80, and 30/70 (mol/mol) DMAEMA/HEMA were prepared with either 3, 6, or 9 mol% TEGDMA [24]. TEM images of 30/70 (mol/mol) DMAEMA/HEMA nanoparticles cross-linked with 3 mol% TEGDMA revealed monodisperse, spherical particles with a 203.1 ± 7.3 nm diameter. Similar sizes were obtained for other DMAEMA/HEMA formulations, confirmed by dynamic light scattering. The zeta potential of pDNA encapsulating 10/90, 20/80, and 30/70 (mol/mol) DMAEMA/HEMA nanoparticles cross-linked with 3 mol% TEGDMA as determined by electrophoresis were −17.2 ± 2.6, −14.1 ± 1.5, and −9.8 ± 0.8 mV, respectively. Quantification of pDNA after extraction from DMAEMA/HEMA nanoparticles exhibited an encapsulation efficiency of 96.4 ± 3.1%. The average diameter and zeta potential were also measured for naked pDNA (62.8 ± 8.1 nm, −8.5 ± 1.4 mV), PEI/pDNA complexes (124.6 ± 11.3 nm, 25.1 ± 6.2 mV), DOTAP/pDNA complexes (176.4 ± 17.1 nm, 31.8 ± 2.4 mV), and pDNA encapsulating PLGA nanoparticles (217.1 ± 11.4 nm, −20.7 ± 1.8 mV). The encapsulation efficiency of pDNA encapsulating PLGA nanoparticles was 76.8 ± 5.1%.

pH-sensitivity was characterized by volumetric swelling (Table 1). Swelling of 10/90, 20/80, and 30/70 (mol/mol) DMAEMA/HEMA nanoparticles cross-linked with 3 mol% TEGDMA was performed as a function of time at pH 5.5. In addition, 30/70 (mol/mol) DMAEMA/HEMA particles prepared with higher cross-linking density (6 and 9 mol% TEGDMA) were evaluated at pH 5.5. The swelling ratio was calculated as the average diameter of the swollen particles divided by the initial average diameter of the particles at pH 7.4. High swelling ratios were achieved at low pH, high DMAEMA content, and low cross-linking density. Maximal swelling was observed after 4 h.

3.2. pDNA release

The release of luciferase pDNA from pH-sensitive DMAEMA/HEMA nanoparticles was monitored at pH 5.5 as a function of time (Fig. 2A). pDNA release increased (by less than 10%) with the addition of 10 mol% DMAEMA. Increasing the cross-linking density to 6 and 9 mol% TEGDMA reduced pDNA release by approximately 30% and 75%, respectively. This was similar to results shown previously with pDNA encoding for green fluorescent protein (GFP) [24]. In comparison, PLGA and 30/70 (mol/mol) DMAEMA/HEMA nanoparticles cross-linked with 3 mol% TEGDMA were observed for pDNA release at pH 5.5 and 7.4 (Fig. 2B). DMAEMA/HEMA nanoparticles had higher pDNA release at pH 5.5 than pH 7.4 whereas PLGA nanoparticles had similar pDNA release at both pH 5.5 and 7.4. DMAEMA/HEMA and PLGA nanoparticles released 96.3 ± 3.1% and 56.1 ± 8.2% of the encapsulated pDNA at pH 5.5 after 48 h, respectively.

The structural integrity of the luciferase pDNA was examined by agarose gel electrophoresis (Fig. 3). Supernatants were collected after polymerization (lane 1) and after three washing steps (lanes 2–4). Encapsulated pDNA, extracted from DMAEMA/HEMA (Fig. 3A) and PLGA nanoparticles (Fig. 3B) confirmed that the pDNA remained intact (lanes 5). No detectable pDNA was released after polymerization of DMAEMA/HEMA nanoparticles or subsequent washing steps. We observed pDNA bands (lanes 1 and 2) after preparation of PLGA nanoparticles and the first washing step. pDNA was released from PLGA nanoparticles during the emulsification process (13.0% determined by the PicoGreen assay) and first washing step (6.4%), causing a lower pDNA loading efficiency than DMAEMA/HEMA nanoparticles.

