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Cation Bulk and pK_a Modulate Diblock Polymer Micelle Binding to pDNA

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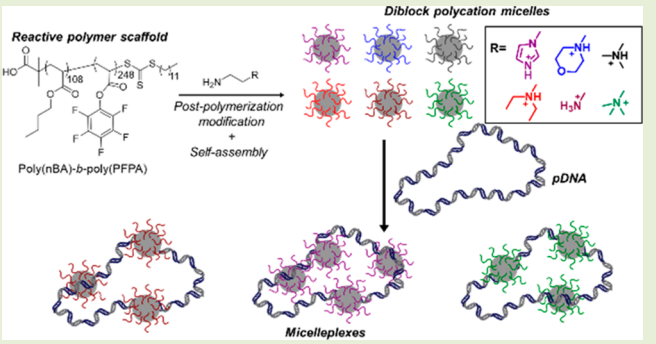
ABSTRACT: Polymer-based gene delivery relies on the binding, protection, and final release of nucleic acid cargo using polycations. Engineering polymeric vectors, by exploring novel topologies and cationic moieties, is a promising avenue to improve their performance, which hinges on the development of simple synthetic methods that allow facile preparation. In this work, we focus on cationic micelles formed from block polymers, which are examined as promising gene compaction agents and carriers. In this study, we report the synthesis and assembly of six amphiphilic poly(*n*-butyl acrylate)-*b*-poly(cationic acrylamide) diblock polymers with different types of cationic groups ((dialkyl)amine, morpholine, or imidazole) in their hydrophilic corona. The polycations were obtained through the parallel postpolymerization modification of a poly(*n*-butyl acrylate)-*b*-poly(pentafluorophenyl acrylate) reactive scaffold, which granted diblock polymers with equivalent degrees of polymerization and subsequent quantitative functionalization with cations of different pK_a. Ultrasound-assisted direct dissolution of the polycations in different aqueous buffers (pH = 1−7) afforded micellar structures with low size dispersities and hydrodynamic radii below 100 nm. The formation and properties of micelle−DNA complexes ("micelleplexes") were explored via DLS, zeta potential, and dye-exclusion assays revealing that binding is influenced by the cation type present in the micelle corona where bulkiness and pK_a are the drivers of micelleplex formation. Combining parallel synthesis strategies with simple direct dissolution formulation opens opportunities to optimize and expand the range of micelle delivery vehicles available by facile tuning of the composition of the cationic micelle corona.



Nucleic acid therapies are becoming ever more present in medicine; however, effective delivery agents still remain a challenge.[1] Cationic polymer vehicles present a promising platform for delivery of biological payloads into cells due to their ease of scale-up, customization, and their lower cost compared to viral delivery vehicles.[2] Research in this field is focused on the preparation of novel polymeric vehicles and understanding the physical properties of their complexes with different genetic cargos.[3] The physical properties of these polymer-pDNA complexes (termed polyplexes) can influence immune activation, organ specificity, and exclusion via the reticuloendothelial system during *in vivo* experiments, therefore affecting their *in vivo* efficacy.[4−7] To advance this field, it is important to understand the fundamental physical properties of nucleic acid−polymer complexes and how complexation and colloidal stability are affected by cationic identity and pK_a.

We and others have identified cationic micelles, formed from self-assembling block polymers, as promising nucleic acid carriers with improved pDNA and ribonucleoprotein transfection when compared to linear polymer analogues that form polyplexes.[8,9] Previous work from our group has centered on micellar vehicles that rely only on one type of cationic monomer, 2-(dimethylamino)ethyl methacylate, with a consistent pK_a (7.4 at 100 mM ionic strength).[10] Other work has shown there is significant importance of cation chemistry in nucleic acid delivery performance. For example, incorporation of cations with pK_a values within biologically relevant ranges (endosomal pH of 4.5−6.5[11] and cytoplasmic pH of 7.4[12]) can improve transfection due to the buffering capabilities known to cause endosomal membrane disruption.[13−16] Furthermore, the pK_a value of a cation is largely dependent on the chemical environment; hence, cationic micelles, with closely packed coronas and therefore many adjacent amine groups, are known to generally have slightly lower pK_a values compared to their linear polymer analogues.[10,17] Incorporation of cationic moieties that can interact with nucleic acids via hydrogen

