The In Vitro, Ex Vivo, and In Vivo Effect of Polymer Hydrophobicity on Charge-Reversible Vectors for Self-Amplifying RNA

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ABSTRACT: RNA technology has the potential to revolutionize vaccination. However, the lack of clear structure–property relationships in relevant biological models mean there is no clear consensus on the chemical motifs necessary to improve RNA delivery. In this work, we describe the synthesis of a series of copolymers based on the self-hydrolyzing charge-reversible polycation poly(dimethylaminoethyl acrylate) (pDMAEA), varying the lipophilicity of the additional co-monomers. All copolymers formed stable polyplexes, showing efficient complexation with model nucleic acids from nitrogen/phosphate (N/P) ratios of N/P = 5, with more hydrophobic complexes exhibiting slower charge reversal and disassembly compared to hydrophilic analogues. The more hydrophobic copolymers outperformed hydrophilic versions, homopolymer controls and the reference standard polymer (polyethyleneimine), in transfection assays on 2D cell monolayers, albeit with significantly higher toxicities. Similarly, hydrophobic derivatives displayed up to a 4-fold higher efficacy in terms of the numbers of cells expressing green fluorescent protein (GFP⁺) cells in ex vivo human skin (10%) compared to free RNA (2%), attributed to transfection enrichment in epithelial cells. In contrast, in a mouse model, we observed the reverse trend in terms of RNA transfection, with no observable protein production in more hydrophobic analogues, whereas hydrophilic copolymers induced the highest transfection in vivo. Overall, our results suggest an important relationship between the vector lipophilicity and RNA transfection in vaccine settings, with polymer biocompatibility potentially a key parameter in effective in vivo protein production.

INTRODUCTION

Since the advent of in vitro transcription, mRNA (mRNA) therapeutics have evolved into a flexible platform, capable of artificially introducing or replacing specific proteins for vaccination and therapeutic applications.1,2 The nonintegrating nature of mRNA means that this technology can be easily inserted into current clinical practice, without the long-term safety risks posed with genome altering DNA alternatives.3–5 As such, several mRNA therapies have undergone, or are currently undergoing clinical trials,6–8 while their manufacturability is currently under investigation.9,10

The activity of RNA therapeutics relies on the efficient delivery of the nucleic acid to the cytosol of the cells at the target site, where it is translated into the active protein. However, naked mRNA suffers from quick pharmacokinetic clearance, poor cellular association and facile degradation, significantly reducing the cytosolic dose and thus its therapeutic activity.11 Given these drawbacks, numerous nonviral delivery strategies such as liposomes and cationic polymers, which protect, improve pharmacokinetics and aid cytosolic delivery of RNA, have been developed.

Strategies exploiting polycations operate by condensing the nucleic acid through electrostatic complexation, forming 100–200 nm polyelectrolyte nanoparticles termed polyplexes that facilitate translocation of the genetic material into the cell. Most nonviral cationic vectors are designed to complex strongly to nucleic acids to afford protection in transit through the body; however for translation, RNA must also be released from the vector and interact with the cellular protein machinery. Vectors can achieve this release process either by introducing chemistries that break the polycationic chain in response to endogenous stimuli (GSH, ROS, pH, enzymes) or through self-immolative charge alteration, which terminates the electrostatic attraction between a vector and nucleic acid.12 Although many of the above endogenous stimuli can be useful for delivery in specific disease states, self-immolative pathways for charge reversal may offer a route for mRNA release in the absence of any specific stimuli.

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At present, there are few examples of gene delivery using self-immolative charge altering vectors. Notable examples include enhanced mRNA delivery utilizing an oligo-(carbonate-β-α-amino ester), which rapidly degrades into a neutral diketopiperazine through an ester–amide cyclization reaction, releasing the mRNA. Another key example is the self-catalyzed hydrolysis of poly(dimethylaminoethyl) acrylate (pDMAEA), which degrades from a polycation into poly-anionic poly(acrylic acid) (pAA) in the presence of water releasing dimethylaminoethanol (DMEA).

From a gene delivery perspective, this delivery system will efficiently condense nucleic acids in their positively charged state and then hydrolyze, charge invert, repel, and release the genetic cargo using a single material. Systems based on the charge-reversing nature of pDMAEA have already been utilized to deliver siRNA and pDNA; however, no studies have yet examined its potential for mRNA delivery.