3.3. Gene transfection

To determine how controlled release of pDNA impacts transfection, condensed or encapsulated pDNA was incubated with cells for different periods of time. Transfection of HeLa (Fig. 4A) and HEK293 (Fig. 4B) cells were quantitatively determined by measuring relative luminescence units (RLUs) of synthesized luciferase after 8, 24, and 48 h after addition of pDNA vehicles. Gene transfection was performed using naked pDNA, pDNA encapsulating DMAEMA/HEMA nanoparticles, pDNA encapsulating PLGA nanoparticles, PEI/pDNA complexes ($N/P = 6$) and DOTAP/pDNA ($N/P = 2$) complexes. The N/P ratios chosen for PEI/pDNA and DOTAP/

Table 1
Volume swelling ratio of DMAEMA/HEMA nanoparticles (10/90, 20/80, and 30/70, mol/mol) cross-linked with TEGDMA (3, 6, and 9 mol%) in phosphate buffer media (pH 5.5, 6.5 and 7.4).

	Swelling time (hour)		
	1	2	4
10/90[a]	1.28 ± 0.09	1.51 ± 0.10	1.73 ± 0.13
20/80[a]	1.38 ± 0.10	1.69 ± 0.11	1.93 ± 0.14
30/70[a]	1.53 ± 0.13	2.04 ± 0.16	2.07 ± 0.17
6[b]	1.25 ± 0.13	1.46 ± 0.10	1.51 ± 0.10
9[b]	1.06 ± 0.06	1.13 ± 0.08	1.17 ± 0.11
pH 6.5[c]	1.23 ± 0.10	1.55 ± 0.12	1.67 ± 0.12
pH 7.4[c]	1.12 ± 0.07	1.22 ± 0.08	1.27 ± 0.13

[a] DMAEMA/HEMA molar ratio (mol/mol); 3 mol% TEGDMA and pH 5.5 swelling buffer.
[b] TEGDMA mol%; 30/70 (mol/mol) DMAEMA/HEMA ratio and pH 5.5 swelling buffer.
[c] Swelling buffer pH; 30/70 (mol/mol) DMAEMA/HEMA ratio and 3 mol% TEGDMA.