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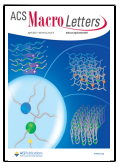

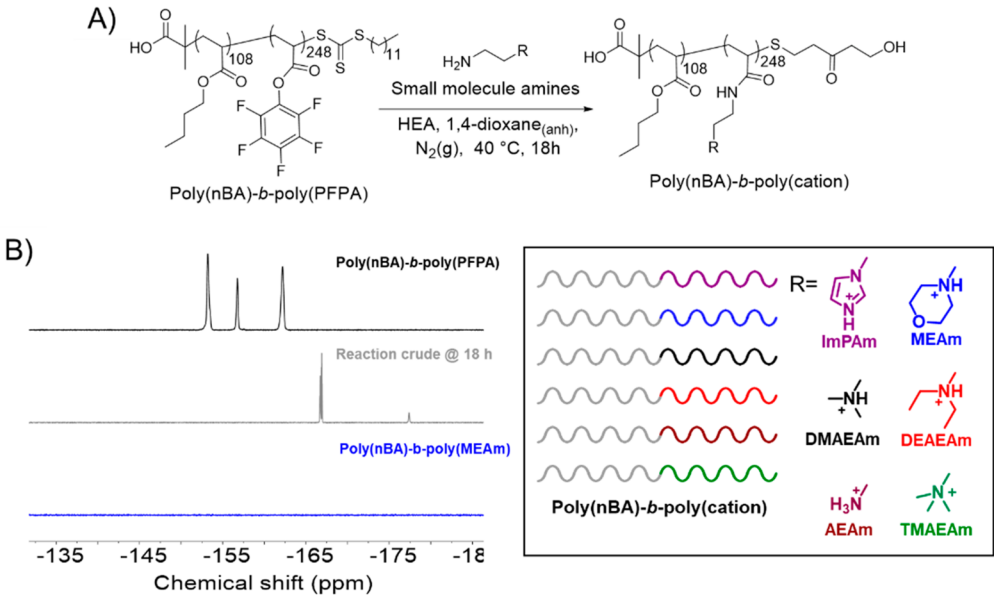

Figure 1. (A) Synthetic scheme for the preparation of the poly(nBA)-*b*-poly(cation) library. (B) ^{19}F NMR spectra of poly(nBA)-*b*-poly(PFPA) scaffold (top), reaction aliquot taken at 18 h (middle), and poly(nBA)-*b*-poly(MEAm) after purification (bottom).

bonding[18,19] or intercalation,[20] rather than solely charged interactions driven by entropic release of counterions, which also leads to different binding and release mechanisms and potential alterations of transfection performance.

Herein, we seek to expand on the understanding of cationic polymer micelles binding to pDNA by specifically incorporating amines with different size/bulkiness and pK_a into the cationic coronas. We demonstrate a synthetic strategy for the preparation of a family of amphiphilic, hydrophobic-*b*-cationic, diblock polymers through a combination of reversible addition−fragmentation chain transfer (RAFT) block polymerization and the postpolymerization modification of a reactive poly(pentafluorophenyl acrylate) (PFPA) ester scaffold. This approach yields a library of six cationic diblock polymers with controlled molar mass and moderate dispersity (Figure 1). This family features incorporation of different nitrogen-based cations with a range of pK_a values, hydrophobicity, and steric bulk as pendant groups in the hydrophilic block.

