Charge-Shifting Polycations Based on N,N-(dimethylamino)ethyl Acrylate for Improving Cytocompatibility During DNA Delivery

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ABSTRACT: Synthetic polycations are studied extensively as DNA delivery agents because of their ease of production, good chemical stability, and low cost relative to viral vectors. This report describes the synthesis of charge-shifting polycations based on N,N-(dimethylamino)ethyl acrylate (DMAEA) and 3-aminopropylmethacrylamide (APM), called PAD copolymers, and their use for in vitro DNA delivery into HeLa cells. PAD copolymers of varying compositions were prepared by RAFT polymerization to yield polymers of controlled molecular weights with low dispersities. Model hydrolysis studies were carried out to assess the rate of charge-shifting of the polycations by loss of the cationic dimethylamino ethanol side chains. They showed reduction in the net cationic charge by about 10−50% depending on composition after 2 days at pH 7, forming polyampholytes comprising permanent cationic groups, residual DMAEA, as well as anionic acrylic acid groups. HeLa cells exposed for 4 h to PAD copolymers with the greatest charge-shifting ability showed comparable or higher viability at high concentrations, relative to the noncharge-shifting polycations PAM and polyethyleneimine (PEI) 2 days post-exposure. Cell uptake efficiency of PAD/60bp-Cy3 DNA polyplexes at 2.5:1 N/P ratio was very high (>95%) for all compositions, exceeding the uptake efficiency of PEI polyplexes of equivalent composition. These results suggest that these PAD copolymers, and in particular PAD80 containing 80 mol% DMAEA, have suitable rates of charge-shifting hydrolysis for DNA delivery, as PAD80 showed reduced cytotoxicity at high concentrations, while still retaining high uptake efficiencies. In addition, the polyampholytes formed during DMAEA hydrolysis in PAD copolymers can offer enhanced long-term cytocompatibility.

INTRODUCTION

Polycations are used extensively in DNA delivery as nonviral vectors because of their ease of production, stability, and low cost relative to lipid-based vectors.1−8 Although recent research has shown much promise in their ability to deliver nucleic acid payloads, there has been growing interest to develop polycations with further increased transfection efficiency and lower toxicity. Many studies involve probing the effect of various derivatives,9−12 polymer architecture,13−15 and polyplex morphology.16 Charge-shifting polycations have emerged as a class of polymers attracting significant interest as transfection agents.19−31 These polycations have high cationic charge densities but undergo a charge-shifting process, making the polymer less cationic. The initial high cationic charge density enables efficient binding and protection of anionic nucleic acids from nuclease, forming polyplexes. Following cellular uptake, the polyplexes can dissociate because of the charge shift of the polycation, releasing the genetic payload for intracellular delivery. Both charge-shifting and reducible polycations based on N,N-(dimethylamino)ethyl (meth)acrylate (DMAE(M)A) have shown promising DNA release capabilities and improved transfection efficiency.32−35 Further, the cytotoxicity of charge-shifting polycations was lower than that of standard synthetic polycations. This was attributed to the degradation of polymeric cationic charge and the release of byproducts such as dimethylaminoethanol (DMAE) that are nontoxic even at high concentrations.32,36 The cytotoxicity of polycations has been attributed to their interactions with critical anionic components of the cell including the phospholipid cell membrane and RNA/DNA, as well as their ability to trigger pro-apoptotic and pro-inflammatory signaling.33,34 Thus, charge-shifting polycations may be desirable alternatives to standard polycations as they can mitigate the accumulation of cytotoxic cationic charges.32,35,36 Recent findings have demonstrated that polycation hydrolysis showed similar antifouling properties to zwitterionic polybetaines35 because of their surface hydration35,36 and have shown promising properties for transfection as the mixed surface charges are reminiscent of certain viruses.37 Thus, charge-shifting polycations that reduce their cationic charge to polycation can be more cytocompat-