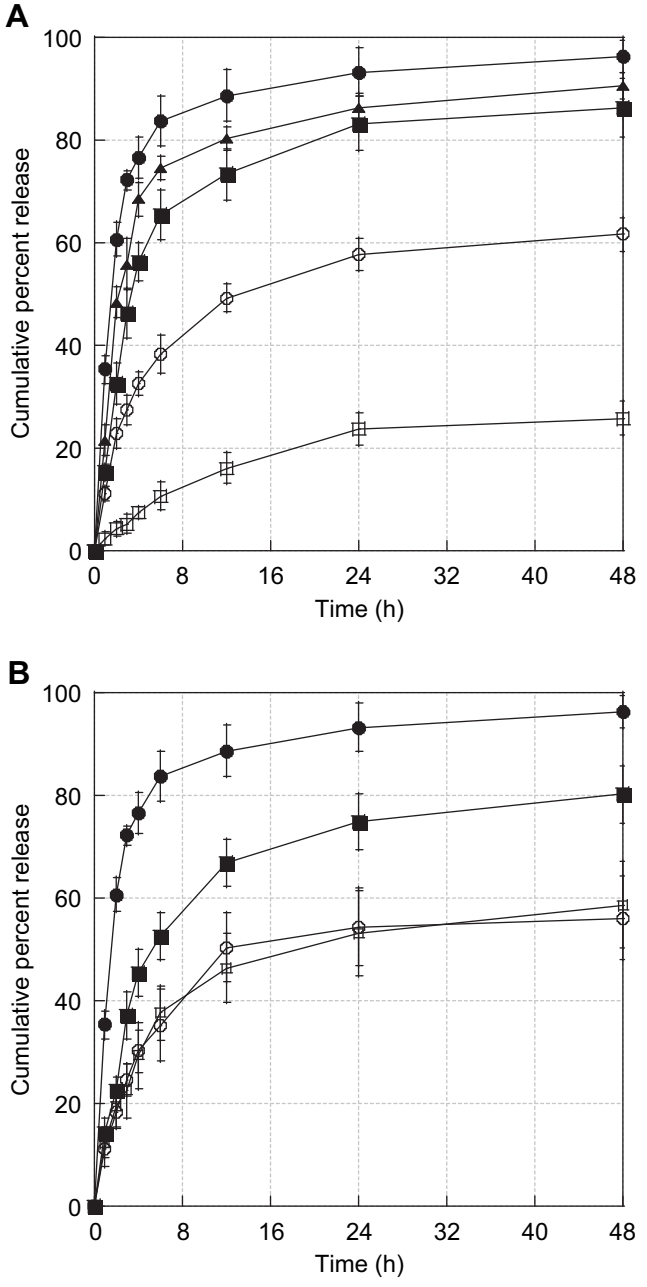

Fig. 2. pH-sensitive luciferase pDNA release. (A) pDNA release was performed from pH-sensitive DMAEMA/HEMA nanoparticles (10/90 (■), 20/80 (▲), and 30/70 (●), mol/mol) cross-linked with 3 mol% TEGDMA and DMAEMA/HEMA nanoparticles (30/70, mol/mol) cross-linked with 6 (○) and 9 (□) mol% TEGDMA at pH 5.5. (B) pDNA release from DMAEMA/HEMA nanoparticles (30/70, mol/mol) cross-linked with 3 mol % TEGDMA at pH 5.5 (●) and 7.4 (■) and PLGA (50:50) nanoparticles at pH 5.5 (○) and 7.4 (□) was obtained. The percentage of cumulative pDNA release was measured at 0, 1, 2, 3, 4, 6, 12, 24, and 48 h, respectively. The error is the standard deviation of the mean, where $n = 3$.

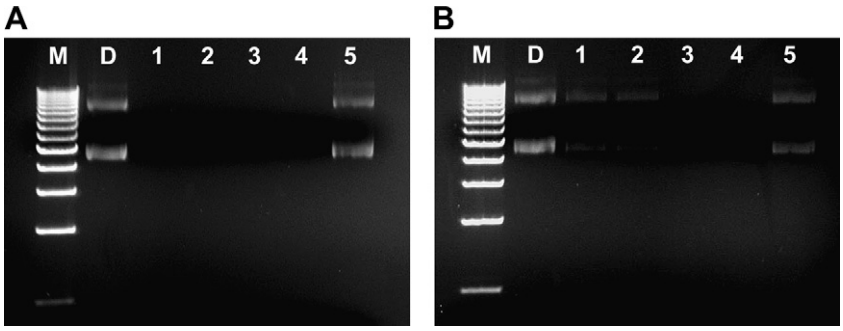

Fig. 3. Gel electrophoresis. Luciferase pDNA encapsulated within (A) DMAEMA/HEMA (30/70, mol/mol) nanoparticles and (B) PLGA (50:50) nanoparticles were examined by gel electrophoresis. Lane M: EZload 1 kb molecular ladder, lane D: plasmid DNA (0.1 μg), lane 1: supernatant after polymerization, lane 2: supernatant after first washing, lane 3: supernatant after second washing, lane 4: supernatant after third washing, lane 5: plasmid DNA extracted from nanoparticles.

pDNA complexes were observed to enhance transfection based on previous reports [15,25,26].