It has recently been reported that the introduction of hydrophilic co-monomers, such as poly(dimethylaminoethyl) hydroxymethacrylate, or the spacing of DMEA units within the chain with nonhydrolyzing DMAEMA can vary the rate and final degree of hydrolysis. Furthermore, to date, most previous examples show a pH-independent hydrolysis mechanism, with few reports showing this can be overcome by hindering the interaction with hydroxide ions. This was achieved by incorporating anionic moieties along the chain; however, such systems would not be beneficial for gene delivery, as they are not net positively charged. Accordingly, we report here a systematic mechanistic study utilizing a library of pDMAEA copolymers, varying the co-monomer lipophilicity to study (a) the effect on hydrolysis rate, (b) if this effects the pH-independent mechanism, and (c) the implications of the co-monomer and charge reversal on mRNA delivery from the perspective of vaccination.

We designed and synthesized a series of pDMAEA copolymers containing varying degrees of a hydrophobic co-monomer, butyl acrylate (BA), and a hydrophilic co-monomer, 2-hydroxyethyl acrylate (HEA), as well as pDMAEA and pDMAEMA (nonhydrolyzing) controls. Hydrolysis was monitored at pH 5.5, 7.4, and 10.1 over 5 days using 1H NMR spectroscopy. All copolymers were formulated into polyplexes with model nucleic acids, and the particle sizes and zeta-potentials were monitored over 7 days, as the polymers charge inverted. Finally, we evaluated the potential for the vectors to deliver mRNA in a vaccine setting, utilizing a 2D cell culture, in ex vivo skin explants and a murine in vivo model.

**MATERIALS AND METHODS**

**Materials.** 2-Hydroxyethyl acrylate (HEA, 96%), butyl acrylate (BA, > 99%), 2-(dimethylamino)ethyl acrylate (DMEA, > 98%), and 2-(dimethylamino)ethyl methacrylate (DMAEMA, 98%) were obtained from Sigma-Aldrich and used for mRNA delivery, as they are not net positively charged. Accordingly, we report here a systematic mechanistic study utilizing a library of pDMAEA copolymers, varying the co-monomer lipophilicity to study (a) the effect on hydrolysis rate, (b) if this effects the pH-independent mechanism, and (c) the implications of the co-monomer and charge reversal on mRNA delivery from the perspective of vaccination.

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Nucleic Acid Synthesis. RNA In Vitro Transcription and Purification. srRNA was prepared as previously described. Briefly, self-amplifying RNA encoding the replicase derived from the Venezuelan Equine Encephalitis Virus (VEEV) and either firefly luciferase (fluc) or enhanced green fluorescent protein (eGFP) was produced using in vitro transcription. DNA was linearized into Escherichia coli, cultured in 100 mL of lysogeny broth (LB) with 100 μg/mL carbenicillin (Sigma-Aldrich, UK), and isolated using a Plasmid Plus MaxiPrep kit (QIAGEN, UK). The concentration and purity of pDNA was measured on a NanoDrop One (ThermoFisher, USA) according to the manufacturer’s protocol. RNA for ex vivo experiments was prepared as previously described. Uncapped RNA transcripts were synthesized using 1 μg of linearized DNA in a MEGAScript reaction (Ambion, UK) according to the manufacturer’s protocol. Transcripts were then purified by overnight LiCl precipitation at −20°C, pelleted by centrifugation at 14000 rpm for 20 min at 4°C, washed 1× with 70% EtOH, centrifuged at 14000 rpm for 5 min at 4°C, and then resuspended in UltraPure H2O (Ambion, UK). Purified transcripts were then capped using a ScriptCap Cap 1 capping system kit (CellScript, Madison, WI, USA) according to the manufacturer’s protocol. Capped transcripts were then purified by LiCl precipitation as described above, resuspended in UltraPure H2O, and stored at −80°C until further use.

Polyplex Formulation. Polymer Nucleic Acid Complexation. Stock solutions of the polymer and nucleic acid were prepared at double the concentration required in the polyplexes in the HEPES buffer (20 mM, pH 7.4) and 5 wt % glucose. Complexes were prepared by mixing the solutions 1:1 to produce the desired N/P ratio. Polyplexes were stirred at 25°C for 30 min to allow formation. For size and zeta-potential studies, polyplexes were prepared with calf thymus DNA and with fluc encoding pDNA for gel electrophoresis. For in vitro transfection studies, stock solutions of the polymers were prepared at a concentration of 5 mg/mL in HEPES buffer (20 mM, pH 7.4) and 5 wt % glucose. The polymer solution was added to the RNA solution at a ratio of 4:1 (v/v) to yield a final RNA concentration of 0.001 mg/mL such that 100 ng was added to each well of a 96-well plate. Particle Size and Zeta-Potential Analysis. The polyplex size and zeta-potential analysis were prepared using a previously reported procedure at N/P ratios of 0.5, 1, 2, 5, 10, 20, and 50 with the polymer fixed at 0.1 mg/mL. The calf thymus DNA concentration varied for each N/P ratio. Size measurements were performed directly with the prepared polyplex solutions for zeta-potential measurements. Polyplex solutions were diluted 1:1 with 10 mM NaCl (aq). Measurements were performed as described in Instrumentation and Analysis. For hydrolysis studies, polyplexes were measured at 24 h intervals and stored at 37°C between measurements.