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Supporting Information
polycations, whereby polyplexes taken up by cells and of the main theories rests on the polyplexes or their DNA payload is still a topic of debate. One reasonably understood, the mechanism of endosomal escape of through defects in the endosomal membrane caused by interactions with the polycation.42,43 The lack of understanding protop-n-sponge effect of polycations, whereby polyplexes taken up by cells and contained within endosomes are acidified by fusion of endosomes with lysosomes. While the proton-sponge effect is commonly used to explain the mechanism of release in many polycation-mediated transfection systems, this hypothesis has been questioned42–44 and there is research indicating that endosomal escape of DNA may instead involve diffusion through defects in the endosomal membrane caused by interactions with the polycation.42,43 The lack of understanding of endosomal escape, including the role of the polycation, further demonstrates a need for new charge-shifting polycations to probe these processes; however, these studies will not be included in the current report. The work of this report will focus on the development and study of polycations based on DMAEA to explore charge-shifting polycations; however, these studies will not be included in the current report. The work of this report will focus on the development and study of polycations based on DMAEA and will probe their potential in improving compatibility with cells while maintaining high uptake efficiencies. As for DMAEA homopolymers previously reported,22,23 these should degrade into nontoxic byproducts while retaining high uptake efficiencies. The effect of charge-shifting hydrolysis on the cytotoxicity in polycation-mediated in vitro DNA delivery will be the focus of this study. In this report, we explore charge-shifting polycations based on DMAEA hydrolysis as potential DNA delivery agents with HeLa cells as a model cell line for which toxicity and delivery studies are commonly reported. In particular, poly(3-aminopropylmethacrylamide-co-N,N-(dimethylamino)ethyl acrylate) (PAD) copolymers with different mol % of DMAEA were used to study the effect of changing the charge-shifting potential of a series of polycations with constant initial charge density, on cytotoxicity and cellular uptake efficiency (Scheme 1).

**EXPERIMENTAL SECTION**

**Materials.** DMAEA, (98%), 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid (CTP, ≥98%), 4,4-azobis(4-cyanovaleric acid) (V-501, ≥97%), deuterium chloride (35% in D2O, 99% D), and WST-1 (Roche) were purchased from Sigma-Aldrich and used as received unless otherwise stated. Polyethyleneimine (PEI, branched, 25 kDa) was purchased from Sigma-Aldrich and used as received. Polyethyleneimine hydrochloride (PEI hydrochloride) was purchased from Polysciences and used as received. Dulbecco’s modified Eagle medium (DMEM) (4.5 g/L glucose, no sodium pyruvate), Opti-MEM (HEPES, l-glutamine), fetal calf serum (FCS) (South American origin), and 1× Dulbecco’s PBS (DPBS) were purchased from Thermofisher Scientific. PE-Annexin V Apoptosis Detection Kit I (BD Pharmingen) was purchased from BD Biosciences.

**Synthesis of PAD Copolymers.** PAD copolymers of 80, 49, 23, and 0 (PAPM) relative mol % of DMAEA were prepared as described previously. Briefly, reversible addition-fragmentation chain-transfer (RAFT) polymerization of APM and DMAEA was conducted using CTP as the RAFT agent and V-501 as the initiator, targeting a molecular weight of 30,000 g/mol. In 20 mL screw cap vials, APM and DMAEA feeds of 15:85, 45:55, 75:25, and 100:0 mol ratios (1.8 g total monomer loading) were sequentially dissolved in 6 mL of a 2:1 water/1,4-dioxane solvent mixture with 1.1 mol equiv of HCl using a 6 M stock solution added relative to DMAEA. The dissolution of the monomers was carried out in an ice-water bath to remove the heat of neutralization of the monomers and to minimize premature hydrolysis of DMAEA. The reaction mixture was adjusted to pH 3–4 by addition of further HCl prior to the addition of CTP and V-501. Small magnetic stirring bars were added, and the vials were capped with septa. The reaction mixture was purged with N2 gas for 45 min at room temperature with stirring and then placed in an 80 °C oil bath. The polymerization proceeded until a targeted total.

