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Diblock copolymers with tunable pH transitions for gene delivery

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Abstract

A series of diblock copolymers containing an endosomal-releasing segment composed of diethylaminoethyl methacrylate (DEAEMA) and butyl methacrylate (BMA) were synthesized via reversible addition-fragmentation chain transfer (RAFT) polymerization. The materials were designed to condense plasmid DNA (pDNA) through electrostatic interactions with a cationic poly(N,N-dimethylaminoethyl methacrylate) (DMAEMA) first block. The pDMAEMA was employed as a macro chain transfer agent (macroCTA) for the synthesis of a series in which the relative feed ratios of DEAEMA and BMA were systematically varied from 20% to 70% BMA. The resultant diblock copolymers exhibited low polydispersity ($PDI \leq 1.06$) with similar molecular weights ($M_n = 19.3 - 23.1$ kDa). Dynamic light scattering (DLS) measurements in combination with 1H -NMR D_2O studies demonstrated that the free copolymers assemble into core-shell micelles at physiological pH. Reduction of the solution pH to values representative of endosomal/lysosomal compartments induced an increase in the net cationic charge of the core through protonation of the DEAEMA residues. This protonation promotes micelle destabilization and exposure of the hydrophobic BMA residues that destabilize biological membranes. The pH value at which this micelle-to-unimer transition occurred was dependent on the hydrophobic content of the copolymer, with higher BMA-containing copolymer compositions exhibiting pH-induced transitions to the membrane-destabilizing state at successively lower pH values. The ability of the diblock copolymers to deliver pDNA was subsequently investigated using a GFP expression vector in two monocyte cell lines. High levels of DNA transfection were observed for the copolymer compositions exhibiting the sharpest pH transitions and membrane destabilizing activities, demonstrating the importance of tuning the endosomal-releasing segment composition.

1. Introduction

Gene therapy and DNA-based vaccines offer significant therapeutic potential but safe, efficacious delivery systems are still needed to enable clinical applications [1,2]. Cationic lipids and polymers have been extensively investigated as non-viral carriers of plasmid DNA (pDNA) due to potential advantages in scalability of production, improved safety profile, and low immunogenicity [3-5]. Cationic polymers include poly(dimethylaminoethyl methacrylate) (pDMAEMA) [6-14], poly(ethylenimine) (PEI) [15-28], and poly(L-lysine) (PLL) [29-35]. The barrier of endosomal escape has been a special challenge for nonviral delivery systems [36], and a variety of pH-responsive polymers [37-39] and lipids [40-42]

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have been developed that exploit the pH gradients formed in the intracellular vesicular trafficking pathways.

Cationic micelles prepared from amphiphilic block copolymers offer a means to preserve the DNA-condensing activity of polycations while introducing pH-sensitive functionalities to overcome the endosomal/lysosomal intracellular barrier [43,44]. Through the use of controlled radical polymerization (CRP) techniques, the synthesis of well-defined polymer architectures can be achieved. Both reversible addition-fragmentation chain transfer (RAFT) polymerization [11,45] and atom transfer radical polymerization (ATRP) [46] have been utilized to develop such multiblock micellar systems. For example, You et al. have designed diblock copolymers consisting of pDMAEMA and poly(N-isopropylacrylamide) (pNIPAM) that assembled into core-shell micelles with pDMAEMA acting as the stabilizing, hydrophilic component [47]. The authors demonstrated that changes in the protonation state of pDMAEMA affected micelle stability as observed by a shift in the phase transition temperature. pDMAEMA exhibits a relatively low charge density, as compared to other polycations, due to the presence of a tertiary amine that is approximately 50% protonated at physiological pH although toxicity issues remain [48-52]. A similar polymer, poly(diethylaminoethyl methacrylate) (pDEAEMA), has a predominately hydrophobic character at physiological pH while retaining a tertiary amine. Tang et al. first demonstrated that pDEAEMA could be used to drive micelle formation of triblock copolymers in an aqueous environment and that destabilization of the particles occurred in a pH-dependent manner [46].

Recently, we described the synthesis of a family of diblock copolymer small-interfering RNA (siRNA) carriers composed of a positively-charged block of pDMAEMA to mediate siRNA binding and a second pH-responsive endosomal releasing block composed of DMAEMA and propylacrylic acid (PAA) in roughly equimolar ratios, and butyl methacrylate (BMA) [11,53]. These materials self-assemble to form micelles at physiological pH values, but upon exposure to the low pH environment of the endosome undergo a pH-induced conformational change rendering them highly membrane destabilizing. Here, we detail the development of a class of copolymer micelles that are capable of mediating endosomal escape of plasmid DNA therapeutics. These materials incorporate DEAEMA as a pH-sensitive switch that activates hydrophobic membrane-interactive BMA residues upon exposure to low pH environments.