Gel Electrophoresis. Gel electrophoresis experiments were performed following a reported protocol. Briefly, polyplexes for the gel retardation assay were prepared with a fixed DNA concentration of 200 ng/mL with polymer concentrations varied for each N/P ratio. Then, 20 μL of polyplex and 4 μL of loading dye (-SDS, 6X) were loaded onto a 1% agarose gel (Promega, USA) that were maintained in culture in complete Dulbecco’s Modified Eagle’s medium (DMEM) (Gibco, Thermofisher, USA) containing 10% (v/v) fetal calf serum (FCS), 5 mg/mL l-glutamine, and 5 mg/mL penicillin and streptomycin (ThermoFisher, UK). Cells were plated at a density of 50000 cells/well in a 96-well plate 24 h prior to transfection. At the time of transfection, the media was completely removed and replaced by 50 μL of transfection media (DMEM with 5 mg/mL l-glutamine). Then, 100 μL of the polypeptide solution was added to each well and incubated for 4 h. Then, the media was replaced with DMEM, and the cells were allowed to culture for 24 h, at which time 50 μL of media was removed, and 50 μL of the ONE-Glo n-luciferin substrate (Promega, UK) was added and mixed well by pipetting. The total volume was transferred to a white 96-well plate (Costar, UK) and analyzed on a FLUOstar Omega plate reader (BMG LABTECH, UK).

Ex Vivo Studies. Flow Cytometry Analysis of eGFP Expression in Human Skin Explants. The transfection of cells in human skin explants and the flow cytometry analysis were performed as previously described. Surgically resected specimens of human skin tissue were collected at Charing Cross Hospital, Imperial NHS Trust, London, UK. All tissues were collected after receiving signed informed consent from patients, under protocols approved by the Local Research Ethics Committee (MED RS_11_014). The tissue was obtained from patients undergoing elective abdominoplasty or mastectomy surgeries and was processed as previously described. Upon arrival in the laboratory, the subcutaneous layer of fat was removed, and the tissue was excised into 1 cm² pieces. Explants were incubated at 37°C with 5% CO2 in 12-well plates with DMEM, which was replaced daily. Explants were injected intradermally (ID) using a micro-fine Demi 0.3 mL syringe (Becton Dickinson, UK) with 5 μg of eGFP srRNA polyplexes. After 72 h, explants were digested into single cell suspensions by incubating in 2 mL of DMEM supplemented with 1 mg/mL collagenase P (Sigma, UK) and 5 mg/mL Dispase II (Sigma, UK) for 4 h at 37°C on a rotational shaker. Digests were then filtered through a 70 μm cell strainer and centrifuged at 1750 rpm for 5 min. Cells were then resuspended in FACs buffer (PBS + 2.5% FCS) and stained with a Fixable Aqua Live/Dead Cell stain (Thermofisher, USA) at 1:400 in FACs buffer for 20 min. Cells were then washed and stained with a panel of antibodies to identify each cell type for 30 min. The antibody panel included CD3-V450 (BioLegend, UK), CD14-Qdot605 (BioLegend, UK), CD19-BV650 (BioLegend, UK), CD56-BV711 (BioLegend, UK), CD1a-PerCP-eFluor710 (BioLegend, USA), CD14-Qdot605 (BioLegend, UK), CD11c-Cy7 (BioLegend, UK), and CD45-AF700 (BioLegend, UK). Cells were then fixed in 1.5% paraformaldehyde and refrigerated until the flow cytometry analysis was conducted. Samples were analyzed on an LSRFortessa (BD Biosciences, UK) with FACSDiva software (BD Biosciences, UK) with 10000 acquired cell events. Gating and
analysis was performed in FlowJo Version 10 (FlowJo LLC, Oregon, USA).

**In Vivo Studies. In Vivo fLuc Expression.** In vivo fLuc saRNA transfection experiments were performed as previously described. All animals were handled in accordance with the UK Home Office Animals Scientific Procedures Act 1986 and with an internal ethics board and UK government approved project (P63FE629C) and personal license (IC37CBB8F). Food and water were supplied ad libitum. Female BALB/c mice (Charles River, UK) 6−8 weeks of age were placed into groups (n = 5) and housed in a fully acclimatized room. In vivo imaging was performed as previously described. Mice were injected either intramuscularly in both hind legs or intradermally with 5 μg of fLuc saRNA complexed with P1−P8 or PEI in a total volume of 50 μL. After 7 days, the mice were injected intraperitoneally (IP) with 100 μL of XenoLight RediJect D-luciferin substrate (PerkinElmer, UK) and allowed to rest for 10 min. Mice were then anesthetized using isoflurane and imaged on an In Vivo Imaging System (IVIS) FX Pro (Kodak Co., Rochester, NY, USA) equipped with Molecular Imaging software version 5.0 (Carestream Health, USA) for 2 min. The signal from each injection site was quantified using Molecular Imaging software and expressed as relative light units (p/s).