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"PAD copolymers, initially highly cationic, complex anionic DNA to form polyplexes. Charge-shifting of PAD copolymers may facilitate intracellular DNA release because of weakening electrostatic interactions. The reduction in net cationic charge may also reduce cytotoxicity."
monomer conversion of approximately 80% was reached as confirmed by $^1$H NMR, which required 1.5–2 h of heating depending on composition. The polymer solutions were dialyzed for 4–5 days against pH 3–4 distilled water using cellulose acetate tubing with a molecular weight cut-off of 3500 g/mol. Purified polymer solutions were freeze-dried, resulting in the polymers as their hydrochloride salt. Typical isolated yields were approximately 70–80%. $^1$H NMR spectra of PAD$_{80}$, PAD$_{49}$, PAD$_{23}$, and PAMP in D$_2$O are shown in Figures S1, S2, S3, and S4, respectively.

NMR Spectroscopy. $^1$H NMR spectra were recorded on a Bruker AV600 or AV500 in D$_2$O using the residual HDO/H$_2$O signal at 4.8 ppm as the chemical shift reference.

Gel Permeation Chromatography. Gel permeation chromatography (GPC) of PAD copolymers was conducted using a Waters GPC consisting of a 717plus Autosampler, 515 HPLC pump, 2414 refractive index detector, Ultrahydrogel (120, 250, and 500) columns (30 cm × 7.8 mm inner diameter); 6 μm particles) using poly(ethylene glycol) standards ranging from 106 Da to 881 kDa (Waters Inc.) for a 15-point, third-order polynomial calibration. The mobile phase was a 1 M acetic acid/sodium acetate buffer at pH 4.8.

Model Hydrolysis Studies. PAD copolymers were dissolved in 100 mM phosphate or acetic buffered solutions at pH 7 and 5, respectively, at a concentration of 0.5 wt %. Samples were maintained at room temperature (22 °C), and $^1$H NMR spectra were recorded at various time intervals. The pH of the PAD$_{80}$, PAD$_{49}$, PAD$_{23}$ and PAMP of the phosphate buffered solutions just after dialysis were adjusted to pH 7.11, 7.06, 7.14, and 7.15, respectively, prior to analysis. The pH of the PAD$_{80}$, PAD$_{49}$, PAD$_{23}$ and PAMP of the acetic-buffered solutions just after dialysis was adjusted to pH 5.03, 5.01, 4.99, and 5.00, respectively, prior to analysis. The pH did not drift significantly (less than 0.09 pH units) after hydrolysis. Hydrolysis percentages were calculated by comparing the integrations of the peaks at 3.9 and 4.5 ppm, according to the supplier’s (Roche) protocol with minor modifications. Absorbance was measured after 1 h incubation at 37 °C using a Tecan Infinite M1000 plate reader at 450 nm, using 690 nm as the reference wavelength. Cells were incubated with 7% (v/v) DMSO in full medium as the positive control.

Preparation of PAD–DNA Polyplexes. Polyplex solutions were prepared at 1:1, 2:1, 5:1, 10:1, and 20:1 mol ratios of polycation to DNA repeat units, resulting in ammonium cation nitrogen to phosphate anion (N/P) ratios of 0.5:1, 1:1, 2.5:1, 5:1, and 10:1, respectively. DNA solutions were prepared in Opti-MEM for a targeted final concentration of 300 nM according to the DNA strand. Polycation solutions were dissolved in DPBS and sterile-filtered through a 0.2 μm syringe filter prior to further dilutions, targeting the desired mol stoichiometry relative to DNA for equivalence mixing. For a typical transfection experiment in a 96-well plate, 50 μL of the DNA solution was added to each polycation solution (50 μL) and pipetted up and down vigorously 10 times using Greiner Bio-One filtered pipette tips. Polycation and polypeptide solutions were prepared freshly prior to all transfection experiments.

Dynamic Light Scattering. freshly prepared polypeptide solutions as-formed (100 μL) were added to ZEN0400 microcuvettes for dynamic light scattering (DLS) measurements (n = 10) at room temperature with a Malvern Zetasizer Nano ZSP using a 173° backscatter angle.

Cell Culture. HeLa cells, purchased from the American Type Culture Collection (ATCC), USA, were cultured in DMEM (4.5 g/L glucose, without sodium pyruvate) containing 10% FCS. Cells were cultured as a monolayer at 37 °C in a humidified atmosphere with 5% CO$_2$.