Gene transfection of pDNA encapsulating DMAEMA/HEMA and PLGA nanoparticles increased with exposure time. In contrast, luciferase synthesis resulting from transfection by PEI/pDNA and DOTAP/pDNA complexes increased between 8 and 24 h but did not change between 24 and 48 h. At 8 h, PEI/pDNA complexes had equivalent performance to 30/70 (mol/mol) DMAEMA/HEMA

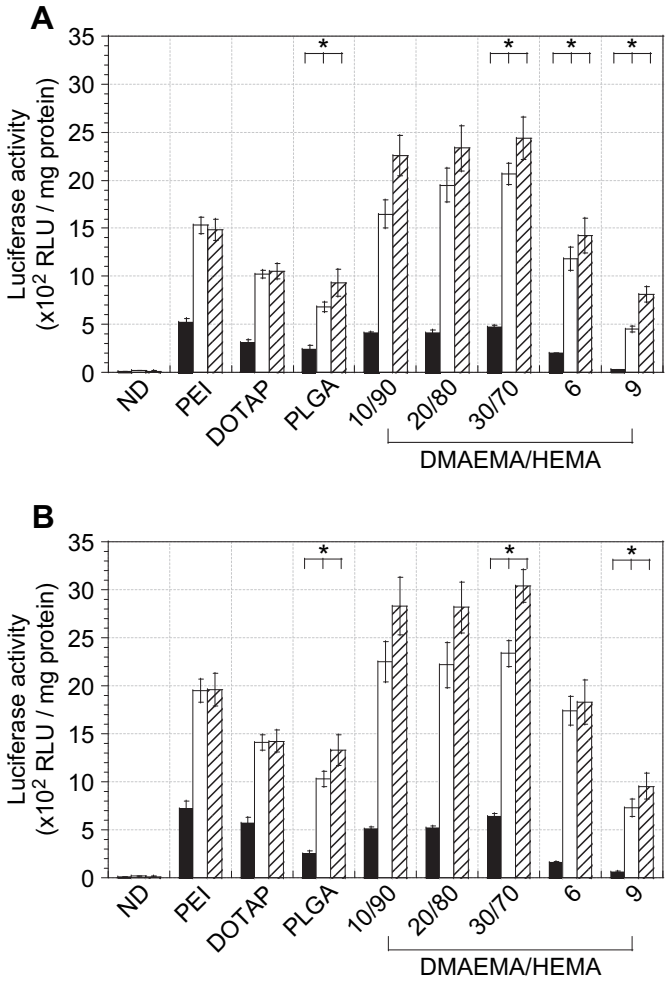

Fig. 4. Impact of DNA release on gene transfection. Gene transfection of (A) HeLa and (B) HEK293 cells was performed by either DNA alone, PEI ($N/P = 6$), DOTAP ($N/P = 2$), PLGA, or DMAEMA/HEMA nanoparticles (10/90, 20/80, and 30/70, mol/mol) cross-linked with 3, 6, and 9 mol% TEGDMA at 8 (black), 24 (white), and 48 h (striped) incubation. The error is the standard deviation from the mean, where $n = 3$. Based on statistical analysis, * means $p < 0.05$ compared to RLU treated by nanoparticles encapsulating pDNA from free-DNA carriers.