Micellization of the block polymers was achieved through ultrasound-assisted dissolution in buffers with pH values ranging from 1 to 7. Micelle size and dispersity were found to be dependent on buffer pH. Micelleplexes were formed at pH values between 5 and 7, where it was found that pH played a role in size and size distribution. Interestingly, cation bulkiness was found to drive complexation strength: micelles with coronas composed of more compact cations bound stronger to pDNA. This work highlights how both the bulkiness and pK_a values of the cationic pendant groups in diblock polymer micelles combine to define how strongly a cationic micelle binds to pDNA, thus affecting the formation, size, and zeta potential of micelleplexes.

A reactive diblock polymer scaffold poly(*n*-butyl acrylate)-*b*-poly(pentafluorophenyl acrylate) (poly(nBA)-*b*-poly(PFPA)) was synthesized via RAFT chain extension of a poly(nBA) macromolecular chain transfer agent (macro-CTA) with PFPA (Figures S1 and S2). The macro-CTA was prepared by bulk RAFT polymerization of nBA at 70 °C (Figure S1) affording a product with narrow molecular weight distribution ($Đ$ < 1.1 by SEC-MALLS). The PFPA monomer was synthesized with good yields (22 g scale, 85% yield), as reported elsewhere,[21] and it was characterized through ^{1}H and ^{19}F NMR (Figure S3). The polymerization of PFPA was performed in 1,4-dioxane at 70 °C at a high monomer concentration (4 M). The ^{1}H NMR spectra of the obtained polymer (Figure S2B) confirmed complete removal of unreacted monomer and granted a poly(nBA)$_{107}$-*b*-poly(PFPA)$_{248}$ scaffold composition. The ^{19}F NMR spectrum showed three distinct signals corresponding to each of the fluorine atoms in the PFPA pendant groups (Figure S2C). The SEC analysis showed a moderately broad distribution with a "tailing" effect (Figure S2D). Although initially this tailing may suggest an incomplete macro-CTA extension, we propose that the tailing is rather an effect of interactions of the PFPA block with the SEC column, as similar dispersities and tailing phenomena were observed in the SEC traces of PFPA homopolymers that were synthesized under similar conditions but with various small-molecule CTAs (Figure S4).

The poly(nBA)$_{107}$-*b*-poly(PFPA)$_{248}$ scaffold was then dissolved in dry, polar aprotic solvents and reacted with six different primary amines (Figure 1A) to obtain a series of acrylamide (Am) polymers. The primary amines contained nitrogen-based cationic moieties connected through an alkyl spacer; (1-imidazolyl)propyl (ImPAm), 2-morpholinoethyl (MEAm), dimethylaminomethyl (DMAEAm), diethylaminoethyl (DEAEAm), *N*-Boc-aminoethyl (AEAm), and trimethylammonium ethylamine (TMAEAm) were used. Guided by the solubility of the small molecule amines either anhydrous 1,4-dioxane or DMF was used as solvent. In the presence of primary amines, the cleavage of the trithiocarbonate end-group in the diblock scaffold is expected; hence, hydroxyethyl acrylate (HEA) was added as a thiol capping agent in a similar fashion to that reported by Woodfield and co-workers[22] to prevent any side reactions stemming from the presence of a reacting thiol end-group. A key advantage of PFPA is the ability to monitor the extent of its postpolymerization modifications via ^{19}F NMR (Figure 1B).[22−24] Under the optimized conditions (i.e., 40 °C and 18 h), the ^{19}F NMR spectra of the reaction crude showed the complete

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disappearance of the three signals of PFPA and the appearance of new signals shifted upfield attributed to the pentafluorophenol product. The complete disappearance of the ^{19}F NMR signals attributed to the PFPA block was confirmed for all six polymers in the library and was used to justify our assumption of complete modification of the PFPA block (*vide infra*). After purification, complete removal of this side product was also seen.