Transfection. HeLa cells were seeded into a tissue-cultured 96-well plate at a density of 10,000 cells per well. Plated cells were incubated for 2 days at 37 °C with 5% CO$_2$ and reached approximately 70–80% confluency prior to transfection. Cells were washed with 100 μL 1× DPBS, and 100 μL of freshly prepared polypeptide solution was added to each well. Each sample was prepared in triplicates. Following the addition of the polypeptide solution, the plate was incubated at 37 °C with 5% CO$_2$ for 4 h. The supernatant was removed, and the cells were washed with 100 μL of 1× DPBS to remove residual polypeptides, and 150 μL of FCS-containing DMEM were added to each well.

Cell Viability. Viability of transfected HeLa cells was measured after 1 and 2 days following transfection using the WST-1 cell proliferation assay. The assay was performed according to the supplier’s (Roche) protocol with minor modifications. Absorbance was measured after 1 h incubation at 37 °C using a Tecan Infinite M1000 plate reader at 450 nm, using 690 nm as the reference wavelength. Cells were incubated with 7% (v/v) DMSO in full medium as the positive control.

Cellular Uptake Efficiency. Cellular uptake of PAD–DNA (60bp-Cy3) polyplexes was measured by flow cytometry using an IntelliCyt iQue Screener. After 4 h of incubation, cells were washed with 1× DPBS to remove residual polypeptides, followed by the addition of 50 μL of 0.05% trypsin—EDTA solution and incubation at 37 °C for 3–5 min for cell detachment. FCS-containing DMEM (200 μL) was added to each well. The cell suspensions were transferred into 1.5 mL tubes and centrifuged at 1150 rpm for 5 min. The supernatant was discarded, and the cell pellet was resuspended in 100 μL of DMEM and then transferred into a 96-well v-bottom plate for analysis by flow cytometry. A minimum of 10,000 event counts was recorded for each sample. Measurements were analyzed using IntelliCyt ForeCyt software. Fluorescence of the populations was measured with the FL2 channel (585/40 nm). As cells uptake fluorescent polypeptides, there is an increase in fluorescence intensity relative to the autofluorescence of...
Untreated cells. Autofluorescence gates were the same for all samples. Cellular uptake efficiency was represented as the percentage of cells showing Cy3 fluorescence relative to the autofluorescence of untreated HeLa cells.

**Apoptosis Assay.** The Annexin V Apoptosis Detection Kit I (BD Pharmingen) was used to identify cell populations as non-, early-, and late-stage apoptotic. During early-stage apoptosis, phosphatidylserine (PS) groups translocate from the inner leaflet to the outer leaflet of the plasma cell membrane. PE-Annexin V selectively binds to PS groups exposed on the outer leaflet of the cell membrane, indicating early-apoptosis. 7-Amino-actinomycin (7-AAD) is a counter stain that binds to double-stranded DNA in late-stage apoptotic cells that have compromised cell membranes. Thus, nonapoptotic cells do not stain positive for either PE-Annexin V or 7-AAD, early-apoptotic cells stain positive for only PE-Annexin V, and late-apoptotic cells stain positive for both PE-Annexin V and 7-AAD. A preliminary test was conducted on HeLa cells 1 day after exposure to 60 bp DNA polyclones with PAD copolymers and PEI at 1:1 and 10:1 N/P ratios. Cells were seeded in a 24-well plate at a density of 150,000 cells per well. Preparation of polyclones and transfection of cells was performed as described for transfection in a 96-well plate. After 24 h, the supernatant of each well was collected into 1.5 mL microcentrifuge tubes prior to washing to retain dead cells for analysis that may be in suspension. Transfected cells were then washed with 1X DPBS and detached with 100 μL of 0.05% trypsin–EDTA after incubation at 37 °C for 3–5 min. FCS-containing DMEM (200 μL) was added to each well. The cell suspensions were transferred into microcentrifuge tubes containing the corresponding supernatant and centrifuged at 1150 rpm for 2.5 min. The cells were washed twice with ice-cold DPBS (100 μL) and resuspended in 100 μL of 1X Annexin binding buffer. The concentration of each cell suspension was determined using a Luna-II automated cell counter with Erythrosin B as the cell stain, and 100,000 cells of each sample were transferred to a 96-well v-bottom plate (total volume 50 μL per well). PE-Annexin V (5 μL) and 7-AAD (5 μL) were added to each well and incubated at room temperature for 20 min in the absence of light. Following incubation, the samples were measured by flow cytometry using an IntelliCyt iQue Screener. PE-Annexin V was detected using the FL2 channel (585/40 nm), and 7-AAD was detected using the FL3 channel (>670 nm) with 25% compensation to adjust for overlapping emission characteristics. Measurements were analyzed using IntelliCyt ForeCyt software. Gates for the apoptosis Annexin V and 7-AAD fluorescence were the same for all samples.