**Statistical Analysis.** Plots and statistics were performed using GraphPad Prism, version 8. Significant differences were identified using either multiple t tests adjusted for multiple comparisons or one-way ANOVA with multiple comparisons.

## RESULTS AND DISCUSSION

**Polymer Synthesis and Characterization.** A library of eight cationic polymers (P1−P8) were synthesized using RAFT polymerization. Six (P1−P6) of these were prepared with 50 mol % charge-reversible monomer DMAEA, while the lipophilicity of the other 50 mol % was varied by increasing and decreasing (in 10 mol % increments) the quantity of HEA and BA, respectively (Scheme 1), with a total DP_{target} = 50. Furthermore, pDMAEA_{25} (P7) and pDMAEMA_{25} (P8) homopolymers were prepared as fully hydrolyzing and nonhydrolyzing controls respectively, with the same cationic content (25 units) as P1−P6. Acrylate polymerizations were performed with the RAFT agent PABTC at 70 °C using thermal initiation with ACVA, while methacrylate polymerizations performed with RAFT agent CPAETC at 90 °C with

![Scheme 1. Schematic Representation for RAFT Polymerization and Monomer Composition of Homopolymers and Copolymers P1−P8](image-url)

**Table 1. Structure and Characterization of the Polymers (P1−P8) Synthesized in This Study**

| polymer | structure | M_n,th (g mol⁻¹)ᵃ | M_n,SEC (g mol⁻¹)ᵇ | Đᵇ |
|---------|-----------|-------------------|-------------------|-----|
| P1      | pDMAEA_{25}−co-BA_{25} | 7000            | 3300             | 1.16|
| P2      | pDMAEA_{25}−co-BA_{20}−co-HEA_{5} | 6950            | 5400             | 1.22|
| P3      | pDMAEA_{25}−co-BA_{15}−co-HEA_{10} | 6900            | 7000             | 1.18|
| P4      | pDMAEA_{25}−co-BA_{10}−co-HEA_{15} | 6850            | 7300             | 1.21|
| P5      | pDMAEA_{25}−co-BA_{5}−co-HEA_{20} | 6800            | 7800             | 1.24|
| P6      | pDMAEA_{25}−co-HEA_{25} | 6700            | 10300            | 1.15|
| P7      | pDMAEA_{25} | 3800            | 3100             | 1.21|
| P8      | pDMAEMA_{25} | 4300            | 5000             | 1.22|

ᵃCalculated using eq 1. ᵇDetermined using DMF-SEC.
VA-088. Full conditions can be found in the Supporting Information (Table S1). \(^1\)H NMR spectroscopy revealed the synthesized polymers had the targeted co-monomer composition, while DMF-SEC analysis revealed narrow (\(D < 1.25\)) and monomodal chromatograms (Table 1, Figure S1, Figure S2). Some deviation between experimental and theoretical molar

Figure 1. (A) Representative \(^1\)H NMR spectrum illustrating the release of DMAE from pDMAEA homopolymer P7 over 5 days. (B) Hydrolysis kinetics derived from \(^1\)H NMR spectra of P1–P8 over 5 days in D\(_2\)O at pH 5.5 (red line), 7.4 (blue line), and 10.1 (green line). (C) pH titration of P1–8, PEI, and 0.1 M NaCl from pH 3 to pH 11, titrated with 0.1 M NaOH (aq). (D) Schematic representation of the partial hydrolysis of pDMAEA copolymers containing either hydrophilic or hydrophobic co-monomers, leading to pH-dependent hydrolysis, due to the hydroxide access for more hydrophilic pDMAEA analogues.
masses was observed, this was more apparent for copolymers with higher HEA mol % likely due to their greater swelling during SEC analysis. The statistical monomer distribution of DMAEA, BA, and HEA ternary copolymers was confirmed through a kinetic polymerization study performed on a mixture containing 33% of each monomer. $^1$H NMR spectra of samples withdrawn periodically through the polymerization revealed identical polymerization kinetics for each co-monomer (Figure S3), suggesting identical reactivity ratios.

