Low Charge Polyvinylamine Nanogels Offer Sustained, Low-Level Gene Expression

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ABSTRACT: Cationic polymer charge and polymer degradability each play a crucial role for packaging and delivering plasmid DNA. High density cationic charge has been shown to enhance transfection efficiency but may give rise to undesirable toxicity. Polyvinylamine (PVAm) nanogels bearing discrete amounts of surface charge were used to systematically examine the balance between transfection efficiency and cytotoxicity. Poly(*N*-vinylformamide) (PNVF) nanogels were prepared via an inverse emulsion polymerization reaction and crosslinked with a nondegradable or acid-labile crosslinker. The nanogels were then hydrolyzed to yield varying degrees of primary amines. The degree of conversion from PNVF to PVAm was controlled using different concentrations of NaOH and hydrolysis times. PVAm nanogel size and charge ranged from 150 to 310 nm, and +3.5 to +18 mV, respectively. These cationic particles were then complexed with pDNA encoding for luciferase. The cytotoxicity of PVAm nanogels and the transfection efficiency of PVAm/DNA complexes were evaluated in carcinomic human alveolar basal epithelial cells (A549). The cytotoxicity of PVAm nanogels increased with increasing accessible charge as expected. Transfection efficiency increased with increasing amounts of amine groups for nondegradable nanogels. Interestingly, acid-labile nanogels bearing low charge demonstrated more sustained gene transfection when compared with the more highly charged nanogels. These observations suggested that the use of degradable particles with less charge may reduce cytotoxicity without compromising overall transfection efficiency. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 118: 1921–1932, 2010

Key words: structure–function relationship; polyvinylamine; pH-sensitive; nanogels; gene delivery

INTRODUCTION

Gene therapy represents a promising method to prevent, treat, or cure diseases. A major challenge of gene delivery is the balance between safety and efficiency. Viruses are highly efficient vectors but often exhibit immunogenicity and mutagenicity. In addition, they can be difficult and expensive to produce. Nonviral vectors including cationic lipids and polymers have received attention because of their simplicity of production and gene-carrying capacity; however, they often have limited transfection efficiency and can exhibit significant toxicity.[1]

Over the past 2 decades, many types of cationic polymers have been developed and studied as an alternative to viral vectors because of their outstanding versatility of physicochemical properties and easy manipulation. Charge is a key parameter of the polymer for DNA binding, interaction with the cell surface, endolysosomal escape, and subcellular localization. The nature of the polymer charge can enhance the transfection efficiency[2–4] but may also result in undesirable cytotoxicity.

Cytotoxicity of polymeric vectors depends upon material composition, exposure time, and dose.[5] Besides charge density, cytotoxicity also depends on molecular weight and degradability of vectors.[5,6] Low molecular weight polyethylenimine (PEI)[7] and polylysine[8,9] exhibited low toxicity in previous studies. As a result, many groups have used low molecular weight PEI to try to avoid toxicity.[10–12] Highly cationic vectors cause cytotoxicity by destabilizing the plasma membrane, interacting with cellular components, and inhibiting normal cellular processes, which can induce necrosis and/or apoptosis.[5,13,14] Many strategies have been used to reduce undesirable toxicity of polymers containing high cation density such as using hydrophilic, tertiary amine-based polymers,[15] reducing the number of primary amines by acetylation,[16] or conjugating with cyclodextrins.[17] Degradability is also an intriguing property which may be incorporated into

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polymers. It is known that some degradable carriers can release free DNA into the cytosol rapidly and some have been shown to exhibit low cytotoxicity.[6] Several groups have used biodegradable polymers such as chitosan[18–20] and poly(lactic-*co*-glycolic acid) (PLGA)[21] as gene carriers. A number of pH-responsive linkers such as imine,[22] diacrylate,[23] ketal,[24,25] or disulfide[26] linkers have also been explored to achieve degradability of synthetic vectors.

The balance between transfection efficiency and toxicity is crucial. For example, fully deacetylated PEI exhibiting only moderate *in vitro* transfection efficiency and low cytotoxicity have shown improved performance *in vivo* when compared with unmodified PEI.[27] Many structure-bioactivity studies have been conducted to find suitable gene carriers. Previous findings revealed structural impacts of vectors on transfection efficiency and cytotoxicity. For example, the proximity of amine units in polymer chains could enhance both gene expression and cytotoxicity.[28] In contrast, liposome: DNA weight ratios had a greater impact on transfection efficiency than lipoplex structures.[29] In another study, polylysine-*graft*-imidazoleacetic acid conjugates bearing high imidazole content exhibited high gene expression.[30] Recent studies show that amino alcohol polymers possessed appreciable *in vitro* gene expression among other poly(β-amino ester)s, and terminal functionality had great effect on *in vivo* transfection efficiency.[31,32] Further structure–function studies should be performed systematically to enlighten efficient, nontoxic, and *in vivo* stable vectors.

Hydrogels have been wildly used as delivery vehicles for vaccine, proteins, peptides, and nucleic acids because of their hydrophilic nature and biocompatibility.[33–35] Here, hydrophilic polyvinylamine (PVAm) nanogels were synthesized with controlled amounts of primary amines. This system provides a means to assess cytotoxicity and transfection efficiency as a function of particle charge. PVAm nanogels were derived from poly(*N*-vinylformamide) PNVF nanogels which were previously reported as protein carriers.[36] The effect of particle degradability on transfection efficiency was also assessed. PVAm nanogels were crosslinked using a nondegradable crosslinker or an acid-labile crosslinker which contains a central ketal subject to rapid degradation in acidic compartments, such as in lysosomes.[25,37] We anticipated that PVAm nanogels with different charge densities and/or degradability would provide different transfection efficiencies. The findings from this well-controlled polymer system will add to literature defining relationships between transfection efficiency and structure, which will aid the development of gene carriers in the future.