The resulting polymers were characterized by using a combination of ^{1}H NMR, DMF-SEC-MALLS, and potentiometric titrations (Table 1). The SEC traces of the amphiphilic polycations (Figure S5) preserved the tailing feature observed in the scaffold traces. Moderate dispersities were also seen. The insolubility of poly(nBA)-*b*-ImPAm (entry 2, Table 1) and poly(nBA)-*b*-TMAEAm (entry 7, Table 1) hindered their SEC characterization. Additionally, poly(nBA)-*b*-AEAm, (entry 6, Table 1) was characterized before removing the Boc-protecting groups. Based on the ^{19}F NMR characterization of the reaction crudes, which showed complete conversion of the PFP pendant groups in the reactive ester block, number-average molar masses (M_n) and the weight fractions of the cationic block (f_{cat}) were calculated for each polymer assuming a poly(nBA)$_{107}$-*b*-polycation$_{248}$ composition. The cationic fractions were around 70%, which would suggest the formation of spherical micelles in aqueous environments.[25,26] The pK_a values of the cationic groups in the amphiphilic diblock polymers were measured through potentiometric titrations, in which the polymers were first dissolved in HCl (0.1 M, 0.5 mg mL^{-1} polymer concentration) and titrated with 0.1 M NaOH. Poly(nBA)-*b*-ImPAm (entry 2, Table 1) precipitated out of solution during the titration process hindering obtainment of a pK_a value for this polymer.[27] The obtained pK_a values range from 5.5 for poly(nBA)-*b*-MEAm to 8.2 for poly(nBA)-*b*-AEAm. It could be assumed, based on values reported for other polycations and cationic small molecules,[27] that the value of pK_a for poly(nBA)-*b*-ImPAm is lower than 5.5.

The self-assembly of the amphiphilic polycations in aqueous environments was explored by using commonly reported methods such as cosolvent assisted dissolution,[28] thin-film hydration,[29] and direct dissolution.[28] The use of the two former methods was prevented by the lack of an organic solvent (or mixtures thereof) that could completely solubilize all polymers. This was particularly difficult for poly(nBA)-*b*-AEAm and poly(nBA)-*b*-TMAEAm. Ultrasound-assisted direct dissolution, in which the polymers were first allowed to stir at room temperature for 7 days and then subjected to an ultrasonication treatment for 1 h, afforded species with mean hydrodynamic radii (R_h) below 100 nm and moderate PDI ($\mu_2/\Gamma^2 < 0.2$), as measured via dynamic light scattering (DLS), in all buffers used (20 mM, IS = 100 mM), which had pH values ranging from 1 to 7 (Figure 2A,B).

A trend was observed correlating a decrease in micelle size as the buffer pH was decreased with most polymer micelle solutions. We suggest that the increase in overall hydrophilicity of the polymers, due to increase charge density in the cationic block, is likely the cause of this trend. We observed this change even in the permanently charged poly(nBA)-*b*-TMAEAm. Additionally, because the micelles were prepared by a direct dissolution method, these supramolecular structures are likely

Table 1. Molecular Characteristics of the Polymer Scaffold and the Diblock Polymer Library

polymer	pK_a[a]	$M_{n,NMR}$ (kDa)	f_{cat}[c]	dn/dc[d]	M_{nSEC}[e] (kDa)	$Đ$[e]
poly(nBA)-*b*-poly(PFPA)	N.A	73.2		0.041	56.9	1.26
poly(nBA)-*b*-ImPAm	–[b]	55.1	0.75	–[f]	–[f]	–[f]
poly(nBA)-*b*-MEAm	5.5	59.8	0.77	0.081	41.9	1.15
poly(nBA)-*b*-DMAEAm	7.6	49.4	0.72	0.060	41.3	1.14
poly(nBA)-*b*-DEAEAm	8.1	56.3	0.75	0.064	38.2	1.16
poly(nBA)-*b*-AEAm	8.2	42.4	0.67	0.063[g]	46.5[g]	1.15[g]
poly(nBA)-*b*-TMAEAm	N.A.	61.9	0.78	–[f]	–[f]	–[f]

[a]From potentiometric titrations of the polymer. [b]Polymer precipitates excessively during titration hindering pK_a determination. [c]f_{cat} = weight fraction of cationic block. [d]dn/dc = from 100% mass recovery calculation in SEC. [e]Values from SEC (MALLS, RI detection) in THF for entry 1 and DMF(LiBr) for entries 3–6. [f]SEC was not performed due to solubility problems (entry 7, TMAEAm) or interactions with the column (entry 2, ImPAm). [g]Data correspond to the Boc-protected polymer.