2. Materials and Methods

2.1. Materials

Materials were supplied by Sigma-Aldrich (St Louis, MO) unless otherwise specified. 2,2'-Azobis(4-methoxy-2,4-dimethyl valeronitrile) (V70) and 1,1'-Azobis(cyclohexane-1-carbonitrile) (V40) were obtained from Wako Chemicals USA, Inc. (Richmond, VA). pDNA gWiz-GFP was obtained from Aldevron LLC (Fargo, ND). Lipofectamine 2000 (LF) was obtained from Invitrogen (Carlsbad, CA). 4-Cyano-4-(ethylsulfanyltiocarbonyl) sulfanylpentanoic acid (ECT) was synthesized as described previously [54]. DMAEMA, DEAEMA, and BMA were distilled prior to use. RAW 264.7 (murine leukaemic monocyte macrophage cell line) (ATCC) cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) High Glucose containing L-glutamine (GIBCO) supplemented with 1% penicillin-streptomycin (GIBCO) and 10% fetal bovine serum (FBS, Invitrogen), JAWSII (murine dendritic cell line) (ATCC) cells were maintained in Minimum Essential Medium α Medium (α MEM, GIBCO) supplemented with 4 mM L-glutamine (Lonza), 5 ng/ml recombinant mouse granulocyte macrophage-colony stimulating factor (Peprotech), 20% heat-inactivated FBS (GIBCO), and 1% penicillin-streptomycin, and passaged using 0.25% trypsin-EDTA (GIBCO). All cells were cultured at 37 °C and 5% CO₂.

2.2. Synthesis of poly(dimethylaminoethyl methacrylate) macro chain transfer agent (pDMAEMA macroCTA)

The RAFT polymerization of DMAEMA was conducted in dioxane at 30 °C under a nitrogen atmosphere for 18 h using ECT and V70 as the chain transfer agent (CTA) and radical initiator, respectively. The initial CTA to monomer molar ratio ($[CTA]_0/[M]_0$) was designed so that the theoretical M_n at 100% conversion was approximately 10,000 g/mol (degree of polymerization (DP) of 65). The initial CTA to initiator molar ratio ($[CTA]_0/[I]_0$) was 20 to 1. The resultant pDMAEMA macro chain transfer agent (macroCTA) was isolated by precipitation into pentane. The polymer was then redissolved in acetone and subsequently precipitated into pentane (×3) and dried overnight in vacuo.

2.3. Block copolymerization of DEAEMA and BMA from a pDMAEMA macroCTA

The desired stoichiometric quantities of DEAEMA and BMA were added to pDMAEMA macroCTA dissolved in dioxane (57 wt% monomer and macroCTA to solvent). For all polymerizations $[M]_0/[CTA]_0$ and $[CTA]_0/[I]_0$ were 100:1 and 20:1, respectively. Following the addition of V40 the solutions were purged with nitrogen for 30 min and allowed to react at 90 °C for 6 h. The resultant diblock copolymers were isolated by precipitation into cold hexanes. The copolymers were further purified by dissolution into ethanol followed by addition into 1X DPBS (final ethanol concentration of 10 vol% and copolymer concentration of ~10 mg/mL). The copolymers were isolated from this solution via chromatographic separation with a PD-10 desalting column (GE Healthcare, Piscataway, NJ), followed by lyophilization to obtain the final copolymer. Aqueous stock solutions were prepared by dissolution of the dry copolymers in ethanol followed by dropwise addition into 10 mM, pH 7.0 sodium phosphate buffer (with 100 mM NaCl) at 2 mg/mL and 5 wt% ethanol. Both the pDMAEMA macroCTA and diblock copolymers were analyzed by 1H -NMR ($CDCl_3$) spectroscopy (Bruker AV 500). Representative NMR spectra can be found in the Supplementary Information (Fig. S-2).