MATERIALS AND METHODS

Material

N-Vinylformamide (NVF; Aldrich), 2,2′-azobis(2,4-dimethylpentanitrile (Vazo-52, DuPont), Tween-80, Span-80, and 25 kDa branched PEI were purchased from Aldrich. All other materials were used as received. 2-(*N*-Vinylformamido) ethyl ether (NVEE) and 2-bis[2,2′-di(*N*-vinylformamido)ethoxy]propane (BDEP) were synthesized according to the procedure previously reported.[38,39]

Synthesis of polyvinylamine nanogel

Polyvinylamine nanogels with controlled charge densities, derived from polyvinylformamide, were synthesized using inverse emulsion polymerization. Briefly, in the aqueous phase, vinylformamide (350 mg) was mixed with 16 mg of low temperature free radical initiator, VAZO-52. Then, 50 mg of crosslinker, NVEE or BDEP, was added and mixed. Water 165 μL (or 160 μL of 10 m*M* phosphate buffer pH 8.0, when BDEP is used) was added and mixed. The water phase then was mixed with 100 mL of hexane, containing VAZO-52 (30 mg), Tween-80 (3.0 g), and Span-80 (4.1 g). The mixture was homogenized to form an inverse emulsion and purged with nitrogen gas. The polymerization reaction was carried out under nitrogen atmosphere at 50°C for 24 h. PNVF nanogels were purified by centrifugation at 15,000 rpm for 45 min, redispersed in water or 10 m*M* phosphate buffer (pH 8.0), and dialyzed (Spectrapor; MWCO 10,000 Da, Spectrum Laboratories Inc., CA). PNVF nanogels were converted to PVAm nanogels by hydrolysis using 0.1 or 0.5*N* NaOH at 80°C for various times as indicated in the results. PVAm nanogels were dialyzed to remove NaOH (Spectrapor; MWCO 2,000 Da, Spectrum Laboratories Inc., CA) and lyophilized (Labconco Corp., MO). Nondegradable and acid-labile PVAm nanogels were redispersed in water or 10 m*M* phosphate buffer (pH 8.0), respectively, to a final concentration of $\sim$ 1 mg/mL. Nanogel size and zeta potential analysis was performed by dynamic light scattering (ZetaPALS; Brookhaven Instruments Corp., NY). To track the conversion of formamide side groups to amines, aliquots of nondegradable and acid-labile nanogels were collected at preselected time points, purified by size exclusion chromatography (Sephadex G-25, Chemsavers Inc., VA) and lyophilized. Dried nanogels were dissolved in D_2O (10 mg/mL), and the ^{1}H-NMR spectra were acquired (Avance 400 MHz with an H/C/P/N QNP gradient probe, probe temperature 25°C, 64 scans).

Cell culture and plasmid DNA preparation

Carcinomic human alveolar basal epithelial cells (A549) were purchased from the American Type Culture Collection (ATCC) and maintained according to ATCC protocol, at 37°C and 5% CO_2. The 5-kilobase pair expression vector pGL3 (Promega Corp., WI), containing the luciferase gene driven by the SV40 promoter and enhancer, was used. Plasmids were grown in *Escherichia coli* cell in Lubris Bertani agar broth supplemented with 60 μg/mL ampicillin and purified using QIAGEN plasmid Giga Kits (Valencia, CA) according to the manufacturer's instructions. The DNA concentration was determined by measuring UV absorbance at 260 nm (Agilent 8453/Agilent 89,090A, Agilent Technologies Inc., CA). The DNA purity was determined by measuring absorbance (A). DNA with an A_{260}/A_{280} ratio of 1.8 or greater was used.

Formation of polyvinylamine /DNA complexes

DNA–nanogel complexes were prepared by adding 10 μL DNA solution (0.1 μg/μL) into 15 μL of nanogel suspension and mixing intensively by repeated pipetting and vortexing. Next, 15 μL of water was added, mixed, and incubated at room temperature for 30 min for complex formation.

Gel electrophoresis study

Complexes were prepared by adding 10 μL DNA solution (0.1 μg/μL) into 15 μL of nanogel suspension as before after which 4 μL of Tris-acetate-EDTA (TAE) buffer (Promega Corp., WI) and 4 μL of SYBR Green I (Invitrogen, CA) was added into the mixture. The mixture was incubated at room temperature for 30 min, and 7 μL of DNA loading buffer (Takara Bio Inc., Japan) was added. Then, 6 μL of the mixture was loaded on to a 1% agarose gel (Fisher, NJ), and electrophoresed (110 V, 30 min). A 1 kb DNA ladder (Promega Corp., WI) was used as a marker. DNA bands were visualized and photographed with an Alpha Imager (Alpha Innotech Corp., CA).

In vitro transfection assay

Cells were cultured in F-12 K medium (Mediatech Inc., VA) supplemented according to ATCC protocol and seeded in 96-well plates at 8,000 cells per well for 24 h before transfection. Right before transfection, the growth medium was removed, cells were washed with 100 μL of phosphate buffer saline (PBS) (MP Biomedicals, LLC, OH), and 100 μL of complexes (500 ng plasmid per well) in serum-free medium was added to each well. Transfection medium was replaced with growth medium 5 h post-transfection. Luciferase expression was measured 48 and 96 h later using a luciferase assay (Promega Corp., WI). Luciferase activity was quantified in relative light units using a microplate reader (SpectraMax M5; Molecular Devices Corp., CA) and normalized by total cell protein which was determined using a bicinchoninic acid assay (Thermo scientific, IL). Experiments were conducted in triplicate.

Particle size and zeta-potential measurements

Nondegradable and acid-labile PVAm nanogels were redispersed in water or 10 m*M* phosphate buffer (pH 8.0), respectively, to a final concentration of $\sim$ 1 mg/mL. Nanogel size and zeta potential analysis was performed by dynamic light scattering (ZetaPALS; Brookhaven Instruments Corp., NY).

The size and stability of complexes was tested in serum free and growth medium. Complex size was determined using a dynamic light scattering system (BT 9000AT; Brookhaven Instruments Corp., Holtsville, NY). Complexes were then diluted with an equal volume of each medium and incubated at 37°C for 4 h. The size of complexes in media over time was determined in the same fashion.

Cell viability assay

Cytotoxicity was characterized using a CellTiter 96 AQueous Cell Proliferation assay kit (Promega Corp., WI). A549 cells were seeded in 96-well plates at an initial density of 8,000 cells per well in 100 μL of growth medium for 24 h. The growth medium was replaced with fresh, serum-free medium containing nanogel samples. Cells were incubated with nanogels for 24 h, and the medium was replaced with complete growth medium. Then, 20 μL of MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]) and PMS (phenazine methosulfate) were added to each well, and the samples were incubated for 2 h. The absorbance was read at 490 nm, relative to blank wells prepared without cells, using a microplate reader (SpectraMax M5; Molecular Devices Corp., CA). Cell viability was expressed as the percentage of absorbance relative to the control (cells not exposed to the nanogels). Experiments were performed in triplicate.