**Chain Lipophilicity Induces pH Dependency on Charge Reversal.** Previous reports studying the charge reversal of pDMAEA into pAA have identified a pH-independent self-catalyzed hydrolysis mechanism. However, most of these studies utilize block copolymers, homopolymers, or statistical copolymers containing co-monomers with complementary functionalities such that there is limited spacing between basic residues within the chain. Given this, we were interested in identifying if the relative lipophilicity of the copolymers impacted the rate of pDMAEA hydrolysis and its pH-independent mechanism. Hydrolysis kinetics of DMAEA side chains in P1−P8 was determined by recording daily $^1$H NMR spectra at pH 5.5, 7.4, and 10.1, monitoring the reduction and increase of integrals of the signals at 4.2 ppm (CH$_2$ next to ester in polymer) and 3.7 ppm (CH$_2$ next to alcohol in DMAE), respectively (Figure 1A and Figure S4). Where copolymers were sparingly soluble at the beginning of the study, a soluble external reference of 1,3,5-trioxane was added to the NMR tubes, and the integrals were back-calculated at the end of the study, at which point

![Figure 2](https://dx.doi.org/10.1021/acs.biomac.0c00698)
deuterium chloride was added to protonate and solubilize the copolymers to quantify the percentage hydrolysis. P1–P6 exhibited comparable hydrolysis rates, reaching ∼50 and 20–30% hydrolysis at pH 7.4 and 5.5, respectively, over 5 days at 37 °C. In contrast, at pH 10.1, the more hydrophobic copolymers (P1 and P2) hydrolyzed significantly slower rates (∼40% over 5 days) than the more hydrophilic derivatives (∼60% over 5 days), with the kinetic profiles revealing a trend of faster hydrolysis with increasing hydrophilicity (Figure 1B). We hypothesize that the slower hydrolysis in P1 and P2 at pH 10.1 may be due to the DMAEA residues being deprotonated at pH 10.1, causing these analogues to be sparingly soluble in the early stages of hydrolysis. However, once a significant portion is hydrolyzed, the resulting acrylic acid residues are deprotonated at this pH, improving the solubility, which may explain the increased hydrolysis rate observed after 72 h. Consistent with the literature, the pDMAEA homopolymer P7 showed almost identical hydrolysis rates at all three pH values, reaching 60% over the course of the experiment, while the nonhydrolyzing pDMAEMA homopolymer P8 did not show any significant signs of hydrolysis.

Interestingly, we observed that the difference in the hydrolysis rate and the final percentage of hydrolysis between pH 5.5 and pH 10.1 increased significantly with the hydrophilic co-monomer composition. For instance, after 5 days, P1 reached 30, 37, and 45% hydrolysis at pH 5.5, 7.4, and 10.2, respectively, which represented a 15% difference between acidic and basic conditions. In contrast, P6 was hydrolyzed to extents of 20, 50, and 65%, a difference of 45% under the same treatment conditions (Figure 1B). While DMAEA homopolymers typically exhibit pH-independent hydrolysis, here we demonstrate a pH-dependent hydrolysis controlled by the hydrophobicity of the co-monomer. We anticipate this behavior may originate depending on the solubility of the zwitterionic complex, which is formed as hydrolysis approaches 50%. In this state, the chains will be partially positive and partially negative, collapsing due to the intramolecular electrostatic attraction. The more hydrophobic derivatives will be less prone to charge reversal due to the poor hydration of these collapsed chains, slowing their hydrolysis (Figure 1D). This behavior may be important in gene delivery, leading to the potential of a pH-dependent controlled release of nucleic acids, or in anatomies and cellular compartments with nonphysiological pH levels.

**Lipophilic Polyplexes Charge Invert Slower than Hydrophilic Derivatives.** Prior to formulating the synthesized polymers with nucleic acids, we investigated the buffering capacity of P1–P8 by titrating dilute polymer solutions with 0.1 M NaOH from pH 2.5 to pH 11. P1–P7 displayed similar titration curves, with two observable pKₐ values, one at ∼pH 5 and the other at ∼pH 10. These were compared to PEI, the reference standard in gene transfection, and P8, the pDMAEMA homopolymer (Figure 1C). Both showed poorer buffering with less defined pKₐ values, likely due to the density of basic residues minimizing the propensity of these analogues for successive protonation events.