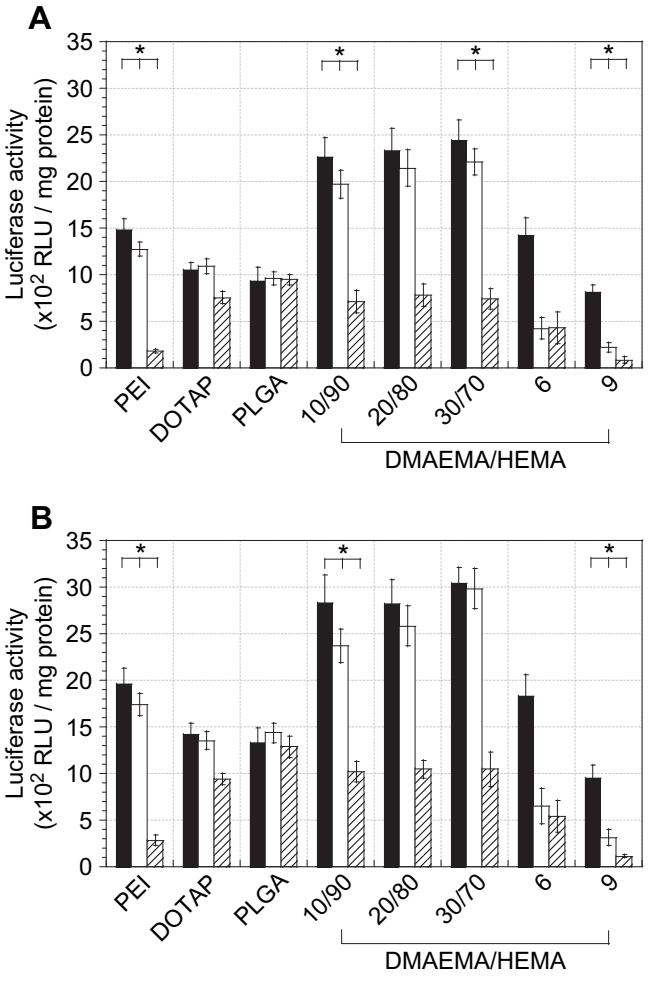

Fig. 5. Impact of swelling on gene transfection by V APTase inhibition. Gene transfection of (A) HeLa and (B) HEK293 cells was performed by either DNA alone, PEI ($N/P = 6$), DOTAP ($N/P = 2$), PLGA, or DMAEMA/HEMA nanoparticles (10/90, 20/80, and 30/70, mol/mol) cross-linked with 3, 6, and 9 mol% TEGDMA in the presence of either 0 (black), 20 (white), or 300 nM (striped) bafilomycin A1 at 48 h. The error is the standard deviation from the mean, where $n = 3$. Based on statistical analysis, * means $p < 0.05$ compared to RLU treated by DNA complexes or nanoparticles encapsulating DNA in the absence of bafilomycin A1.

nanoparticles cross-linked with 3 mol% TEGDMA. At 24 and 48 h post-transfection, DMAEMA/HEMA nanoparticles cross-linked with 3 mol% TEGDMA outperformed PEI/pDNA complexes, DOTAP/pDNA complexes, and pDNA encapsulating PLGA nanoparticles. It is noted that gene transfection of HEK293 cells was higher than HeLa cells but showed a similar trend. After 48 h, transfection of HeLa and HEK293 cells by pDNA encapsulating 30/70 (mol/mol) DMAEMA/HEMA nanoparticles cross-linked with 3 mol% TEGDMA was 2440 ± 220 and 3040 ± 170, respectively.

Gene transfection was also examined in the presence of 0, 20, and 300 nM bafilomycin A1 to inhibit pH-induced swelling (Fig. 5). Bafilomycin A1 inhibited endosome acidification and consequently transfection from PEI/pDNA complexes and pDNA encapsulating DMAEMA/HEMA nanoparticles. PEI/pDNA complexes and pDNA encapsulating 30/70 (mol/mol) DMAEMA/HEMA nanoparticles cross-linked with 3 mol% TEGDMA showed an 87% and 70% decrease in transfection, respectively, in the presence of 300 nM bafilomycin A1 relative to transfection without bafilomycin A1. We did not observe significant differences in transfection from pDNA encapsulating PLGA nanoparticles with addition of bafilomycin A1. DOTAP/pDNA complexes had a smaller reduction (29%) in transfection efficiency with 300 nM bafilomycin A1 relative to PEI/pDNA complexes and 30/70 (mol/mol) DMAEMA/HEMA cross-linked with 3 mol% TEGDMA.

Monitoring of the endosomal pH was performed by the addition of LysoSensor DND-160 into the growth medium after transfection with pDNA encapsulating 30/70 (mol/mol) DMAEMA/HEMA nanoparticles cross-linked with 3 mol% TEGDMA and PEI/pDNA complexes (Fig. 6). In the absence of bafilomycin A1, pDNA encapsulating 30/70 (mol/mol) DMAEMA/HEMA nanoparticles and PEI/pDNA complexes had a greater number of endosomes relative to the control without a transfection agent. The number of endosomes decreased with the addition of 300 nM bafilomycin A1.