RESULTS

Structure–transfection relationships of polycationic gene delivery vectors have received significant attention because of the massive demand to rationalize efficient vector structure.[16,17,23] Charge density

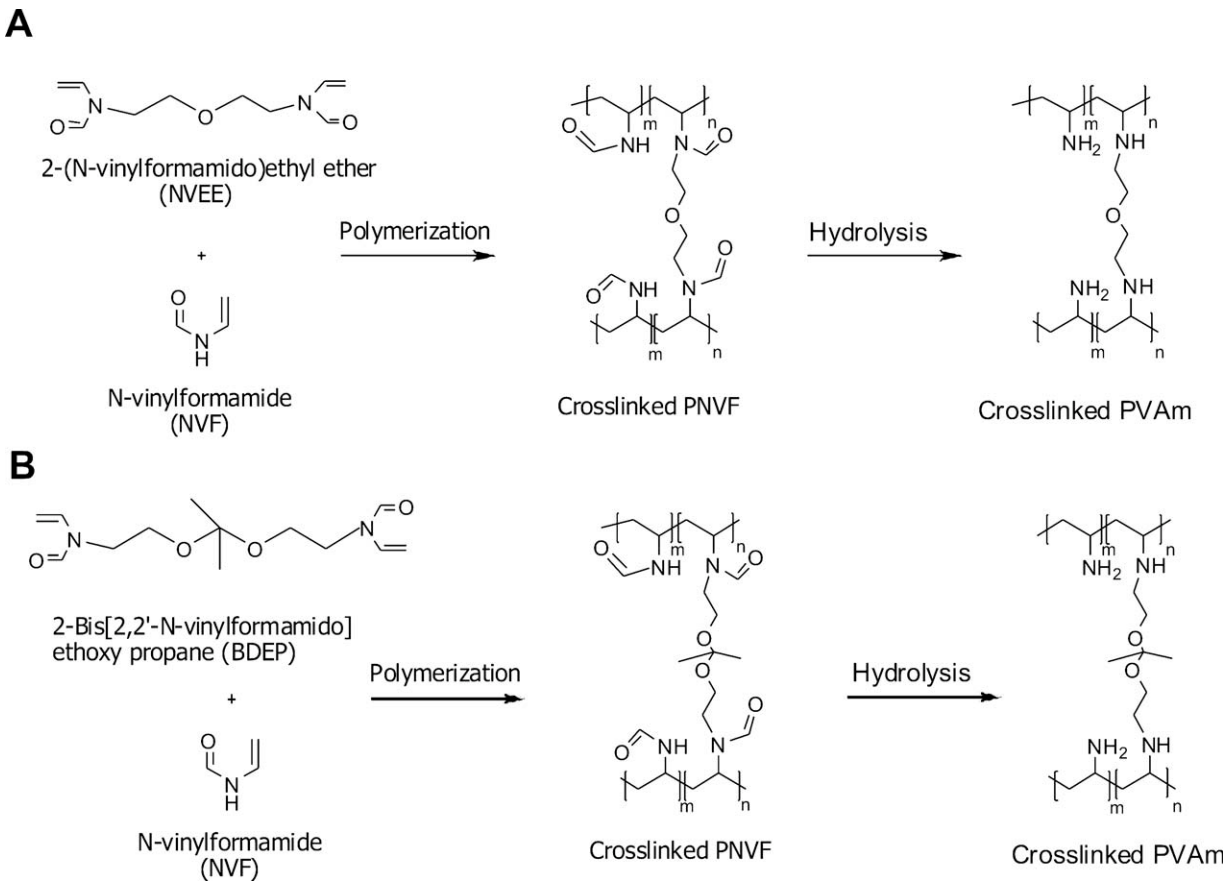

Figure 1 Reaction scheme for (A) nondegradable and (B) acid-labile PVAm nanogel synthesis.

and degradability are well known effectors of transfection efficiency and cytotoxicity. PVAm nanogels represent an attractive model for systematically studying DNA delivery. An important feature is that PVAm nanogels can be hydrolyzed from PNVF nanogels to yield different charge densities. This allows one to examine transfection efficiency of vectors as a function of particle charge, which also plays a primary role in cytotoxicity. The effect of degradability on transfection efficiency may also be assessed by crosslinking PVAm nanogels with nondegradable or acid-labile crosslinkers. Two sets of PVAm nanogels, nondegradable and acid-labile, with various charge densities were examined to correlate these chemical properties to transfection efficiency and cytotoxicity.

PVAm nanogel synthesis

Nondegradable and acid-labile PNVF nanogels were synthesized using inverse emulsion polymerization under nitrogen atmosphere at 50°C for 24 h. The size of nanogels and the hydrolysis rate of acid-labile nanogels was controlled by the monomer: crosslinker ratio.[36,38] The monomer: crosslinker ratio 7 : 1 and high temperature (50°C) were selected based on previous experimentation.[36] These conditions yield small and stable nanogels and offer high yields at short reaction times. After purification, PNVF nanogels were converted to PVAm nanogels by hydrolysis of formamide side groups using NaOH at 80°C. Nanogels were collected at different hydrolysis times to produce PVAm nanogels bearing different charge densities. The overall synthesis schemes for nondegradable and acid-labile PVAm nanogels are shown in Figure 1.

The size, size distribution, and surface charge of PNVF and PVAm nanogels were measured by dynamic light scattering in water or 10 m*M* phosphate buffer pH 8 for nondegradable and acid-labile PVAm nanogels, respectively (Table I). Particle size and zeta potential of PVAm nanogels hydrolyzed with 0.1 and 0.5*N* NaOH at 80°C were monitored over time (Fig. 2). Zeta potential measurements signified the conversion from PNVF to PVAm nanogels. The conversion rate was controlled by the concentration of NaOH and hydrolysis time. PVAm nanogel charge and size tended to increase as a function of hydrolysis time. The increase of osmotic pressure inside the polymer network because of counter ions coordinating with an increasing number of positively charged amines

TABLE I
Representative Properties of PNVF and PVAm Nanogels

Nanogels	Type	Hydrolysis condition	Effective diameter (nm ± SD)[a]	Polydispersity	Zeta potential (mV ± SD)[a]
PNVF	Nondegradable	–	129 ± 2.7	0.09	−11 ± 1.7
PVAm a	Nondegradable	20 min, 80°C, 0.5*N* NaOH	158 ± 4.3	0.17	+8.2 ± 1.7
PVAm b	Nondegradable	3 h, 80°C, 0.5*N* NaOH	116 ± 6.9	0.01	+18 ± 3.7
PNVF	Acid-labile	–	177 ± 11	0.23	−0.45 ± 2.8
PVAm c	Acid-labile	1 h, 80°C, 0.1*N* NaOH	308 ± 40	0.32	+3.5 ± 0.8
PVAm d	Acid-labile	6 h, 80°C, 0.1*N* NaOH	260 ± 48	0.43	+10 ± 0.4

[a] SD, Standard deviation.