**Statistical Analysis.** Sample means were compared using a one-way ANOVA analysis with the Bonferroni post hoc test for multiple comparisons. Differences for which there was calculated under the assumption that the means of the normal distribution were compared using a t-test. Differences with p < 0.05 were considered statistically significant.

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**RESULTS AND DISCUSSION**

**Synthesis of PAD Copolymers.** PAD80, PAD49, PAD23, and PAD0 with 80, 49, 23, and 0 mol % DMAEA, respectively, were synthesized using methods described previously. Herein, PAD0 will be referred to as PAPM and was synthesized as a control polycation that does not undergo charge-shifting hydrolysis. It was hypothesized that polyplexes made with charge-shifting polycations would be less toxic to HeLa cells relative to standard polycations such as PAPM and PEI, the latter is currently the gold standard polycation transfection agent. In particular, branched PEI of 25 kDa from Sigma-Aldrich was fractionated to obtain a lower molecular weight fraction (4–10 kDa) of PEI, which has shown higher transfection efficiency and lower toxicity in several cancer cell lines. Therefore, the relative order of cytotoxicity was anticipated to be as follows: PAPM > PEI > PAD23 > PAD49 > PAD80. In addition, the polyampholytes with cationic/anionic charge ratios approaching 80/20, 49/51, and 23/77 formed by hydrolysis may be even more cell compatible than the corresponding polyacrylic acids formed during hydrolysis of DMAEA homopolymers described by Monteiro.

RAFT polymerization to form the PAD copolymers resulted in reasonably low dispersions (Table 1), as well as controlled molecular weights as shown by their GPC traces in Figure 1. Properties of the PAD copolymers are summarized in Table 1, and their 1H NMR spectra are shown in Figures S1–S4.

![Figure 1.](https://dx.doi.org/10.1021/acsomega.9b03734)

The calculated end group analysis for PAD80 was higher than those for the other copolymers, which may be due to degradation of the end group during polymer purification. In this case, results obtained by GPC analysis would provide more accurate molecular weight information for polymer comparisons. In this study, polymer end groups were not removed because the toxicity of the cationic polymer typically outweighs the effect of the end group. However, it should be noted that end group removal might provide marginal improvements in cytocompatibility.
monitored at pH 7 and room temperature (22 °C) by 1H NMR spectroscopy, and the results are shown in Figure 2 in terms of % hydrolysis of their respective initial DMAEA units. These conditions are approximations as in true transfection experiments; DMAEA hydrolysis will be affected by the presence of lipases, being part of a polyplex, and by the local pH ranging from pH 7 in media and cytosol to as low as pH 5 within lysosomes. In addition, an increase in temperature to 37 °C will also increase the rate of hydrolysis as shown previously for PAD copolymers and related DMAEA polymers.31,38

The hydrolysis of PAD copolymers at pH 7 is rapid initially, with all compositions showing first-order kinetics over the first day corresponding to half-life times of 2–3 days (Figure S6). After about one day, however, the rates of hydrolysis slow down for all compositions. This slower, second stage of hydrolysis was noted previously and was attributed to charge repulsion of hydroxide anions from anionic AA units formed after each DMAEA unit hydrolyses (Donnan exclusion).6,38,39,47,48

The data are replotted in terms of polymer net cationic charge % in Figure 3. The net cationic charge is defined here as the mol % cationic monomer—mol % anionic monomer or % APM + % DMAEA − % AA. For example, after approximately 2 days, all 3 polymers showed around 30% hydrolysis of their respective DMAEA groups (Figure 2), which corresponds to net cationic charges of about 55, 67, and 84% for PAD80, PAD49, and PAD23, respectively. After one week, the net cationic charges dropped to 31, 45, and 72% (Figure 3), respectively. As expected, PAPM did not show any hydrolysis even after 20 days (data not shown), as methacrylamides are much more hydrolytically stable than acrylates.