2.4. Gel permeation chromatography

Gel permeation chromatography (GPC) was used to determine molecular weights and polydispersities (M_w/M_n , PDI) of both the pDMAEMA macroCTA and diblock copolymer samples. SEC Tosoh TSK-GEL R-3000 and R-4000 columns (Tosoh Bioscience, Montgomeryville, PA) were connected in series to a Agilent 1200 series (Agilent Technologies, Santa Clara, CA), refractometer Optilab-rEX and triple-angle static light scattering detector miniDAWN TREOS (Wyatt Technology, Santa Barbara, CA). HPLC-grade DMF containing 0.1 wt% LiBr at 60 °C was used as the mobile phase at a flow rate of 1 mL/min. The molecular weights of each polymer were determined using a multi-detector calibration based on dn/dc values calculated separately for each homopolymer and copolymer composition.

2.5. Formation of copolymer/pDNA polyplexes and lipoplexes

Copolymer/pDNA polyplexes were formed by combining equal volumes of pDNA (0.1 mg/ml in molecular biology grade water) and copolymer solutions (in Dulbecco's phosphate-buffered saline, pH 7.4 (PBS)) for 30 min at room temperature. Lipoplexes were formed by combining pDNA with Lipofectamine 2000 at a 3:1 v/w LF:DNA ratio in serum-free media in accordance with the manufacturer's protocol.

2.6. Gel retardation assay

The charge ratio (+/-) at which the diblock copolymers mediate complete pDNA condensation was determined via a gel retardation assay. The charge ratio is the molar ratio between protonated DMAEMA tertiary amines (assuming 50% protonation at physiological

pH) and phosphate groups along the pDNA backbone. Copolymer/pDNA polyplexes were formulated with 0.5 μg pDNA for 30 min followed by 30 min incubation in the presence of FBS (final FBS concentration of 10%). A 0.7% (w/v) agarose gel was loaded with each lane containing a separate treatment and subsequently run at 90V for one hour. The gels were stained with SYBR Gold prior to fluorescence visualization.

2.7. Dynamic light scattering (DLS)

The sizes of free diblock copolymer micelles and copolymer/pDNA polyplexes were determined by DLS measurements using a Malvern Zetasizer (Worcestershire, UK). Free copolymer measurements were performed at a polymer concentration of 100 $\mu\text{g}/\text{mL}$ while polymer/pDNA particles were analyzed at a pDNA concentration of 5 $\mu\text{g}/\text{mL}$. All measurements were performed in the presence of 150 mM NaCl at 37°C. Mean diameters are reported as the number average.

2.8. Hemolysis assay

The potential for the free copolymer to disrupt endosomal membranes was assessed by a hemolysis assay. The protocol followed here has been described previously [38]. Briefly, polymer was incubated for one hour in the presence of erythrocytes at 20 $\mu\text{g}/\text{mL}$ in 100 mM sodium phosphate buffers (supplemented with 150 mM NaCl) of varying pH (7.4, 7.0, 6.6, 6.2, and 5.8) intended to mimic the acidifying pH gradient to which endocytosed material is exposed. The extent of cell lysis (i.e. hemolytic activity) was determined by detecting the amount of released hemoglobin via absorbance measurements at 492 nm.

2.9. ^1H -NMR D_2O titration

^1H -NMR spectroscopy (Bruker AV 500, D_2O) was used to probe solvation of the diblock copolymer segments as a function of pH, thereby discriminating micelle and unimer conformations. Aqueous copolymer solutions were diluted into D_2O from ethanol stocks and titrated separately to three pD values (7.4, 6.6, and 5.8) using sodium deuteroxide and deuterium chloride. pD values were calculated using the following correlation: $\text{pD} = \text{pH} \cdot 1.06831$ [55].

2.10. In vitro transfections

RAW 264.7 or JAWSII cell lines were seeded in 24-well plates in 1 mL complete media ($2 \cdot 10^5$ cells/well in DMEM/10% FBS/antibiotics or $1.5 \cdot 10^5$ cells/well in αMEM /20% FBS/antibiotics, respectively) and cultured for 24 h to 70% confluency. Polyplexes and lipoplexes were formulated as described above. Cells were washed once with PBS and incubated with polyplexes/lipoplexes at 1 μg DNA/well in 200 μL antibiotic-free media for 4 h at 37 °C. Cells were then washed and the media replaced with 500 μL complete media for an additional 44 h prior to analysis.