likely accounted for the marked size increase.[40] Of course, the protonation of amine groups as pH decreases further propagates the observed size increase.[41] Gradual charge increase was obtained during formamide hydrolysis using 0.1*N* NaOH for both nondegradable and acid-labile PVAm. In comparison, 0.5*N* NaOH yielded a more rapid charge increase resembling a second order reaction, where the rate of reaction tended to strongly depend on the concentration of reactants. Nondegradable nanogels appeared to be less prone to size change than acid-labile nanogels. The larger observed size increase may be due to some cleavage of the acid-labile crosslinker during the purification.[36] As previously reported, the half-lives of acid-labile nanogels were 10, 90 min and ~ 57 h in solution pH 4.7, 5.8, and 7.4, respectively. Even though the pH of the dialysis medium was controlled (pH ~ 8) in the purification step, acid-labile crosslinkers still hydrolyzed slowly which might have led to the larger observed size of degradable nanoparitcles. The conversion of formamides to amines was confirmed by ^{1}H-NMR for both nondegradable and acid-labile PVAm nanogels (Fig. 3). The decrease in intensity of the formamide chemical shift (—C**H**O,

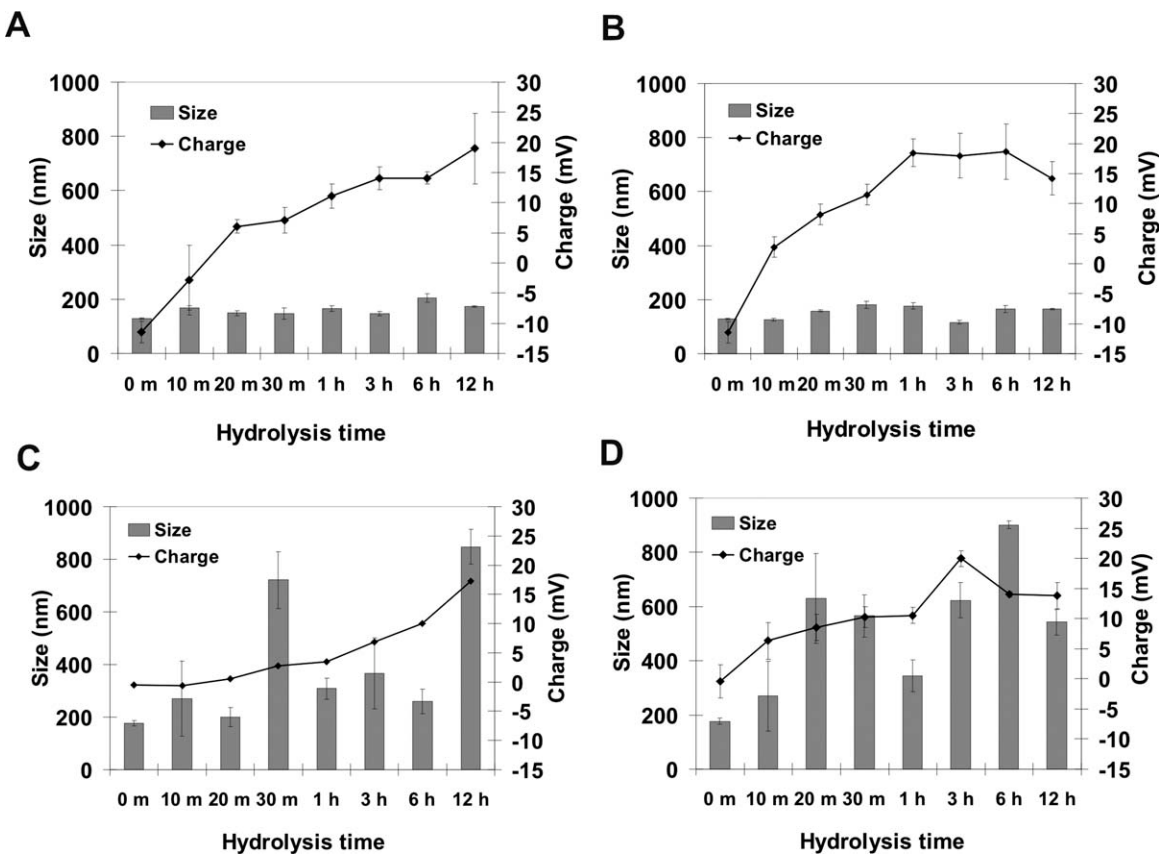

Figure 2 Conversion rate of nondegradable PVAm nanogels with (A) 0.1*N* NaOH (B) 0.5*N* NaOH and degradable PVAm nanogels with (C) 0.1*N* NaOH (D) 0.5*N* NaOH at 80°C over time.