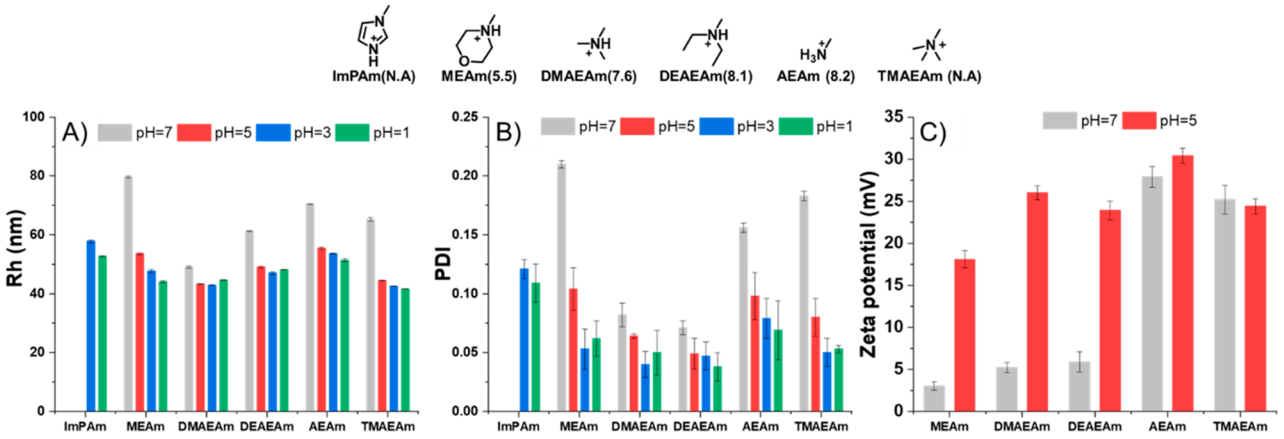

Figure 2. (A) Mean hydrodynamic radii (R_h), (B) polydispersity index (PDI, μ_2/Γ^2), and (C) zeta potential of micelles formulated at 0.1% w/w via direct dissolution with ultrasonication as measured by DLS. The micelles were formulated in buffers with various pH values between 1 and 7. The buffer concentration was 20 mM, and the ionic strength was adjusted to 100 mM with NaCl.

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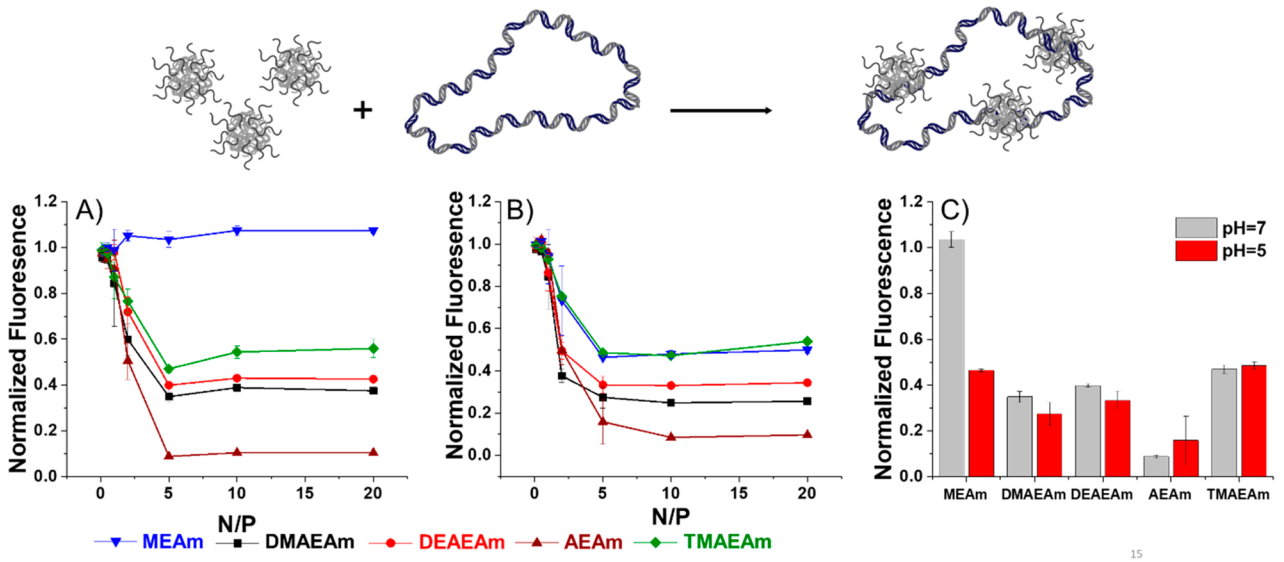