The hydrolysis of PAD copolymers should be slow enough to allow for prior complexation with DNA to form polyplexes, followed by cellular uptake by interaction of the cationic polyplex with the cellular membrane. The latter would require the polymer to remain net cationic for at least 4 h, which is in line with the results obtained in Figures 2 and 3 that show less than 5% hydrolysis. During incubation after uptake of the polyplexes, the endosomes become more acidic (pH 4–5) by uptake of HCl and fusion with lysosomes. Under these conditions, DNA should ideally remain condensed in polyplexes for protection from lysosomal nucleases.21,23,30,31

This would make DMAEA hydrolysis ideal because we recently showed that DMAEA ester hydrolysis is slowed in the range of pH 3–5.38,39 The rate of hydrolysis of PAD copolymers at pH 5 was markedly slower than at pH 7, with all compositions showing approximately 16% hydrolysis after nearly 10 days (Figure S7). PAD copolymers may thus retain enough amino groups, both from APM as well as from DMAEA groups, for pH buffering as well as for disruption of the endosomal membrane to enable to escape by out-diffusion.24 Once the polyplexes have escaped from the endosomes, the environment of the cytosol with a pH of 7 should allow for accelerated hydrolysis of DMAEA units within PAD copolymers. We recently showed that in the presence of adequate amounts of buffer, the rates of hydrolysis of DMAEA units in such copolymers increase significantly with pH.38,39 The release of DNA from the polyplexes may then occur in the cytosol, during transport to the nucleus, or in the nucleus itself. In addition to providing a potential release mechanism for DNA, charge-shifting hydrolysis may reduce the long-term cytotoxicity of the polymer by decreasing the polymeric cationic charge. In the present work, the size and cellular uptake efficiencies of DNA polyplexes incorporating a range of charge-shifting PAD copolymers were assessed, and the viability of cells after exposure to charge-shifting PAD copolymers determined using standard polycations as controls. The hydrolysis experiment serves as a model to better understand the effect of pH in the complex intracellular environment and provides useful insights toward polymer stability for further mechanistic studies.

**Size Determination of Polyplexes.** DLS was used to measure the size of polyplexes made from PAD copolymers and 60bp DNA. Polyplex solutions were prepared using the same procedure as a transfection experiment with no further dilutions. Polyplexes made with PAD copolymers and PEI all showed broad size distributions, as shown in a representative graph for the 2.5:1 N/P ratio (Figure 4). The results for the range of concentrations are summarized in Figure 5.

The average diameters of the polyplexes ranged from 400 to 1400 nm. At the 1:1 N/P ratio, the polyplexes were similar
after exposure to polyplexes, viability of HeLa cells shows an
viability than the PAD copolymers. Interestingly, two days
post-exposure to the polyplexes was measured using the WST-
1 proliferation assay (Figures 6 and 7, respectively).

Cell Viability. Cell viability of HeLa cells after 1 and 2 days
post-exposure to the polyplexes was measured using the WST-
1 proliferation assay (Figures 6 and 7, respectively).