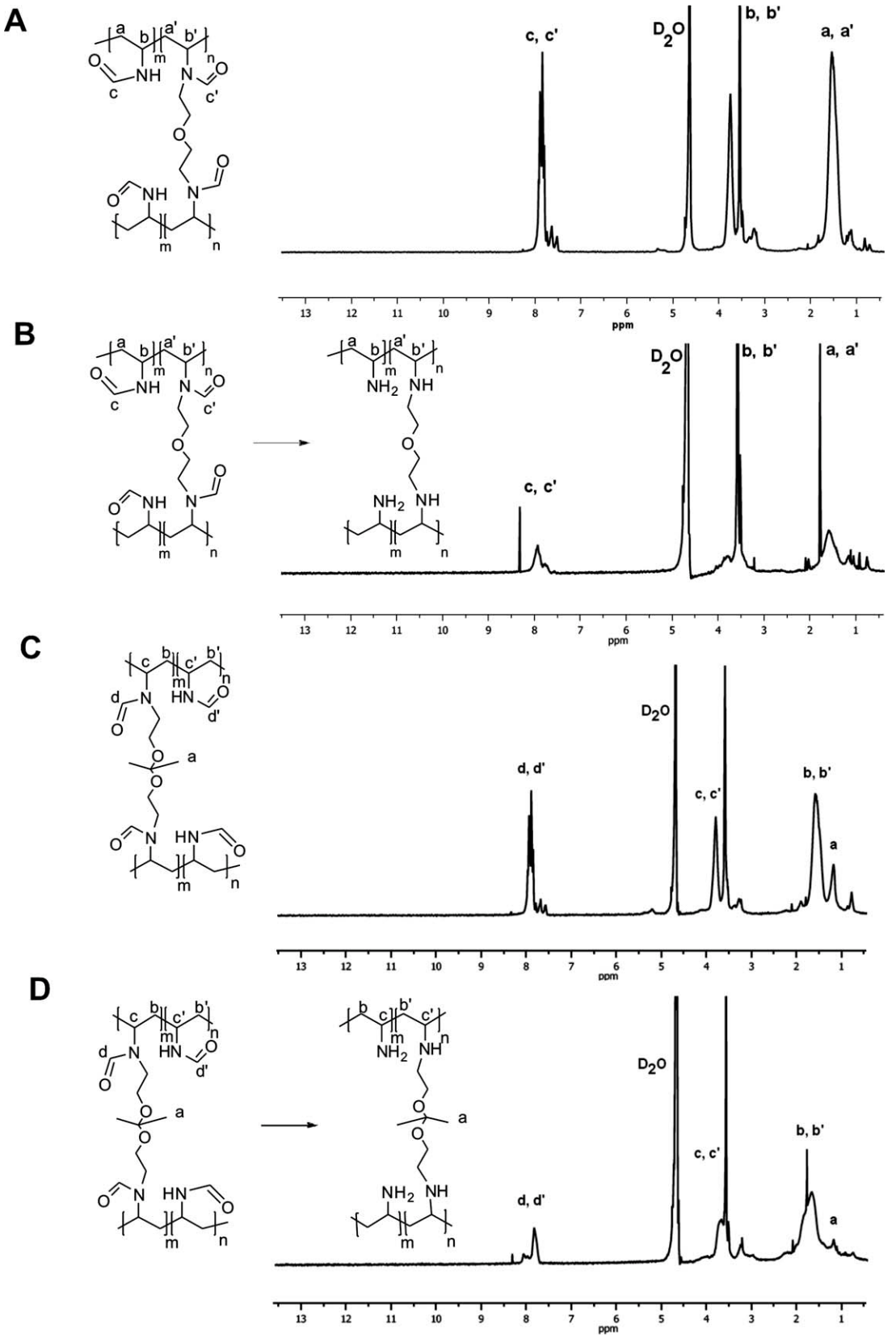

Figure 3 ^{1}H-NMR spectra of (A) nondegradable PNVF and (B) nondegradable PVAm nanogels after hydrolysis with 0.1*N* NaOH at 80°C for 6 h and (C) acid-labile PNVF and (D) acid-labile PVAm nanogels after hydrolysis with 0.5*N* NaOH at 80°C for 3 h.

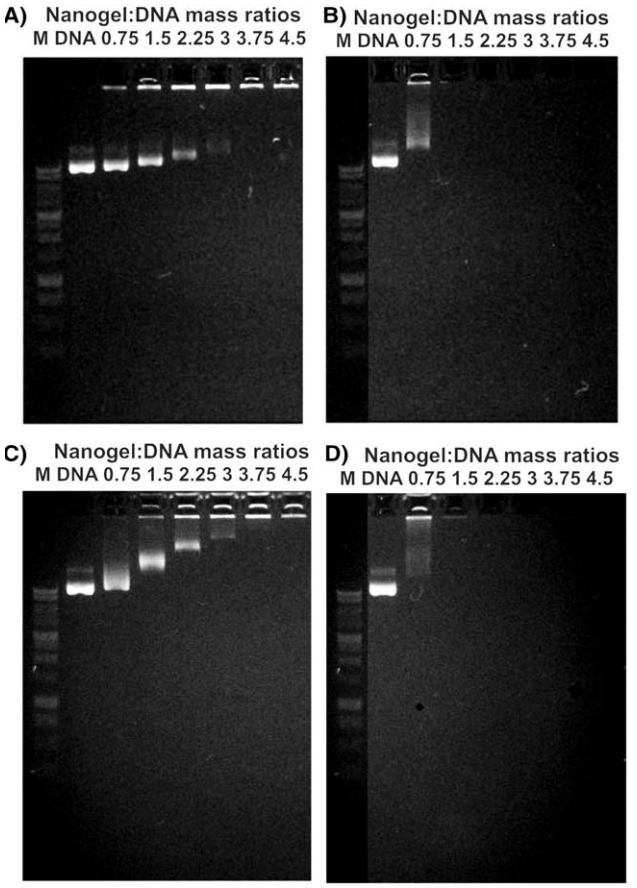

Figure 4 Gel electrophoresis of nondegradable PVAm/DNA complexes made from (A) 158 nm, +8.2 mV, (B) 116 nm, +18 mV, and acid-labile PVAm/DNA complexes made from (C) 308 nm, +3.5 mV (D) 228 nm, +11 mV.

~ 8 ppm) was observed in both nondegradable and acid-labile nanogel formulations.

Complex formation

The electrostatic interaction between DNA and different nanogel formulations as a function of nanogel: DNA mass ratio was examined by gel electrophoresis. In general, both nondegradable and acid-labile PVAm with low and high surface charge formed complexes efficiently with plasmid DNA at nanogel: DNA ratios greater than 3. The higher nanogel: DNA ratios bound the DNA more completely, and, eventually, eliminated the electrophoretic mobility of the DNA (Fig. 4). Nondegradable and acid-labile nanogels bearing the more highly charged surface showed improved DNA condensation when compared with nanogels bearing lower charge as expected.

Dynamic light scattering was used to determine the size of the complexes. PVAm complexes exhibited sizes from 180 to 560 nm in diameter (Fig. 5). For polymer: DNA complexes (e.g., PEI: DNA), the complex sizes have been reported to be a function of the DNA: polymer ratio.[42,43] Data for PVAm complexes suggested that the size was related to the original size of the nanogels, thus suggesting that the DNA was adsorbing to the nanogel surface and not causing significant flocculation. The stability of the complexes in serum-free and growth medium was also monitored over time (Fig. 6). The complexes with high nanogel: DNA ratios, bearing high zeta potential, exhibited an increase in particle size in growth medium. The ionic interaction between serum protein and excess positive charge was a likely cause of the observed size increase and agglomeration.[44] Interestingly, PEI complexes and PVAm complexes with low nanogel: DNA ratios retained small sizes in growth medium for longer periods of time.