Figure 3. Relative binding strength of micelle formulations to pDNA analyzed through PicoGreen dye exclusion assays. DNA-intercalated PicoGreen dye (plotted as normalized intensity) upon complexation with micelle formulations at different N/P values on (A) MOPS buffer (pH = 7) and (B) acetate buffer (pH = 5). (C) Comparison of the binding strength for all micelles at N/P = 5.

to be nonergodic[28] and their size to be greatly influenced not only by the polymer features, but by the preparation pathway.

The imidazole-based micelles, prepared from poly(nBA)-*b*-ImPAm, were insoluble in buffers with pH > 5—even after 7 days of stirring and the ultrasound treatment. This polymer was thus excluded from the following studies since our ultimate goal is to formulate micelleplexes with pDNA, as this process is commonly done at pH values at or above 5.[8,9,30] Figure 2C displays the zeta potential values of micelles prepared in MOPS and acetate buffers (pH 7 and 5, respectively). As expected, the polymer with the higher pK_a value, poly(nBA)-*b*-AEAm, and the permanently charged poly(nBA)-*b*-TMAEAm showed positive zeta potential values >25, and that did not change appreciably when the pH was changed. In contrast, the three polymers with lower pK_a values show small positive zeta potential values (∼5) when prepared at pH = 7, which increase dramatically when the micelles are prepared in a buffer of pH 5.

Micelleplexes formed between the diblock micelles and pDNA (4.7 kbp) were first explored through dye exclusion assays performed with PicoGreen as the fluorophore at pH 5 and 7. The micelle−pDNA binding strength (interpreted as a decrease in the normalized fluorescence of PicoGreen) was measured as a function of the N/P ratio for all micelles. At pH 7, all polymers, except for poly(nBA)-*b*-MEAm, exhibit an increase in binding strength with increasing N/P ratio up to a value of 5, after which no significant change is observed (Figure 3A). Poly(nBA)-*b*-MEAm is a pH-responsive polymer with a low pK_a value; the MEAm corona-forming block presents a low charge density at pH 7, and thus it is not able to bind pDNA effectively and does not exclude PicoGreen from intercalating with it, even at N/P ratios of 20. The ability of MEAm copolymers to stay in solution (or dispersion when in the form of micelles), even when uncharged, makes these polymers attractive endosomal escape promoters,[13,31,32] in comparison with low-pK_a monomers such as ImPAm which are impaired because of their low solubility. At pH 5 (Figure 3B), all polymers exhibit a similar trend to that observed at pH 7, poly(nBA)-*b*-MEAm included. From the normalized fluorescence values obtained at N/P ratios >5, we observed that the binding strength increases when going from bulkier cations (i.e., MEAm and TMAEAm, with cation molecular volumes of 127 and 112 Å^3, respectively, Table S2) to more compact ones (i.e., AEAm, with a cation molecular volume of 58 Å^3, TableS2) (Figure 3C). Hence, when the polymers are sufficiently charged (i.e., the pH of the buffer is lower than the pK_a of the micelle's cationic corona), the micelle−pDNA binding strength is dictated by how hindered the cations are, and this should be a selection criterion when synthesizing designer polymers for pDNA protection and delivery applications. Within the polymer family, the DEAEAm polymers exhibited a medium binding strength; even though these cations exhibit a large molecular volume (129 Å^3, Table S2), these are the more hydrophobic of the set (highest log *P* value), which might account for this observation.