3.4. Cell viability

The cytotoxicity of naked luciferase pDNA, pDNA encapsulating PLGA and DMAEMA/HEMA nanoparticles, and PEI/pDNA and DOTAP/pDNA complexes was examined in HeLa cells (Fig. 7). The cell viability of pDNA encapsulating 30/70 (mol/mol) DMAEMA/HEMA nanoparticles in HeLa had 93.1 ± 3.3% viability compared to PEI/pDNA complexes with 88.7 ± 5.8%. The cell viability of HEK293 was similar to HeLa cells.

4. Discussion

The aim of this study was to evaluate the mechanism of endosomal delivery by pH-sensitive nanocarriers. DMAEMA/HEMA vehicles were originally designed to swell in response to small pH changes (>0.2 pH units [27]). Increasing the content of DMAEMA simultaneously increased the nanocarrier cationicity and swelling ratio which may alter cellular uptake, DNA release, and endosomal escape. Differences in the swelling ratio and DNA release for the 10/90, 20/80, and 30/70 (mol/mol) DMAEMA/HEMA

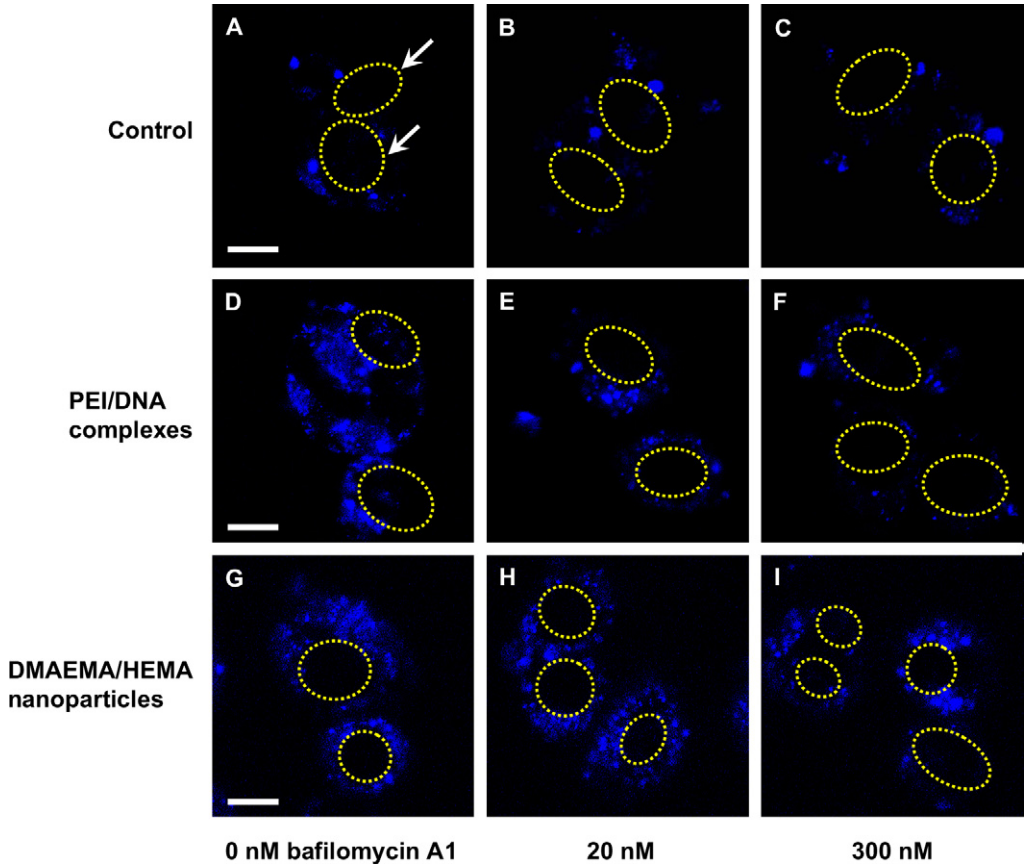

Fig. 6. Micrographical images of HeLa cells with endosomal staining. HeLa cells were cultured with PEI/DNA complexes (D–F) and DNA-encapsulating DMAEMA/HEMA nanoparticles (G–I) under 0 (A, D, and G), 20 (B, E, and H), and 300 nM (C, F, and I) bafilomycin A1. After 4 h incubation, endosomes were stained with 5 μM of LysoSensor DND-160 dye to evaluate endosomal pH change. *Ns* and white arrows represent nuclei and yellow circles show boundaries of nuclei. The control micrographs (A–C) show HeLa cells at different concentration of bafilomycin A1 without any transfection agent. Scale bar = 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

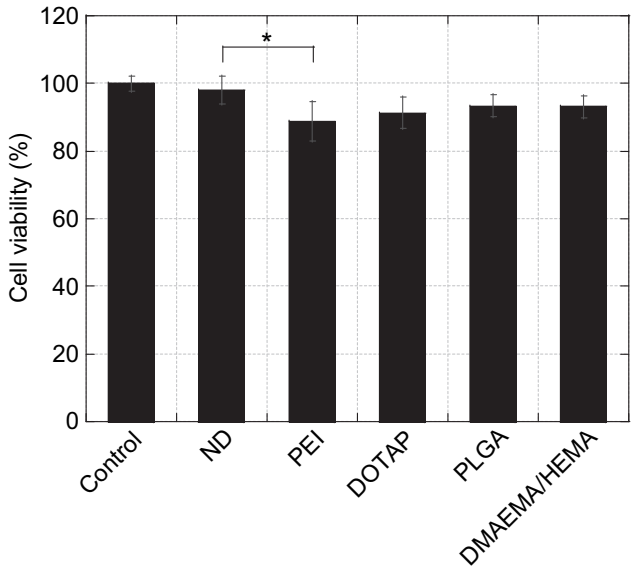