Cytotoxicity test

The *in vitro* cytotoxicity of nondegradable and acid-labile PVAm nanogels each exhibiting low or high charge was measured using an MTS assay. Results indicated that cytotoxicity increased with increasing accessible charge (Fig. 7). The IC_{50} of PEI was extremely low (~ 5 μg/mL). The IC_{50} of nondegradable PVAm nanogels bearing charge +8.2 and +18 mV were ~ 400 and 10 μg/mL, whereas acid-labile PVAm nanogels bearing charge +3.5 and +10 mV were ~ 300 and 10 μg/mL, respectively. Acid-labile nanogels bearing lower charges (e.g., +10 mV)

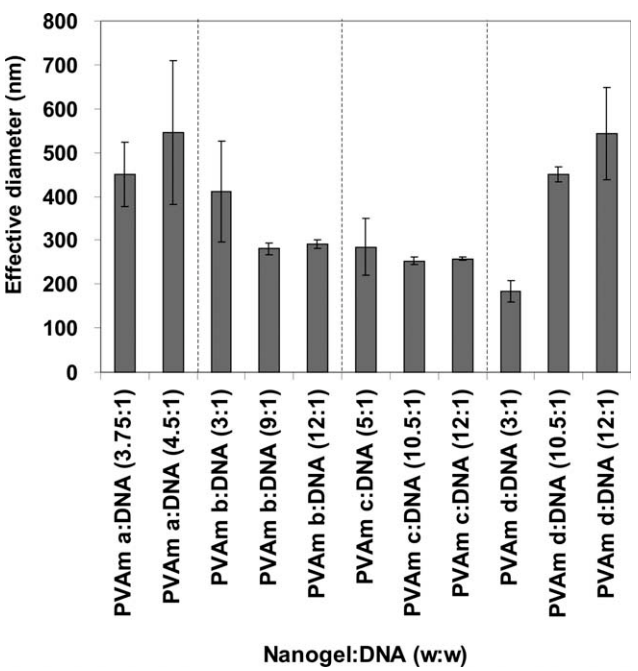

Figure 5 Effective diameter of nondegradable (a, b) and acid-labile (c, d) PVAm/DNA complexes at different polymer-to-DNA ratios ($n = 3 \pm$ SD).

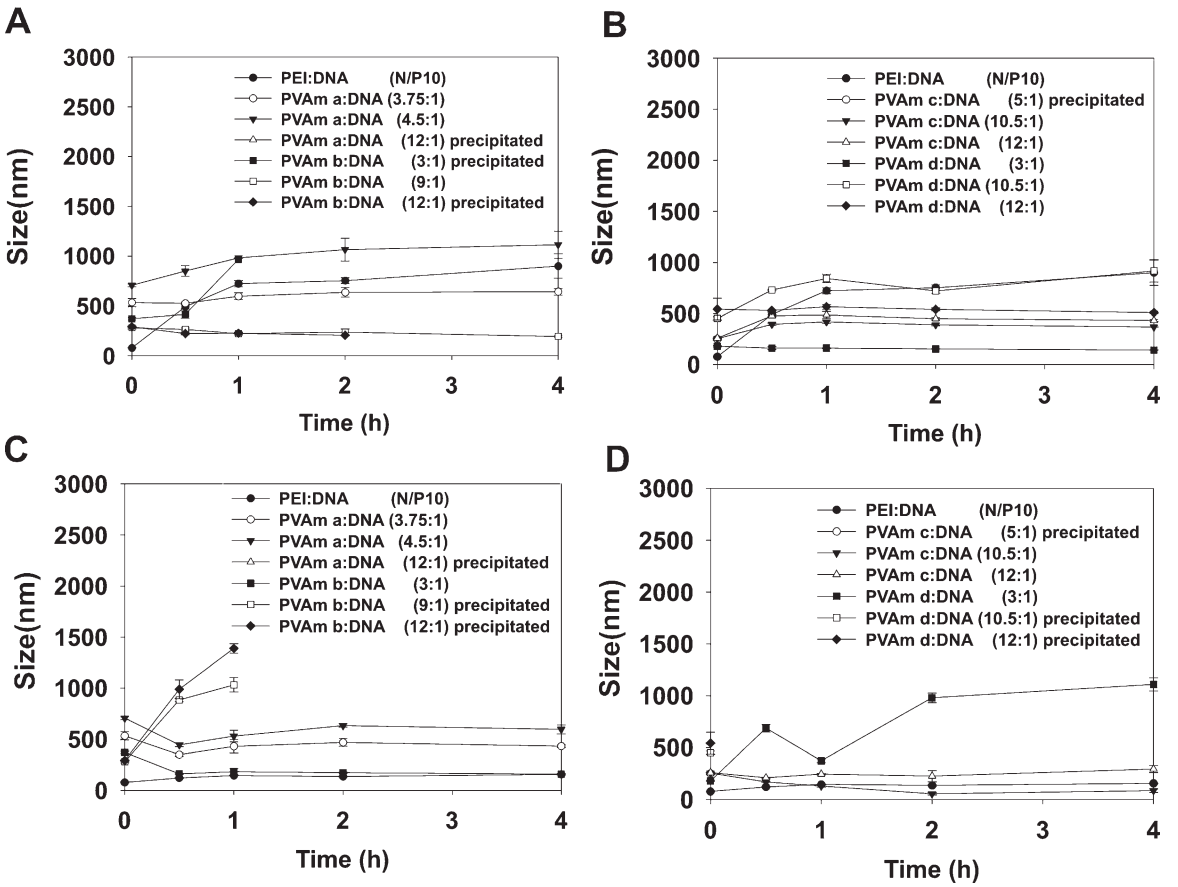

Figure 6 Stability of (A) nondegradable and (B) acid-labile PVAm/DNA complexes at different nanogel-to-DNA ratios in serum-free medium and (C) nondegradable and (D) acid-labile PVAm/DNA complexes at different nanogel-to-DNA ratios in growth medium over time.

had almost the same level of cytotoxicity when compared with nondegradable nanogels bearing higher charges (e.g., +18 mV). Charge density resulting from the number and the arrangement of cationic residues is a key factor for cytotoxicity.[5] Nondegradable nanogels have a globular shape, whereas acid-labile nanogels gradually degrade to linear oligomers as dictated by the pH of the microenvironment over time. As reported previously, the half-lives of acid-labile nanogels were 10, 90 min and ~ 57 h in solution pH 4.7, 5.8, and 7.4, respectively, and the size of oligomers after degradation was ~ 14,000 Da.[36] Therefore, acid-labile nanogels were expected to be degraded rapidly once they were endocytosed. Upon degradation, PVAm oligomers are linear and have more accessible cationic charges than nondegradable nanogels. Therefore, highly flexible cationic oligomers (degradation products) could interact and crosslink anionic microtubules or motor proteins and perhaps lead to the observed cytotoxicity.[45] In addition, flexible molecules have potentially improved interactions with membranes than rigid molecules.[46] Our results also supported this hypothesis; globular nondegradable nanogels bearing almost the same charge (+8.2 mV, IC_{50} ~ 400 μg/mL) had lower cytotoxicity when compared with acid-labile nanogels (+10 mV, IC_{50} ~ 10 μg/mL). The results suggested that accessible cationic charge density dictated the relative cytotoxicity of nanogels in this system.