2.11. Flow cytometry analysis of gene expression

RAW 264.7 cells were incubated for 10 min at room temperature in PBS-based cell dissociation buffer (GIBCO) and collected by vigorous washing. JAWSII cells were collected by trypsinization. Cells were resuspended in PBS containing 2% FBS and 0.2 $\mu\text{g}/\text{mL}$ propidium iodide (PI, Invitrogen). GFP expression data was acquired on a BD FACScan flow cytometer (BD Biosciences). 10,000 events gated on viable PI-negative cells were collected per sample and analyzed in FlowJo (TreeStar).

2.12. Lactate dehydrogenase cytotoxicity assay

Cytotoxicity was evaluated by a lactate dehydrogenase (LDH) cytotoxicity detection kit (Roche). Cells were seeded at 8×10^4 cells/well (RAW 264.7) or 6×10^4 cells/well

(JAWSII) in 48-well plates in 400 μ L complete media and cultured for 24 h. Cells were then washed and polyplexes/lipoplexes at 0.4 μ g DNA/well were added in 200 μ L antibiotic-free media. Cells were incubated for 24 h, washed, then lysed in 400 μ L of RIPA Lysis Buffer (Pierce) at 4 $^{\circ}$ C for at least 1 h. Lysates were diluted 2:3 in PBS in a 96-well plate (total volume 100 μ L), combined with 100 μ L of LDH substrate solution, incubated for 10-20 min at room temperature, and absorbance measurements recorded at 490 nm (reference 650 nm).

2.13. Statistical Analysis

ANOVA was used to test for treatment effects, and Tukey's test was used for post hoc pairwise comparisons between individual treatment groups.

3. Results

3.1. Diblock copolymer synthesis and characterization

A series of diblock copolymers were synthesized according to Scheme 1 consisting of two blocks: a DMAEMA homopolymer and a statistical copolymer composed of BMA and DEAEMA at varying monomer feed ratios, from 20% to 70% BMA. The pDMAEMA homopolymer (7,400 g/mol; DP 47) was employed as a macroCTA in the subsequent copolymer synthesis (Table 1, Fig. S-1). The diblock copolymer series exhibited low polydispersity ($PDI \leq 1.06$) with similar molecular weights ($M_n = 19.3 - 23.1$ kDa) and compositions close to the monomer feed ratios.

3.2. pH-responsive transitions of diblock copolymers

The diblock copolymers were designed to undergo a pH-triggered phase transition, shifting the equilibrium from a micelle to unimer conformation as pH decreased. The tunability of the pH transition was controlled by varying the hydrophobic content of the second block. Particle size measurements were conducted for solutions of the diblock copolymers across a pH range of 7.4 to 5.8 (Fig. 1). These pH values were selected to mimic the physiological trafficking of the materials from the extracellular space to acidic endosomal/lysosomal compartments. At physiological pH, each copolymer formed particles of approximately 20 nm in diameter. As the copolymers were exposed to more acidic conditions they were found to sharply transition to unimers. The pH at which this transition occurred was found to be strongly dependent on the relative amounts of BMA and DEAEMA present in the core. Higher BMA content shifted the transition to lower pH values. The copolymer that contained 20% BMA underwent a structural transition from micelles at pH values of 7.4 to unimers at pH 7.0 and below, while the 60% BMA copolymer transitioned at a pH values of 6.2. The copolymer with 70% BMA content did not undergo a phase transition from micelles to unimers over the pH range investigated (i.e. pH 7.4 – 5.8). The optimal pH-induced phase transition behavior was observed for the 40% BMA copolymer, where micelle-sized particles were observed at pH values near physiological conditions (i.e. pH 7.4 and 7.0) with a sharp transition to unimers at early (pH = 6.6) and late endosomal (pH 5.8) pH values.

The hemolytic activity of the diblock copolymers as a function of pH was found to be highly correlated with the intrinsic structural transition properties (Fig. 2). In this assay, human red blood cells were incubated with the copolymer in buffers over a pH range of 7.4 – 5.8. For all copolymers investigated, negligible hemolytic activity was observed at pH 7.4, which suggests that the micellar architecture does not display membrane-destabilizing segments in the necessary protonation state. The pH at which high levels of membrane destabilization were observed correlated well with the phase transition pH values determined by DLS. These studies taken together demonstrate that the highest levels of membrane destabilization occur at or below the transition pH from micelles to unimers where significant interaction of

the copolymer core segment with the biological membrane is possible. Consistent with DLS measurements, the 40% BMA copolymer showed optimal pH-dependent hemolysis with a remarkably sharp transition from non-hemolytic at pH values above 7.0 to strongly membrane disruptive at pH 6.6 and below. A similar class of pH-responsive materials, methacrylic acid copolymers, also exhibit improved membrane destabilizing activity upon the incorporation of hydrophobic units, e.g. ethyl acrylate, further illustrating the importance of polymer hydrophobicity on biological activity [56].