The hydrodynamic radii distributions of micelleplexes formed in buffers at pH 5 and 7 at an N/P of 5 are shown in Figure 4 (red lines) and are compared with the distributions measured from their parent micelle dispersions (black lines). At pH 7, micelles assembled from poly(nBA)-*b*-MEAm and their mixture with pDNA show an almost identical distribution, suggesting an absence of pDNA−micelle complexation, which agrees with the binding strength experiments (Figure 3A) for these micelles. For all other diblock polymers at pH 7, a broadening and shift toward larger sizes (or multimodal distributions in the case of poly(nBA)-*b*-TMAEAm), were observed. These changes are characteristic of micelleplex formation with the increase in size, suggesting that multiple-micelle micelleplexes were obtained.[31] At pH 5 the shift toward larger particle sizes was observed for all micelles during micelleplex formation. These results coupled with the dye-exclusion data show how complexation can be tailored through the selection of the cationic moiety and suggest how the MEAm can be used as a pH-responsive trigger.

The zeta potential of the micelle dispersions and their complexes with pDNA was measured at N/P of 5 in both MOPS and acetate buffer. At pH 5 micelleplexes and micelles

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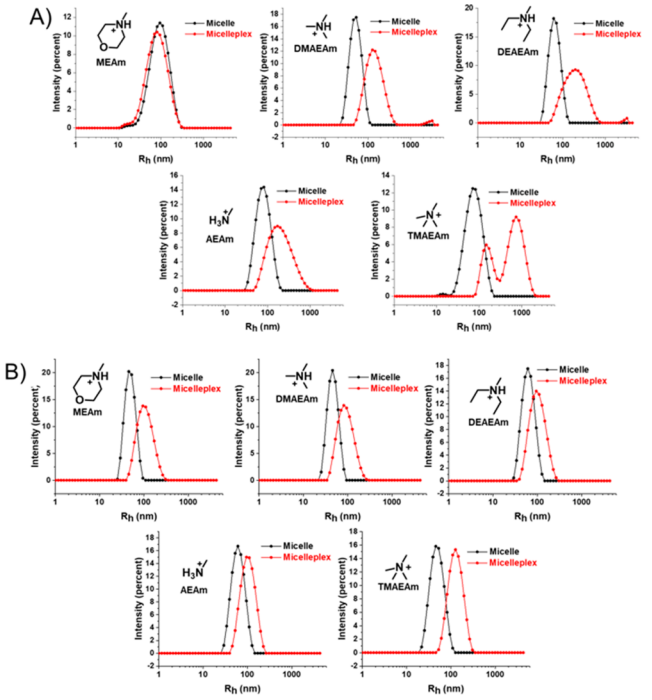

Figure 4. Hydrodynamic radius (R_h) distributions of micelles (black) and micelleplexes (red) formulated at 0.02% w/w in (A) MOPS buffer (pH = 7) and (B) acetate buffer (pH = 5) measured by DLS immediately after pDNA complexation.