Fig. 7. Cell viability assay. PLGA nanoparticles and DMAEMA/HEMA (30/70, mol/mol, abbreviated DMA/HEMA) nanoparticles cross-linked with 3 mol% TEGDMA were incubated with HeLa cells for 24 h and compared to a control without nanoparticles, naked DNA, PEI/DNA complexes ($N/P = 6$), and DOTAP/DNA compexes ($N/P = 2$). The error is the standard deviation from the mean, where $n = 3$. $^{*}p < 0.01$, Student's t-test.

formulations cross-linked with 3 mol% TEGDMA resulted in similar transfection efficiencies. To exploit differences between the different DMAEMA/HEMA formulations, we investigated transfection as a function of incubation time (Fig. 4) and as a function of cationicity (Fig. 5).

Transfection was assessed as a function of time to evaluate the impact of the amount of DNA delivered on transfection. All modes of delivery showed an increase in luciferase expression between 8 and 24 h post-transfection. Increasing the incubation time from 24 to 48 h had no effect on transfection by PEI/pDNA and DOTAP/pDNA complexes; however, transfection by pDNA encapsulating DMAEMA/HEMA and PLGA nanoparticles showed an increase in luciferase synthesis. Increased luciferase expression was not a direct result of swelling, as swelling ceased after 4 h (Table 1). Enhanced luciferase expression was dependent on pDNA release; an increase of approximately 5% of encapsulated pDNA was observed from DMAEMA/HEMA and PLGA nanoparticles between 24 and 48 h (Fig. 4).

Triggered release of pDNA from pH-responsive DMAEMA/HEMA nanoparticles cross-linked with 3 mol% TEGDMA resulted in twice the amount of DNA being released in comparison to PLGA at pH 5.5 (Fig. 2B). PLGA nanoparticles, which degrade by acid hydrolysis, did not increase pDNA release with decreasing pH. Based on the similar pDNA release profiles at pH 7.4 and 5.5, it is not surprising that transfection by PLGA nanoparticles remained the same with or without the addition of bafilomycin A1 (Fig. 5).