Figure 5. Diameters of Polyplexes of PAD copolymers, PAPM, and
PEI with 60bp DNA at 1:1, 2.5:1, 5:1, and 10:1 N/P ratios,
determined using DLS. Error bars represent the standard deviation
from 2 polyplex batches with 10 measurements each.

between all of the polymers around 1100 nm. However, at
higher polymer loadings, polyplexes with DMAEA-rich PAD
copolymers (i.e., \( \text{PAD}_{80} \)) resulted in smaller particles (~700
nm) than those with DMAEA-poor PAD copolymers and
PAPM (~1400 nm). This trend may be due to the different
interactions of tertiary and primary ammonium cations of the
polymers with phosphate anions of DNA. The relatively large
size of polyplexes obtained may also be due to the overall
concentration used during complexation and can be changed
to obtain smaller particles. Although the size of polyplexes is
known to affect cellular uptake, many other variables can affect
cellular uptake that go beyond the scope of this report.49 Thus,
the effects of polyplex diameters on cell uptake and viability
were not further explored in this study.

Cell Viability. Cell viability of HeLa cells appeared to decrease with increasing polycation
concentrations for all of the polymers. There is no discernible
effect of APM/DMAEA ratios, while PEI shows higher cell
viabilities than the PAD copolymers. Interestingly, two days
after exposure to polyplexes, viability of HeLa cells shows an
effect of PAD composition: HeLa cells treated with \( \text{PAD}_{80} \)
showed higher viabilities than cells treated with \( \text{PAD}_{49} \), \( \text{PAD}_{23} \),
and PAPM. This effect was more pronounced for polymer/
DNA ratios of 5:1 and 10:1 N/P.

Similar viability results across the range of compositions of
PAD copolymers after one day are not surprising given the
relatively slow rate of charge-shifting hydrolysis. Because PAD
copolymers maintain enough cationic charge to bind DNA, it
is likely that the initial net cationic charge of the polyplexes
that cells are exposed to would be similar for all PAD
copolymers. Thus, the initial cytotoxicity for the polyplexes,
and any excess free polycation, should be similar for the
different compositions. This seems to be the challenge in the
field of synthetic polymers for transfection as the cationic
charge is necessary for the condensation and uptake of DNA;
however, the polymers are also cytotoxic at high concen-
trations due to disruption of the cellular membrane.

After two days post-exposure, viability appeared to be higher
for cells exposed to polyplexes made from PAD copolymers
with greater charge-shifting ability. In some cases, particularly
at lower N/P ratios, cells treated with polyplexes showed
viabilities greater than untreated cells (i.e., viability > 100%). It
is unclear why this was the case; however, the trends observed
between polymer composition and concentration (N/P ratios)
remain evident. The results suggest that cells are better able to
recover after exposures to these copolymers, which may be due
to the degradation of the cytotoxic cationic charge. Hydrolysis
data showed that after two days, the net charge of \( \text{PAD}_{49} \),
\( \text{PAD}_{80} \), and \( \text{PAD}_{23} \) were approximately 55, 67, and 84% net
cationic, respectively (Figure 2). Thus, it is likely that improved recovery of cell viability may be due to the charge-
shifting hydrolysis of the PAD copolymers. This trend matches
that observed by other researchers for related charge-shifting
copolymers.22,27 Given the poor cell viability observed at the
10:1 N/P ratio for all polycations, subsequent transfection
experiments were conducted at more optimal concentrations
(1:1, 2.5:1, and 5:1 N/P ratios).

Cellular Uptake. HeLa cells were exposed to polyplexes
made from PAD copolymers, PAPM, and PEI with DNA
fluorescently labeled with Cy3 (60bp-Cy3). The cells were
analyzed by flow cytometry following the 4 h incubation period
with polyplexes. Gating examples of the dot plots obtained are
provided in the Supporting Information (Figures S8–S13),
and the results are summarized in Figure 8.

Uptake efficiency of 60bp-Cy3 DNA at the 1:1 N/P ratio is
higher for PAPM than the PAD copolymers. The nominal net
charge of the polyplex with the 2:1 PAD to DNA mol ratio
should be neutral; however, as some hydrolysis has occurred, it
may actually be slightly negative. This may be why the uptake

Figure 6. HeLa cell viability one day post-exposure to 60bp DNA
with PAD copolymers, PAPM, and PEI at 1:1, 2.5:1, 5:1, and 10:1 N/
P ratios. HeLa cells were treated with DMSO [7% (v/v) in FCS-
containing DMEM] as a positive control.