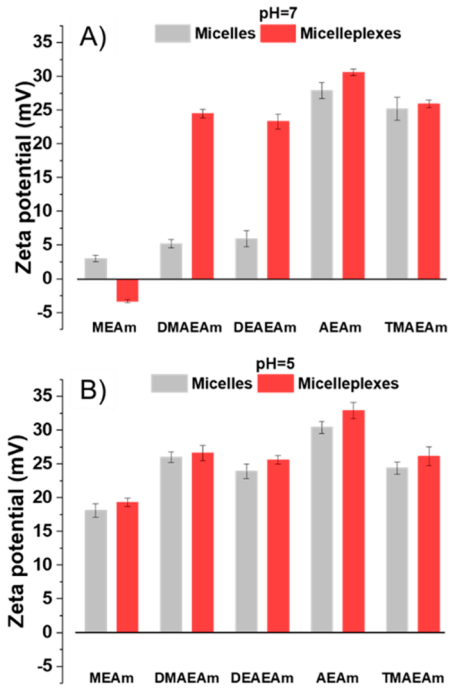

Figure 5. Zeta potential of micelles (gray bars) and micelleplexes (red bars) formulated at 0.02% w/w in (A) MOPS buffer (pH = 7) and (B) acetate buffer (pH = 5) measured by DLS immediately after pDNA complexation.

showed similar positive values of the zeta potential. The N/P value of 5 coupled with a high charge density expected due to the low pH value can explain why no significant change is observed and how the zeta potential of the complex is dominated by the species present in excess.[33,34]

At pH 7 three different cases were seen. For micelles and micelleplexes formed with poly(nBA)-*b*-MEAm a zeta potential close to zero was observed, again suggesting that the low charge density of the corona of these micelles is responsible for their inability to effectively complex pDNA at this pH value. For highly charged poly(nBA)-*b*-AEAm and poly(nBA)-*b*-TMAEAm the zeta potentials of micelles and micelleplexes are almost identical, suggesting that for these high-pK_a moieties a change of pH from 5 to 7 does not impair their ability to bind pDNA. Micelles formed from poly(nBA)-*b*-DMAEAm and poly(nBA)-*b*-DEAEAm showed a small positive zeta potential, which increased toward more positive values during micelleplex formation. Combined with the binding strength and DLS (Figures 3 and 4), experiments show evidence of pDNA complexation by these micelles at pH = 7; the increase in zeta potential is attributed to the formation of complexes with larger sizes than the micelles and, again, where the excess component on the complex is driving the zeta potential.

In conclusion, our work highlights the potential of using different cationic chemistries to tailor the physical and chemical properties of micelleplexes as promising gene packaging agents and carriers. We demonstrated a synthetic approach combining controlled radical polymerization with a postpolymerization modification to obtain a small library of poly(*n*-butyl acrylate)-*b*-poly(cationic acrylamide) diblock polycations with identical numbers of repeating units in each block. The assembly of these diblock polymers into nanometer-sized structures was achieved through an ultrasound-assisted direct-dissolution method, performed in buffers of pH values from 1 to 7. The average size and size distribution of the obtained micelles were a function of the buffer's pH.

A combination of pDNA binding, size, and zeta potential measurements during the micelleplex formation process revealed how the combination of buffer pH, micelle pK_a, and bulkiness of the cations controls the ability of these micelles to bind pDNA. Micelles formed with poly(*n*-butyl acrylate)-*b*-poly(MEAm) micelleplexes were only achieved at pH = 5. Thus, having a buffer pH lower than the micelle's pK_a is a requirement for successful complexation. For all other micelles, where buffer pH was always lower that the micelle's pK_a, the cation bulkiness in the micelle corona was the parameter that controlled pDNA binding and micelleplex formation. We observed an inverse correlation where micelles with coronas composed of more compact cations bound stronger to pDNA. This work highlights how both the bulkiness and pK_a values of the cationic pendant groups in diblock polymer micelles combine to define how strongly a cationic micelle binds to pDNA, thus affecting the formation, size, and zeta potential of micelleplexes. Ongoing efforts are aimed to explore the binding of these novel micellar vectors with other biological payloads of these novel micellar vectors with other biological payloads along with biological delivery experiments.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmacrolett.2c00015.

^{1}H NMR spectra of representative polymers, SEC-MALS, gel electrophoresis, dye exclusion, and experimental procedures and data (PDF)

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Notes

The authors declare no competing financial interest.

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