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# Development of a novel endosomolytic diblock copolymer for siRNA delivery

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## Abstract

The gene knockdown activity of small interfering RNA (siRNA) has led to their use as target validation tools and as potential therapeutics for a variety of diseases. The delivery of these double-stranded RNA macromolecules has proven to be challenging, however, and in many cases, is a barrier to their deployment. Here we report the development of a new diblock copolymer family that was designed to enhance the systemic and intracellular delivery of siRNA. These diblock copolymers were synthesized using the controlled reversible addition fragmentation chain transfer polymerization (RAFT) method and are composed of a positively-charged block of dimethylaminoethyl methacrylate (DMAEMA) to mediate siRNA condensation, and a second endosomal-releasing block composed of DMAEMA and propylacrylic acid (PAA) in roughly equimolar ratios, together with butyl methacylate (BMA). A related series of diblock compositions were characterized, with the cationic block kept constant, and with the ratio of DMAEMA and PAA to BMA varied. These carriers became sharply hemolytic at endosomal pH regimes, with increasing hemolytic activity seen as the percentage of BMA in the second block was systematically increased. The diblock copolymers condensed siRNA into 80–250 nm particles with slightly positive Zeta potentials. SiRNA-mediated knockdown of a model protein, namely glyceraldehyde 3-phosphate dehydrogenase (GAPDH), in HeLa cells generally followed the hemolytic activity trends, with the most hydrophobic second block (highest BMA content) exhibiting the best knockdown. This pH-responsive carrier designed to mediate endosomal release shows significant promise for the intracellular delivery of siRNA.

### Keywords

pH-responsive polymers; siRNA delivery; RAFT polymerization; intracellular delivery

## Introduction

The efficient and safe delivery of small interfering RNA (siRNA) is a significant challenge to its development as a clinical therapy [1–4]. Carriers for siRNA delivery usually consist of cationic polymers, peptides or lipids that form complexes with the nucleic acid, protecting it from nuclease attack, and facilitating cell uptake through electrostatic interactions with negatively-charged phospholipid bilayers or through specific targeting moieties [5–13]. A variety of synthetically and biologically-derived polymers have been investigated for use as nucleic acid carriers including poly(dimethylaminoethyl methacrylate) (pDMAEMA) [14–17], poly(L-lysine) [18–23], polyethylenimine (PEI) [24–29], and chitosan [30–32]. While many cationic polymers are highly efficient at nucleic acid delivery, significant cytotoxicity

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is observed [33–35]. In addition, anionic serum proteins can interact with net positively-charged siRNA/polycation complexes and cause aggregation or decomplexation, significantly reducing or ablating siRNA efficacy [36].

Once siRNA is endocytosed, the predominant fate is enzymatic degradation in the lysosome or recycling and extracellular clearance [37]. In order to circumvent this fate, several strategies have been employed to enhance endosomal escape. pH responsive lipid or lipid-like molecules and viral fusogenic proteins and peptides promote endosomal escape by becoming membrane destabilizing through a pH-dependent shift in their conformation [5, 8–13, 38–40]. In an effort to mimic viral endosomal escape mechanisms that trigger membrane destabilization at acidic pH, polymers that possess pH-sensitive chemical functionalities, such as carboxylate groups, have been explored [41–45]. Poly(propylacrylic acid) (PPAA) undergoes a hydrophilic-to-hydrophobic transition at endosomal pHs, mediating membrane disruption [46]. This conformational shift is triggered by the gradual protonation of carboxylic acid residues along the polymer backbone and can be tuned to occur at specific pHs by copolymerization with hydrophobic monomers [47].

The modular design of diblock polymers allows the incoporation of both cationic segments that complex nucleic acids and other segments that become membrane disruptive at endosomal pH values. Diblock polymers have been widely explored as materials as nucleic acid delivery carriers [48–53]. The synthesis of these materials was simplified with the advent of controlled radical polymerization (CRP) techniques, including reversible addition-fragmentation chain transfer (RAFT) polymerization [54–56]. These new polymerization techniques enable precise control over molecular weight polydispersities, while eliminating the need for stringent reaction conditions, and expand the scope of monomer components. A variety of compositions have been investigated for the respective block segments. However, neutral hydrophilic monomers such as poly(ethylene glycol) (PEG) and hydroxypropyl methacrylamide (HPMA) are most often chosen as stabilizing blocks because of their water solubility and low toxicity [57–60]. In addition, Zhao et al. recently reported the synthesis of block copolymers stabilized by inclusion of a zwitterionic block. This system, consisting of 2-(methacryloyloxy)-ethylphosphorylcholine and 2-(diethylamino)-ethyl methacrylate, was shown to efficiently deliver antisense oligodeoxynucleotide to human cervical carcinoma cells [61].