Copolymers containing low BMA content showed negligible hemolytic activity even at pH values where the copolymers were unimers. Conversely, the copolymer with 70% BMA content does not undergo a phase transition from micelles to unimers and does not show significant hemolytic activity at any of the pH values examined. These results are consistent with our previous micellar delivery systems where a minimum hydrophobic content is required to disrupt biological membranes [11].

3.3. $^1\text{H-NMR}$ D_2O titration

To further evaluate the structure copolymers adopt in solution as a function of pH, $^1\text{H-NMR}$ was performed on the 40% and 70% BMA copolymer compositions and pDMAEMA homopolymer in D_2O at three pD values: 7.4, 6.6, and 5.8 (Fig. 3). The pDMAEMA homopolymer exhibits three distinctive resonances between 2.6-4.7 ppm at pD 7.4: δ 2.8-2.9 methyl (CH_3NHCH_3), δ 3.4 methylene (CH_2NH), and δ 4.3 methylene (OCH_2). Upon decreasing the solution pD to 6.6 and 5.8, the peaks at δ 2.8-2.9 and 3.4 shift downfield to δ 3.0 and 3.6, respectively, as a result of less effective shielding of the hydrogen atoms from the electron pair of the protonated nitrogen atom [57]. At pD 7.4, the copolymer spectra resembles that of the DMAEMA homopolymer. When the pD is decreased to 6.6, a peak attributed to DEAEMA methyl protons (δ 3.3, CH_3NHCH_3) evolves for the 40% BMA composition but is absent for the 70% BMA composition suggesting solvation of the copolymer core for the former composition. At pD 5.8, this peak is present for both copolymers. These results demonstrate that the copolymers adopt a core-shell micelle conformation in an aqueous environment which destabilize in a pH-responsive, composition-dependent manner due to solvation of the core-forming segment.

3.4. Copolymer/pDNA polyplex characterization

The ability of the diblock copolymers to condense plasmid DNA into serum-stable nanoparticles as a function of charge ratio (+/-) was assessed by performing a gel retardation assay (Fig. S-2) and by measuring particle size by dynamic light scattering (DLS, Fig. 4). At all charge ratios investigated (+/- = 1 to 4), the copolymers were able to completely condense plasmid DNA. At charge ratios of 4, the following diameters (nm) were reported for copolymer compositions from 20-70% BMA, respectively: 250 ± 60 , 200 ± 50 , 260 ± 80 , 180 ± 50 , 250 ± 60 , and 260 ± 80 . There appeared to be no significant influence of composition on the ability of the copolymers to condense pDNA. This finding is likely a result of the common pDMAEMA condensing segment shared by the materials and provides strong evidence that the DEAEMA component does not electrostatically interact to a significant degree with the plasmid DNA at physiological pH values.

3.5. Evaluation of plasmid DNA transfection activity

The transfection activities of the diblock copolymer carriers in antigen-presenting cells were evaluated in RAW 264.7 murine macrophages and JAWSII murine dendritic cells (Fig. 5). Cells were treated with copolymer/DNA polyplexes formulated at theoretical charge ratios of 2 in the presence of serum (10% for RAW 264.7, 20% for JAWSII cells) and GFP expression was quantified 48 h post-transfection by flow cytometry. In RAW 264.7 cells, all copolymers incorporating less than 60% BMA produced high transfection efficiencies

comparable to or exceeding that of the commercial liposomal agent, Lipofectamine 2000. The highest efficiencies were obtained with the 30% and 40% BMA copolymers, which elicited more than double the transfection efficiency obtained using Lipofectamine (44% and 48% vs. 17%). Copolymers with 60% and 70% BMA produced very low levels of GFP expression. Absolute transfection efficiencies were lower in the JAWSII cells, but all of the copolymer carriers outperformed Lipofectamine. The 30% BMA polymer mediated the highest transfection activity with a 9-fold increase in efficiency relative to Lipofectamine. Overall, copolymers that underwent micelle to unimer transitions at endosomal pH values (20-50% BMA) were the most effective vehicles for intracellular pDNA delivery, while copolymers that remained micellar at these pH values (60-70% BMA) were deficient in mediating transfection.