Figure 7. HeLa cell viability two days after exposure to polyplexes of
60bp DNA with PAD copolymers, PAPM, and PEI at 1:1, 2.5:1, 5:1,
and 10:1 N/P ratios. HeLa cells were treated with DMSO [7% (v/v)
in FCS-containing DMEM] as the positive control. Statistical
significance for PAD copolymer samples at 5:1 and 10:1 N/P ratios
are shown. *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.0001 \).
efficiency of PAPM polyplexes is higher at this low polycation/DNA ratio, as endocytosis of neutral particles would be more likely than of anionic particles.

At the 2.5:1 N/P ratio, uptake efficiency appeared to be very high (>95%) for all PAD copolymers and PAPM. At 5:1 N/P, uptake efficiency of PAD80 polyplexes remained high at 92%; however, uptake efficiency began to decrease for PAD copolymers containing less DMAEA. Similarly, only 70% of the cells showed fluorescence when treated with PAPM polyplexes at 5:1 N/P. These decreases in cellular uptake efficiency correlate with the cytotoxicity data obtained using the WST-1 assay. HeLa cells treated with PAD copolymer polyplexes richer in APM showed a subpopulation that was thought to correspond to apoptotic cells. This was confirmed by conducting an Annexin V apoptosis assay at 1:1 and 10:1 N/P ratios that showed a high percentage of apoptotic cells (>80%) in this subpopulation (Figures S14–S16). As this subpopulation was more prominent at higher concentrations (10:1 N/P), this is in agreement with the increased cytotoxicity observed at higher polymer concentrations from the WST-1 assay. Thus, the decrease in cellular uptake efficiency of PAD copolymers correlates with the toxicity of the corresponding polyplexes. This could be due to cationic charges of PAPM and PAPM-rich polyplexes that are unable to undergo charge-shifting hydrolysis, causing cell death and thereby reducing the number of cells in the region of healthy, fluorescent cells. Similarly, PAD80 polyplexes showed the highest uptake efficiency at the 5:1 N/P, which may be due to PAD80 having the greatest charge-shifting ability and hence a greater proportion of healthy, Cy3-positive cells.

Cellular uptake efficiency of PEI polyplexes appeared to be significantly lower than PAD copolymer polyplexes at 1:1 and 2.5:1 N/P ratios (Figure 8). At the highest, 5:1 N/P ratio, PEI shows comparable uptake efficiency to PAD copolymers used at the 2.5:1 ratio. These results for PAD copolymers are promising as PEI is currently the gold-standard polycation used in the field because of its high transfection efficiency.

**CONCLUSIONS**

PAD copolymers of varying compositions were synthesized by RAFT polymerization to obtain polymers with controlled molecular weights and dispersities. Model hydrolysis studies of the polymers using 1H NMR spectroscopy provided useful information on the evolution of the net charge of the polymers at pH 7, suggesting a route for potential intracellular dissociation of complexed DNA for controlled release from PAD80 and PAD49 polyplexes. HeLa cells transfected with PAD copolymer polyplexes showed very high viability at the 1:1 and 2.5:1 N/P ratios of all of the compositions. However, at higher polymer loadings, HeLa cells transfected with PAD copolymers of higher DMAEA composition showed higher viability than cells treated with non-charge-shifting PAPM. The higher viability associated with PAD copolymers is correlated to the charge-shifting ability of the polymers which likely reduces cytotoxicity, particularly long-term, as cells recovered well 2 days after exposure. Cellular uptake efficiency of PAD copolymer polyplexes was very high (>95%) at the 2.5:1 N/P ratio, out-performing PEI polyplexes which required double the polycation loading to achieve a similar level of uptake efficiency. Overall, PAD copolymers of higher DMAEA content (PAD80 and PAD49) used at the 2.5:1 N/P ratio appear as promising candidates for further cell transfection experiments including gene expression.

In conclusion, the findings in this report suggest that charge-shifting cationic copolymers are a promising class of synthetic polymers suitable for in vitro DNA delivery. Future work will involve exploring gene transfection efficiency and mechanism of intracellular release of DNA from polyplexes with these charge-shifting copolymers, both with HeLa and other cell lines.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.9b03734.

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