In the work reported here, we have designed a polymer carrier for siRNA that combines DMAEMA as a siRNA-condensing block with a PAA-containing terpolymer-stabilizing block to provide endosomolytic activity. We employed RAFT polymerization to prepare a series of well-defined block copolymers that vary in composition of the PAA block. The diblock copolymers and resulting polymer/siRNA complexes have been fully characterized with respect to size and charge ratio. In addition, their pH-dependent membrane destabilization and ability to promote siRNA internalization have been investigated. Finally, efficacy of siRNA delivery has been investigated both at the mRNA and protein levels as a function of carrier/siRNA charge ratio and siRNA concentration.

## Materials and Methods

### Materials

Chemicals and all materials were supplied by Sigma-Aldrich unless otherwise specified.

### Synthesis of RAFT chain transfer agent

The synthesis of the chain transfer agent (CTA), 4-Cyano-4-(ethylsulfanylthiocarbonyl) sulfanylvpentanoic acid (ECT), utilized for the following RAFT polymerizations, was adapted from a procedure by Moad et al. [62]. Briefly, ethanethiol (4.72 g, 76 mmol) was

added over 10 min to a stirred suspension of sodium hydride (60% in oil) (3.15 g, 79 mmol) in diethyl ether (150 ml) at 0 °C. The solution was then allowed to stir for 10 min prior to the addition of carbon disulfide (6.0 g, 79 mmol). Crude sodium S-ethyl trithiocarbonate (7.85 g, 0.049 mol) was collected by filtration, suspended in diethyl ether (100 mL), and reacted with Iodine (6.3 g, 0.025 mol). After 1 h the solution was filtered, washed with aqueous sodium thiosulfate, and dried over sodium sulfate. The crude bis(ethylsulfanylthiocarbonyl) disulfide was then isolated by rotary evaporation. A solution of bis(ethylsulfanylthiocarbonyl) disulfide (1.37 g, 0.005 mol) and 4,4′-azobis(4-cyanopentanoic acid) (2.10 g, 0.0075 mol) in ethyl acetate (50 mL) was heated at reflux for 18 h. Following rotary evaporation of the solvent, the crude ECT was isolated by column chromatography using silica gel as the stationary phase and 50:50 ethyl acetate hexane as the eluent. $^1$H NMR ($CDCl_3$) δ 1.36 t ($SCH_2C\mathbf{H_3}$); δ 1.88 s ($CCNC\mathbf{H_3}$); δ 2.3–2.65 m ($C\mathbf{H_2}C\mathbf{H_2}$); δ 3.35 q ($SC\mathbf{H_2}CH_3$).

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

The RAFT polymerization of DMAEMA was conducted in DMF at 30 °C under a nitrogen atmosphere for 12 hours using ECT and 2,2′-Azobis(4-methoxy-2.4-dimethyl valeronitrile) (V-70) (Wako chemicals) as the radical initiator. The initial monomer to CTA ratio $([CTA]_o/[M]_o$ was such that the theoretical $M_n$ at 100% conversion was 10,000 (g/mol). The initial CTA to initiator ratio ($[CTA]_o/[I]_o$) was 10 to 1. The resultant pDMAEMA macro chain transfer agent was isolated by precipitation into 50:50 diethyl ether/pentane. The resultant polymer was redissolved in acetone and subsequently precipitated into pentane (x3) and dried overnight in vacuo.

## Block copolymerization of DMAEMA, PAA, and BMA from a pDMAEMA macroCTA

The desired stoichiometric quantities of DMAEMA, PAA, and BMA were added to pDMAEMA macroCTA dissolved in N,N-dimethylformamide (25 wt % monomer and macroCTA to solvent). For all polymerizations $[M]_o/[CTA]_o$ and $[CTA]_o/[I]_o$ were 250:1 and 10:1 respectively. Following the addition of V70 the solutions were purged with nitrogen for 30 min and allowed to react at 30 °C for 18 h. The resultant diblock copolymers were isolated by precipitation into 50:50 diethyl ether/pentane. The precipitated polymers were then redissolved in acetone and precipitated into pentane (x3) and dried overnight in vacuo. 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 in DMF with respect to polymethyl methacrylate standards (SEC Tosoh TSK-GEL R-3000 and R-4000 columns (Tosoh Bioscience, Montgomeryville, PA) connected in series to a Viscotek GPCmax VE2001 and refractometer VE3580 (Viscotek, Houston, TX). 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. Both the pDMAEMA and diblock copolymers were analyzed via $^1$H NMR spectroscopy (Bruker DRX 499). Representative NMR spectras for pDMAEMA and the diblock copolymers can be found in the Supplementary Information.