3.6. Copolymer/pDNA polyplex cytotoxicity

Carrier cytotoxicity was evaluated by incubating the cells with polyplex solutions for 24 h, then quantifying cell viability by measuring the total LDH content relative to untreated cells (Fig. 6). All copolymer carriers displayed comparable or improved toxicity compared to Lipofectamine in both cell lines. In RAW 264.7 cells, viability was high ($\geq 70\%$) across the series of copolymer compositions while Lipofectamine exhibited 55% cell viability. In JAWSII cells, high toxicity was observed in Lipofectamine-treated cells ($<10\%$ viability), while polyplexes were associated with moderate to low toxicity (54-85% viability). Polyplex toxicity was generally found to increase with decreasing BMA content in both cell lines, a trend that inversely follows transfection activity.

4. Discussion

The diblock copolymer design investigated in these studies is comprised of two distinct polymeric segments with discrete functions (Scheme 1, Table 1, Fig. S-1). The first block is a homopolymer of DMAEMA, a monomer possessing a tertiary amine that is approximately 50% protonated at physiological pH when it is within a polymeric backbone [49]. The cationic nature of this block allows for electrostatic interactions to be made with anionic phosphate groups of nucleic acids, an activity which has been demonstrated previously with DMAEMA polymers [6,7,9,11]. The pDMAEMA segment also provides significant hydrophilic stabilization of the resultant polyplexes. The second block is a statistical copolymer containing DEAEMA and BMA with pH-responsive endosomolytic activity. This block is intended to possess a predominantly hydrophobic character due to the BMA component in addition to deprotonated DEAEMA residues.

The inclusion of DEAEMA in the core-forming segment serves as a pH-sensitive trigger. Upon a decrease in pH, the tertiary amine on this residue protonates to increase the positive charge density within the micelle interior. This leads to electrostatic repulsion between adjacent polymer chains, resulting in micelle destabilization once a sufficient charge density is reached. Vamvakaki et al. demonstrated that hydrophilic block copolymers containing a pDEAEMA segment lost micellar structure upon increasing the degree of amine group ionization past 10% [57]. The relative proportion of hydrophobic components and protonatable amines within the core-forming segment of our copolymer design strongly tunes micelle stability as a function of pH. As the hydrophobic content in the second block increased, greater acidic conditions were necessary to destabilize micellar particles (Fig. 1). With the exception of the 70% BMA composition, all copolymers had lost particulate structure by pH 5.8.

The hemolytic activity closely correlated with the copolymer structural transition as significant red blood cell lysis was only observed at pH values where micellar particles were not detected (Fig. 2). These findings are indicative of an enhanced exchange between a

micelle and unimer conformation as the copolymers are exposed to acidifying conditions. At physiological pH, the copolymers were unable to mediate significant hemolysis but once exposed to pH environments supporting unimer evolution, significant lysis was observed.

The proposed core-shell micelle conformation of these copolymers was further validated by $^1\text{H-NMR}$ D_2O titration studies (Fig. 3). At pD 7.4, the diblock copolymer spectra resemble that of the DMAEMA homopolymer suggesting that the pDMAEMA block forms a solvated corona while the second block is collapsed due to entropically-driven hydrophobic interactions. At an intermediate pD value of 6.6, a peak attributed to DEAEMA residues within the core block emerges in the 40% BMA copolymer but is absent in the 70% BMA copolymer. The previous DLS and hemolysis findings provide evidence that the former composition adopts a unimeric conformation in this environment while the latter retains micelle characteristics. This result confirms that solvation of the core block is driving this pH-dependent micelle destabilization into unimers.

Based on these initial findings, we anticipated that the activity of these copolymers could be exploited to facilitate intracellular delivery of pDNA by condensing the nucleic acid into serum-stable nanoparticles via electrostatic interactions with the DMAEMA homopolymer block. DLS sizing data showed that at charge ratios of 2 and greater, approximately 200 nm particles were formed for each of the copolymer compositions (Fig. 4). We found that copolymer compositions that were not membrane-interactive at intermediate pH values (7.0 to 6.2) were unable to mediate significant GFP expression. Copolymers that destabilized into unimers at these pH values exhibited not only significant expression but were also more active than the commercial standard, Lipofectamine 2000. The 30% and 40% BMA compositions were found to be effective at transfecting RAW 264.7 cells while the latter copolymer had the highest activity in JAWSII cells. These findings suggest that optimizing the specific pH at which the copolymer carriers transition from inert to endosomolytic states is critical to attaining maximal transfection activity. Furthermore, the copolymer design allows for the pH-responsive profile to be modulated by adjusting the relative monomer feeds in the second block polymerization. This tunability could be advantageous in optimizing carriers for cell types that exhibit different endosomal/lysosomal pH evolution.