Polyplex formulations were optimized using model nucleic acids, either with calf thymus DNA or luciferase-encoding plasmid DNA. Particle size, zeta-potential measurements, and gel-retardation assays were performed on polyplexes at N/P ratios of 0.5, 1, 2, 5, 10, 20, and 50. Stable complexes, displaying narrow particle size distributions (PDI < 0.3), positive zeta-potentials above 20 mV, and full DNA-binding abilities were observed above N/P = 5 (Figure S5). All polymers yielded polyplexes of similar sizes, exhibiting average diameters between 120 to 200 nm (Figure 2A). The particle size (Figure 2B) and zeta-potentials (Figure 2C) of polyplexes formulated at N/P = 10 utilizing the charge-reversible polymers (P1–P7) evolved steadily over 1 week as hydrolysis progressed. Interestingly, instead of decreasing in size as polymers in polyplexes hydrolyzed, the polyplex diameters increased from ∼150 nm to over 1000 nm (Figure 2B). However, at this time point, the scattering intensity of the solutions rapidly decreases (Figure 2D), indicating partial or full disassembly of the polyplex, but the increase in particle size, visible via DLS (Figure 2B), is likely due to a few aggregated particles when the zeta potentials reach ∼0 mV, skewing the light scattering analysis. The charge-reversible nature of P1–P7 was evidenced by the steady decrease in zeta-potentials, decreasing from positive (∼30 mV) to negative (∼6 mV) as the cationic DMAEA units hydrolyzed to anionic acrylic acid residues (Figure 2C). The nonhydrolyzing pDMAEMA homopolymer P8 exhibited some aggregation, increasing from 110 to 145 nm diameters between 1 and 7 days, while the zeta-potentials showed minimal change (Figure 2B). The zeta-potentials and scattering intensity of the polyplexes derived from each of the hydrophobic copolymers decreased significantly slower than hydrophilic analogues, likely due to the lower hydration potential of these complexes, slowing the self-catalyzed hydrolysis process. These results indicate that polyplexes formulated with self-hydrolyzing analogues should be effective at releasing nucleic acids, and the particles themselves will dissociate, potentially avoiding bioaccumulation.

**Hydrophobic Polyplexes Are the Most Cytotoxic yet Induce the Highest Protein Expression In Vitro.** We assessed the membrane interactions of polyplexes derived from our library of polymers by evaluating their propensity to lyse erythrocytes (hemolysis, Figure 3A). Polyplexes derived from P1–P8 and PEI were formulated at N/P = 0.5, 5, and 50. Their hemolytic activity was compared to Triton-X (positive control), HEPES buffer (vehicle control), free DNA and PBS (negative control). The observable increasing trend in hemolytic activity with increasing N/P ratios is likely due to the higher concentration of free polymer not residing in the polyplex. Previous studies have indicated that, although the nominal N/P ratio in the solution typically exceeds 10, the actual N/P ratio within the complex usually does not exceed 2; thus, substantially more free polymer is present at high N/P ratios. At N/P = 50, a stepwise increase in the hemolytic activity was observable with more hydrophobic analogues, starting with negligible hemolysis in P6 polyplexes and rising to 97% hemolysis, which is almost identical to the positive control, in P1 polyplexes (Figure 3A). It is well documented that polycations incorporating hydrophobic residues are exceptionally membrane active due to their attraction to the phospholipid bilayer and disruptive hydrophobic interactions. The hemolytic activity has also been a key marker for high endosomal escape efficiencies and thus may give insight into the mechanisms of RNA delivery for these polymers. Following this, the in vitro transfection efficiency and cytotoxicity of polyplexes comprising fLuc-encoding saRNA and polymers P1-P8 at N/P ratios of 2, 10, and 50 in HEK293T cells after 24 h of incubation were also assessed (Figure 3B). From a vaccination perspective, saRNA poses significant advantages over conventional mRNA, as these constructs self-replicate upon their arrival in the cytoplasm,
represented as the mean previously optimized PEI formulation (dotted gray line). Data are with polyplexes formed of P1 (C) luciferase expression in HEK 293T cells 24 h after transfection sheep blood. Cell viability as a function of (B) metabolic activity and absence of BA units and the faster hydrolysis may account for derivatives, and the pDMAEA homopolymer were the least likely due to the higher concentration of noncomplexed the polyplexes derived from all of the synthesized polymers at negative potentials. As in the case of the hemolytic activity, zeta potentials, while low molar mass analogues showed high molar mass polycations at high N/P ratios display positive consistent with a previous report indicating complexes with negative zeta potentials than DNA analogues, potentially attributable to the poorer condensation of the larger nucleic acid type via traditional intramuscular or intradermal injections. RNA vaccines to be easily incorporated into current clinical practices, it has been posited that these would be administered through traditional intramuscular or intradermal injections. We therefore examined the transfection efficiency of a subset of the formulations tested in vitro in an ex vivo human skin explant model (Figure 4A). Polymers P1, P3, P6, P7, and P8 were formulated into polyplexes with saRNA encoding for green fluorescent protein (GFP) at N/P = 10. Nonformulated RNA yielded expression in ∼2% of cells, which did not significantly increase upon complexation with P3 (p = 0.92), P6 (p > 0.99), P7 (p = 0.94), and P8 (p = 0.99). However, formulations with P1, the most effective polymer in vitro, showed a significant increase in GFP+ cells (∼11%) compared to RNA alone (p = 0.022, Figure 4A). The numbers of GFP+ cells in these studies were similar to those in previous studies with mannansylated PEI-saRNA complexes but were higher than those with cationic lipid-saRNA formulations. Although many of the formulations did not increase the percentage of GFP+ cells, skin is a complex tissue comprising many cell types in their native tissue architecture. We therefore sought to identify which cells were expressing the GFP. Transfected skin explants were enzymatically homogenized, and each cell type was labeled using a panel of fluorescently labeled antibodies. Then, GFP+ cells were categorized by cell population compared to PEI formulations.