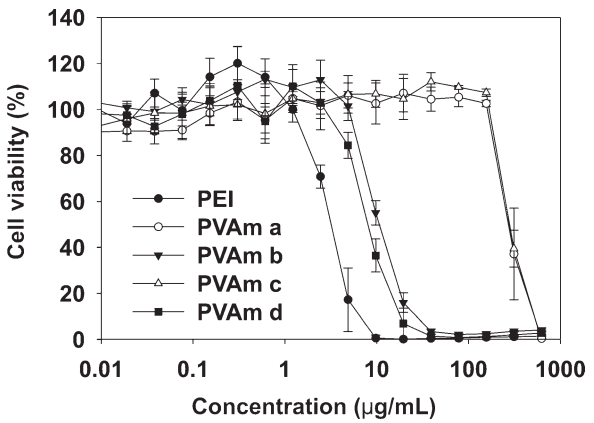

Figure 7 Cytotoxicity of PEI, nondegradable (a, b) and acid-labile (c, d) PVAm nanogels in A549 cells.

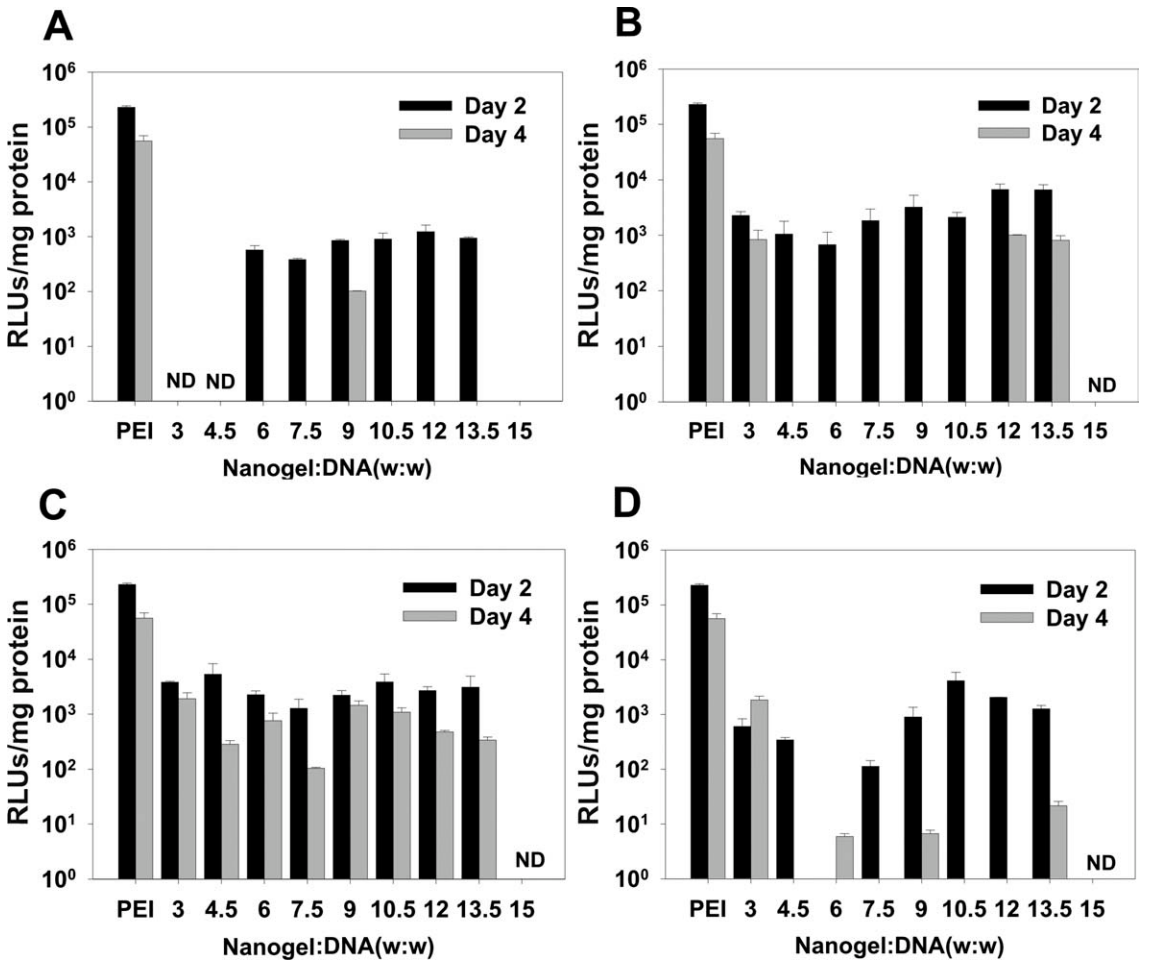

Figure 8 Transfection efficiency of nondegradable PVAm complexes made from (A) PVAm, 158 nm, +8 mV (B) PVAm, 116 nm, +18 mV and acid-labile complexes made from (C) PVAm, 308 nm, +3.5 mV (D) PVAm, 228 nm, +11 mV at different nanogel-to-DNA ratios 2 and 4 day post-transfection in A549 cells.

Transfection study

In vitro transfection efficiency in A549 cells was systematically studied as a function of the nanogel: DNA ratio. Overall, the transfection efficiencies of nondegradable and acid-labile PVAm nanogels were moderate when compared with PEI (Fig. 8). However, this system; nanogels with similar structure, but different charge densities and degradability, provided a tunable model to track the relationship between these properties and transfection efficiency. Most PVAm nanogels exhibited the highest transfection efficiency after 48 h of incubation. The nanogels bearing higher surface charge typically yielded higher transfection efficiencies at 48 h. This is presumably a result of the improved electrostatic interaction with cell surfaces.[47] Acid-labile complexes made from PVAm bearing a lower surface charge exhibited higher transfection efficiencies on Day 4 compared to all other formulations. Acid-labile PVAm nanogels bearing high surface charge mediated markly lower gene expression. The observed reduction in gene expression for highly charged particles may have resulted from ineffective dissociation of DNA and relatively high cytotoxicity. Negligible transfection efficiency was observed at Day 4 for all complexes made from nondegradable nanogels. Other rate limiting processes such as nuclear localization or transcription may also be affected by the relative ability of these complexes to release DNA.[48–51]

Size of the complexes is known to be an important parameter effecting the endocytic pathway. It is reported that latex beads $\leq$200 nm (50, 100 and 80% of 200 nm) are exclusively taken up by clathrin-mediated endocytosis. With increasing size, the caveolae-mediated pathway becomes apparent. Larger beads (500 nm) are internalized predominantly via caveolae-mediated endocytosis, a nondegradative pathway reported to result in productive transfection.[52–54] Even though nondegradable nanogels were smaller than 200 nm, both nondegradable and acid-labile nanogels were 200–500 nm in size when complexed with DNA, thus suggesting both endocytosis pathways.