5. Conclusions

A series of diblock copolymers containing a pH-responsive endosomal-releasing segment composed of various ratios of diethylaminoethyl methacrylate (DEAEMA) and butyl methacrylate (BMA) were synthesized via reversible addition-fragmentation chain transfer (RAFT) polymerization. These diblock copolymers self-assemble into micelles at physiological pH but undergo a pH-induced phase transition at lower pH values. The pH at which this phase transition occurs can be precisely tuned by modification of the BMA content. Diblock copolymers with 30 – 40 % BMA content exhibited phase transitions at pH values that are similar to those encountered in the early and late endosomes. These materials showed significant levels of red blood cell lysis at these pH values but negligible cell lysis under physiological conditions. High levels of DNA transfection were observed for these materials highlighting their potential to exploit unique endolysosomal trafficking pathways specific to individual cell types, thereby providing a tunable gene delivery platform.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Particle size measurements of free copolymers as a function of pH via DLS. All measurements were performed at a copolymer concentration of 1 mg/mL in 100 mM sodium phosphate buffer with 150 mM NaCl. Diameter values were determined from the lognormal number average. Error bars represent the standard deviation as calculated from the polydispersity index (PDI) of the particles.

Figure 2. Hemolytic activity of diblock copolymers at a concentration of 20 µg/mL. Hemolytic activity is normalized relative to a positive control, 1% v/v Triton X-100, and the data represent a single experiment conducted in triplicate $\pm$ standard deviation.

Figure 3.
¹H-NMR spectra of pDMAEMA homopolymer, 40%, and 70% BMA diblock copolymers in D₂O from 4.7 to 2.6 ppm at three different pD values.

Figure 4.

Particle size measurements of diblock copolymer/pDNA polyplexes as a function of charge ratio (+/-) by DLS. Mean diameter was determined from the lognormal size distribution. Data are compiled from one experiment with each sample run in triplicate. Error bars represent the standard deviation as calculated from the polydispersity index (PDI) of the particles.

Figure 5.

In vitro transfection efficiencies in RAW 264.7 (A) and JAWSII (B) cells. Copolymer/pDNA polyplexes were formulated at theoretical charge ratios of 2. LF corresponds to treatment with Lipofectamine/pDNA. Data are from a single experiment run in triplicate with the error bars representing the standard deviation. Statistical significance was evaluated at a level of $p < 0.05$ with the following symbols indicating significance as compared to LF, 20%, 30%, 40%, 50%, 60%, and 70% BMA copolymers, respectively: *, @, #, \$, %, ^, &.

Figure 6.

Cytotoxicity of polyplexes in RAW 264.7 (A) and JAWSII (B) cells. Polyplexes were prepared at theoretical charge ratios of 2. Data are from a single experiment run in triplicate with the error bars representing the standard deviation. Statistical significance was evaluated at a level of $p < 0.05$ with the following symbols indicating significance as compared to LF, 20%, 30%, 40%, 50%, 60%, and 70% BMA copolymers, respectively: *, @, #, \$, %, ^, &.

Scheme 1.

RAFT-mediated synthesis of a diblock copolymer consisting of a cationic poly(DMAEMA) block and an endosomolytic hydrophobic block incorporating DEAEMA and BMA at varying molar feed ratios.

Table 1

Molecular weights, polydispersities, and monomer compositions for polymer designs.

Polymer	Theoretical ^a % BMA	2 nd block	Experimental ^b % BMA	2 nd block	2 nd block M_n^c (g/mol)	Total M_n^c (g/mol)	PDI ^c (M_w/M_n)
P1	20		20		12600	20000	1.06
P2	30		27		15700	23100	1.04
P3	40		39		13200	20600	1.06
P4	50		48		14700	22100	1.03
P5	60		57		13200	20600	1.03
P6	70		70		11900	19300	1.04

^a Calculated molar feed ratio

^b As determined by ¹H-NMR (CDCl₃) spectroscopy (Bruker AV 500) (Fig. S-1)

^c As determined by GPC.