**Hydrophobic Polymers Enhance the Number of saRNA-Expressing Cells in Human Skin Explants.** For RNA vaccines to be easily incorporated into current clinical practices, it has been posited that these would be administered through traditional intramuscular or intradermal injections. We therefore examined the transfection efficiency of a subset of the formulations tested in vitro in an ex vivo human skin explant model (Figure 4A). Polymers P1, P3, P6, P7, and P8 were formulated into polyplexes with saRNA encoding for green fluorescent protein (GFP) at N/P = 10. Nonformulated RNA yielded expression in ∼2% of cells, which did not significantly increase upon complexation with P3 (p = 0.92), P6 (p > 0.99), P7 (p = 0.94), and P8 (p = 0.99). However, formulations with P1, the most effective polymer in vitro, showed a significant increase in GFP+ cells (∼11%) compared to RNA alone (p = 0.022, Figure 4A). The numbers of GFP+ cells in these studies were similar to those in previous studies with mannansylated PEI-saRNA complexes but were higher than those with cationic lipid-saRNA formulations.

Although many of the formulations did not increase the percentage of GFP+ cells, skin is a complex tissue comprising many cell types in their native tissue architecture. We therefore sought to identify which cells were expressing the GFP. Transfected skin explants were enzymatically homogenized, and each cell type was labeled using a panel of fluorescently labeled antibodies. Then, GFP+ cells were categorized by cell type via flow cytometry (Figure 4B). We observed that the cell phenotype in the skin used was predominantly epithelial (∼60%), with leukocytes (∼8%) and fibroblasts (∼11%), and dendritic (∼10%) with a much smaller proportion of Langerhans cells (∼0.8%), natural killer (NK) cells (∼1%), monocytes (∼3%), B cells (∼4%), and T cells (∼0.5%). In skin treated with nonformulated RNA, the majority of GFP expression was found in the commonly found epithelial cells, leukocytes, and fibroblasts; however, some enrichment was observed in NK cells, B cells, and Langerhans cells compared to the cell composition of the skin (Figure 4B). The profile of GFP+ cells was generally similar for skin treated with polyplexes derived from the faster hydrolyzing hydrophilic...
yielding an average luminescence of \( \text{~5.0 \times 10^5} \) RLU. Mice administered with P1, P3, P6, P7, and P8 had a rather variable translational machinery. In a site of high blood vessel and tight-binding of nucleic acids to a polymer-saRNA formulation and protection against nucleases following injection of PEI (jetPEI). PEI formulation outperformed all polyplexes, obtained in the optimized PEI formulation (jetPEI). In contrast to the data presented above, P1 yielded no RNA expression across all positive legs with an average luminescence of \( \sim 10^{-4} \), roughly 10-fold lower than that of PEI (Figure 5A and B). It is possible that the poor translation of P1 for in vitro to in vivo protein expression arose due to the previously observed toxic effects of P1, P3, and P6 in vivo and ex vivo studies. Mice were injected intramuscularly on each leg with polymer-saRNA formulations, as determined by flow cytometry. The selected polymers were formulated for saRNA complexation at N/P = 10 for consistency with the nonhydrolyzing pDMAEMA homopolymer (P7) which both show slow or negligible charge reversal, had similar expression profiles with a significantly higher expression in epithelial cells, closely matching the resident skin cell population (Figure 4B). From these observations, we can conclude that P1 polyplexes at N/P = 10 enhance the number of GFP+ cells in human skin explants due to an increase in the expression from epithelial cells.