Transfection by PEI/pDNA complexes and pDNA encapsulating DMAEMA/HEMA nanoparticles were sensitive to endosomal pH, as shown by decreasing luciferase expression with increasing concentration of bafilomycin A1 (Fig. 5). This agreed with previous reports that PEI operates by acting as a 'proton sponge' [2,28]. Gene delivery based on endosomal acidification (pH 5–6.5 from early to late endosome) [29,30] has been a popular strategy used in several drug and gene delivery applications [2,31–33]. Use of biodegradable or less cationic polymers relative to PEI has been a focus for reducing cell toxicity [34]. DMAEMA/HEMA nanoparticles have a low N/P ratio (1.4 N/P for 10/90 DMAEMA/HEMA nanoparticles) which may reduce cytotoxic effects.

Two distinct differences between the four nanocarriers were their size and zeta potential. These two attributes can influence particle uptake by cells [35,36]. Positively and negatively charged vehicles have exhibited differences in their rate of uptake in vitro [37]. In addition, particle size has been shown to influence the mode of uptake [38]. Differences in the rate of uptake or endocytotic pathway may impact transfection [39]. Though there exist obvious differences between the four vectors, the objective was to investigate pH-sensitive and pH-insensitive formulations that utilize pDNA complexation and encapsulation methods for transfection.

DMAEMA/HEMA nanoparticles with different DMAEMA content had similar uptake despite changes in zeta potential; however, increasing the cross-linking density from 3 to 9 mol% reduced uptake by two-thirds [24]. PEI/pDNA complexes were taken up readily by cells [40]. Likewise, PLGA nanoparticles were endocytosed by cells [41–43]. Although uptake was not a focus of this study, it could influence transfection. We kept the size of particles constant for pDNA encapsulating vehicles, but differences in charge could affect uptake. Negatively-charged DMAEMA/HEMA nanoparticles may exhibit lower cellular uptake than cationic vehicles.

As shown in Fig. 6, an increase in the number of endosomes was observed when a transfection agent was administered, which may also allude to the uptake of particles. Surprisingly, low pH endosomes were detected visually in HeLa cells with the addition of 300 nM bafilomycin A1. This may contribute to the low transfection observed in the presence of bafilomycin A1.

pDNA encapsulating DMAEMA/HEMA vehicles enhanced transfection without the adverse effects of cytotoxicity, outperforming PEI/pDNA complexes, DOTAP/pDNA complexes, and pDNA encapsulating PLGA nanoparticles at 24 and 48 h (Fig. 4). Based on our findings, the endosomal pH played a primary role in pH-sensitive gene delivery. DMAEMA/HEMA nanoparticles were not reliant on the cationic character of the vehicle for cellular uptake, DNA release, or endosomal disruption. Instead, triggered pDNA release by particle swelling enhanced luciferase expression. PLGA nanoparticles did not efficiently release pDNA relative to DMAEMA/HEMA nanoparticles. We demonstrated that enhanced transfection may be achieved by utilizing pH-induced swelling.

5. Conclusions

We identified that pH-triggered pDNA release and not cationic character was responsible for enhanced transfection by DMAEMA/HEMA nanoparticles in two cell lines. PEI/pDNA and pDNA encapsulating DMAEMA/HEMA nanoparticles were sensitive to changes in endosomal pH. This was confirmed by decreasing transfection upon V ATPase inhibition by bafilomycin A1. Transfection by pH-sensitive vehicles was modestly affected by increasing DMAEMA content (i.e., cationic character) in the absence or presence of bafilomycin A1. Triggered DNA release from DMAEMA/HEMA nanoparticles enhanced gene transfection relative to controlled release by PLGA nanoparticles. Gene transfection strategies may be improved by addressing methods for unencumbered pDNA release.

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