Relatively high transfection efficiencies were observed at low and high nanogel: DNA ratios compared to medium ratios. It may be that moderate nanogel: DNA ratios (e.g., 6 : 1 and 7.5 : 1) formed tight complexes, impeding the dissociation of DNA for transcription. At low polymer: DNA ratios (e.g., 3 : 1 and 4.5 : 1), it is probable that looser complexes were formed allowing DNA to dissociate from the complexes more easily. At high nanogel: DNA ratios (e.g., 10.5 : 1), it may be that the increased positive charge improved recruitment of the complexes to the negatively charged cell surface resulting in higher transfection efficiencies despite the tight DNA binding. Similar results have been reported for cationic lipid/ DNA complexes, and the results have been explained in terms of the interactions between complexes and cells.[29,55]

Transfection efficiency of nondegradable complexes seemed to be directly related to the toxicity of nanogels, especially at high nanogel: DNA ratios. Nondegradable PVAm nanogels having higher cytotoxicity yielded higher transfection efficiencies. Conversely, there was no apparent correlation between cytotoxicity and transfection efficiency for acid-labile complexes. Acid-labile nanogels bearing low charge exhibited higher transfection efficiency than acid-labile nanogels bearing high charge. In previous reports, particles bearing degradable bonds tended to release DNA rapidly regardless of the charge density.[6] The ability to release DNA and overall low cytotoxicity were likely key factors leading to sustained gene expression levels at Day 4.

DISCUSSION

Gene delivery vectors should have high transfection efficiency and low toxicity to facilitate translation into the clinic. These properties have rarely been found in one vector. Polymers or lipids bearing high charge have improved DNA packaging, enhanced cell surface interaction that facilitates uptake by endocytosis, and improved endolysosomal escape.[6,56] All of these features lead to high gene expression. However, it is well known that highly charged materials often have high cytotoxicity, aggregate readily in bodily fluids, and may induce an inflammatory response. For example, systemic administration of lipoplexes activates the innate immune system rapidly and induces proinflammatory cytokines.[57]

Several approaches have been reported to produce less toxic vectors. Generally, lower molecular weight polymers are preferred because of their corresponding reduction in toxicity. Many chemical modifications have been assessed to produce highly efficient and safe vectors. Forrest et al. acetylated primary and secondary amines of branched, 25 kDa PEI to secondary and tertiary amides, respectively.[16] The 43% acetylated PEI showed a 26-fold higher transfection efficiency when compared with unmodified PEI. The decrease in the number of protonable nitrogens would be expected to decrease endolysosomal escape via the proton-sponge mechanism. However, it was suspected that the improved performance was compensated by more DNA release from looser complexes and an overall reduction in cytotoxicity.

Polymer degradability is another important factor for translating gene delivery vehicles. Crosslinking strategies have also been utilized for producing a number of degradable polymers. Taking advantage of pH differences between physiological pH ($\sim$ 7) and lysosomal pH ($\sim$ 4–5), pH-responsive crosslinkers have been synthesized and incorporated into polymer backbones yielding a variety of degradable vectors.[22,24,25] Using naturally degradable polymers offers an alternative way to reduce toxicity. Cationic polysaccharides are water-soluble and biodegradable. Two main groups that have been investigated for gene delivery are chitosan derivatives and cationic dextrans (e.g., dextran-spermine). These materials have generally shown good DNA condensing ability and relatively low cytotoxicity when compared with traditional vectors. In one study, chitosan and trimethylated chitosan showed appreciable transfection in two cell lines when compared with PEI.[20] In addition, dextran-spermine polyplexes showed more mild tissue and systemic toxicity in mice in comparison to PEI.[58]

In this study, hydrophilic PVAm nanogels bearing different cationic charge exhibited different gene expression levels. For nondegradable nanogels, high charge densities yielded higher gene expression when compared with nanogels with low charge densities as expected. The observed gene expression may result from properties of these nanogels, for example, charge density which effects complex size and stability as well as cell binding. Highly charged nondegradable nanogels were able to condense DNA into smaller complexes when compared with nondegradable nanogels with lower charge. The small size and complex stability under physiological conditions are important factors for efficient gene delivery.[59] The overall net positive surface charge also enhances interaction with cellular membrane which promotes endocytosis. For example, highly cationic polymers were previously described to possess high transfection efficiency.[5,60,61] Nagasaki et al. reported cationic derivatives of Schizophyllan, a natural polysaccharide, showing superior transfection efficiency with an increased degree of amination.[62] These materials also showed higher cytotoxicity as expected.

Interestingly, acid-labile nanogels with low charge densities yielded extended gene expression while maintaining lower cytotoxicity. The primary effector of the observed transfection efficiencies for complexes made from low charge nanogels may be the

small complex size. A secondary effector for sustained gene expression might be the low toxicity of the low charge density nanogels. Even though higher charge polymers had higher efficiency at the same dose, they were more toxic and had lower efficiency with increased dose (presumably due to increased cytotoxicity).[60] Our results are also in accordance with findings by Miyata et al., where poly (ethylene glycol)–poly(L-lysine) block copolymer with reduced charge density by thiolation was found to have high transfection efficiency when compared with high charge density copolymers with the same degree of thiolation.[63]

The data reiterated that degradability and cationic charge are important properties of gene vectors. Balancing the two features, low charge and degradability can give rise to improved transfection efficiency without compromising cytotoxicity. Finally, the data reiterate the need to study gene expression for longer periods of time, when compared with the typical 1–2 day transfection and 1 day cytotoxicity studies that are common today.

Gene therapy continues to hold promise to cure a number of diseases and to improve disease management. However, clinical applications are not yet realized as there are several confounding barriers. Overcoming one barrier (e.g., cytotoxicity) has typically amplified another barrier (e.g., transfection efficiency). Understanding polymer structural effects on transfection will help us to optimize nonviral vector formulations. It is probable that carefully balancing transfection efficiency and toxicity is a key that should be further explored to develop suitable gene vectors.

CONCLUSIONS

PVAm nanogels having similar chemical structure and bearing discrete combinations of charge densities and degradability offered a means to assess structure–transfection relationships for this material. PNVF nanogels were synthesized and crosslinked with nondegradable or acid-labile crosslinkers via an inverse emulsion polymerization reaction. PNVF nanogels were then hydrolyzed to PVAm nanogels exhibiting different charge densities. The cytotoxicity of PVAm nanogels increased with increasing charge accessibility. Nondegradable nanogels yielded high initial gene expression. Interestingly, acid-labile nanogels bearing lower charge exhibited more sustained gene expression offering the highest cumulative gene expression of any nanogel formula. These observations suggested that transfection efficiency may be improved by balancing low charge and degradability without compromising cytotoxicity.

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