**In Vivo Studies.** Following the successful transfection of 2D cell monolayers and human skin explants mediated by our charge-reversing polymers, we sought to evaluate the trends in saRNA vaccination using an in vivo murine model. Mice were injected intramuscularly on each leg with polyplexes composed of charge-reversing polymers P1, P3, P6, P7, and P8 with fLuc-encoding saRNA at N/P = 10. These polymers were chosen such that we could examine the trends in polymer hydrophobicity (P1, P3, and P6) and the effect of charge reversal (P7 and P8) with a minimal number of animals. The selected polymers were formulated for saRNA complexation at N/P = 10 for consistency with in vitro and ex vivo skin studies. The luminescence was monitored 7 days after injection at the expected time point for peak expression (Figure 5A and B). Charge-reversible formulations were compared to the pDMAEMA nonhydrolyzing control P8 and an in vivo optimized PEI formulation (jetPEI). In contrast to the data obtained in the in vitro and ex vivo experiments, in the murine model, the PEI formulation outperformed all polyplexes, yielding an average luminescence of \( \sim 5.0 \times 10^5 \) RLU. Mice administered with P1, P3, P6, P7, and P8 had a rather variable luminescence, with no groups exhibiting RNA expression on every injected leg. In contrast again to the in vitro and ex vivo results above, P1 yielded no RNA expression across all five animals, while mice treated with P6 polyplexes exhibited 7/10 positive legs with an average luminescence of \( \sim 4 \times 10^5 \), roughly 10-fold lower than that of PEI (Figure 5A and B). It is possible that the poor translation of P1 for in vitro to in vivo protein expression arose due to the previously observed toxic membrane interactions, causing local cell death proximal to the injection site, or to local self-association/aggregation of the more hydrophobic complexes in the protein-rich muscle regions, of which both factors would have reduced transfection. In contrast, polyplexes derived from P6, P7, and P8, which were the least cytotoxic or membrane active, yielded the highest efficacy in vivo, which may have been a consequence of the high vascularization in muscle and rapid transport of polyplexes following injection. In addition, animals treated with the charge-reversible pDMAEA homopolymer (P7) displayed similar luminescence values to those injected with the nonhydrolyzing pDMAEMA control (P8). These data perhaps indicate that the unpackaging of RNA from the polyelectrolyte complexes is not the rate-limiting step in the high vascularization in muscle and rapid transport of polyplexes following injection. In addition, animals treated with the charge-reversing pDMAEA homopolymer (P7) displayed similar luminescence values to those injected with the nonhydrolyzing pDMAEMA control (P8). These data perhaps indicate that the unpackaging of RNA from the polyelectrolyte complexes is not the rate-limiting step in transfection and expression of the target protein in vivo, at least for these types of polymers injected into this anatomical site (Figure 5A and B). There is always a trade-off between the tight-binding of nucleic acids to afford colloidal stability in a formulation and protection against nucleases following injection and the ability to enter target cells and address the translational machinery. In a site of high blood vessel and...
protein content, such as a site following intramuscular injection, the polyplexes have the potential to bind to many other biomolecules prior to reaching a target cell. In addition, the processing of RNA in vivo involves multiple competitive binding interactions intracellularly, and these may have been sufficient to the unpackaging of RNA from the complexes, irrespective of whether the side-chains in the polyplexes were hydrolyzing.

Finally, it should be noted that the polymers in this study were designed as probes of transfection rather than materials to be adopted in the clinic, as the acrylate-based chain is not biodegradable. Nevertheless, as they are chemically well-defined, the polymers could be used to test systematically the effects of side-chain hydrophilicity and hydrophobicity in conjunction with side-chain hydrolysis and charge-reversal in a manner not possible for main-chain biodegradable polymers. Our future studies will focus on further probing the mechanisms by which the RNA polyplexes are transported and unpackaged in as close to clinically relevant in vivo models as possible.

**CONCLUSIONS**

In conclusion, we designed a systematic library of charge-reversible polymeric gene delivery vectors, varying the relative lipophilicity of copolymers based on the self-catalyzed hydrolysis of pDMAEA. By spacing DMAEA units with hydrophobic units, we were able to produce self-hydrolyzing polymers with pH-dependent hydrolysis. In contrast, hydrophobic units, we were able to produce self-hydrolyzing polymers with pH-dependent hydrolysis. By spacing DMAEA units with hydrophobic units, we were able to produce self-hydrolyzing polymers with pH-dependent hydrolysis. Alternatively hydrophobic polymers with pH-dependent hydrolysis. In contrast, hydrophobic units, we were able to produce self-hydrolyzing polymers with pH-dependent hydrolysis. Alternatively hydrophobic polymers with pH-dependent hydrolysis. Alternatively hydrophobic polymers with pH-dependent hydrolysis.

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biomac.0c00698.

**ASSOCIATED CONTENT**

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Supporting Information contains detailed information on the polymerization conditions and characterization data for the synthesized polymers, 1H NMR spectra for hydrolysis studies, and physical characterization of polyplexes (PDF).

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**Notes**

The authors declare no competing financial interest.

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