## siRNA/polymer complex characterization

After verification of complete, serum-stable siRNA complexation via agarose gel retardation (see Supplementary Information), siRNA/polymer complexes were characterized for size and zeta potential using a ZetaPALS detector (Brookhaven Instruments Corporation, Holtsville, NY, 15 mW laser, incident beam = 676 nm). Briefly, polymer was formulated at concentrations of 0.1–10 mg/ml in Dulbecco's Phosphate Buffered Saline (PBS, without calcium and magnesium, Gibco) and complexes were formed by addition of polymer to GAPDH siRNA (50 μM, Qiage n, H s _ G A P D H _ 3 H P, s e n s e: 5 ′-GGUCGGAGUCAACGGAUUU-3′, antisense: 5′AAAUCCGUUGACUCCGACC-3′). The

theoretical charge ratios (+/−) are calculated using only the positively-charged DMAEMA block, which is 50% protonated at pH=7.4, and the negatively-charged siRNA. The zwitterionic second block is ignored for charge ratio calculations as it is uncharged at pH=7.4. The ratio of DMAEMA to PAA in this block is generally within error of 1:1 and these moieties are 50% positively-charged and 50% negatively-charged, respectively, at pH=7.4. In general, the appropriate volume of siRNA was added to a tube and diluted in PBS to a concentration at 6-10x of the intended testing concentration (for particle size and Zeta measurements, the final siRNA concentration was 25 nM). The required volume of polymer was then added to bring the total complex concentration to ~5x. Particles were allowed to condense for 20 min at room temperature then were diluted to 1x in PBS, as this salt concentration is relevant to the subsequent *in vitro* and hypothetical *in vivo* testing performed, and measured. Correlation functions were collected at a scattering angle of 90°, and particle sizes were calculated using the viscosity and refractive index of water at 25 °C. Particle sizes are expressed as effective diameters assuming a log-normal distribution. Average electrophoretic mobilities were measured at 25 °C using the ZetaPALS zeta potential analysis software, and zeta potentials were calculated using the Smoluchowsky model for aqueous suspensions.

### HeLa cell culture

HeLa cells, human cervical carcinoma cells (ATCC CCL-2), were maintained in minimum essential media (MEM) containing L-glutamine (Gibco), 1% penicillin-streptomycin (Gibco), and 10% fetal bovine serum (FBS, Invitrogen) at 37 °C and 5% $CO_2$.

### pH-dependent membrane disruption of carriers and siRNA/polymer complexes

Hemolysis [38, 42] was used to determine the potential endosomolytic activity of both free polymer and siRNA/polymer conjugates at pH values that mimic endosomal trafficking (extracellular pH = 7.4, early endosome pH = 6.6, and late endosome pH = 5.8). Briefly, whole human blood was collected in vaccutainers containing EDTA. Blood was centrifuged, plasma aspirated, and washed three times in 150 mM NaCl to isolate the red blood cells (RBC). RBC were then resuspended in phosphate buffer (PB) at pH 7.4, pH 6.6, or pH 5.8. Polymers (10 μg/ml) or polymer/siRNA complexes were then incubated with the RBC at the three pH values for 1 hour at 37 °C. Intact RBC were then centrifuged and the hemoglobin released into supernatant was measured by absorbance at 541 nm as an indication membrane disruption.

### Measurement of carrier-mediated siRNA uptake

Intracellular uptake of siRNA/polymer complexes was measured using flow cytometry (Becton Dickinson LSR benchtop analyzer). Helas were seeded at 15,000 cells/$cm^2$ (6-well plates) and allowed to adhere overnight. FAM labeled siRNA (Ambion) was complexed with polymer at a theoretical charge ratio of 4:1 for 30 min at room temperature and then added to the plated HeLas at a final siRNA concentration of 25 nM (1000 μl volume). After incubation with the complexes for 4 h, the cells were trypsinized and resuspended in PBS with 0.5% BSA and 0.01% trypan blue. Trypan blue was utilized as previously described for quenching of extracellular fluorescence and discrimination of complexes that have been endocytosed by cells [63]. 10,000 cells were analyzed per sample and fluorescence gating was determined using samples receiving no treatment and treated with polymer alone.

### siRNA/polymer complex cytotoxicity

siRNA/polymer complex cytotoxicity was determined using and lactate dehydrogenase (LDH) cytotoxicity detection kit (Roche). HeLas were seeded in 96-well plates at a density of 12,000 cells/$cm^2$ and allowed to adhere overnight. Complexes were formed by addition of

polymer (0.1 mg/ml stock solutions) to GAPDH siRNA at theoretical charge ratios of 4:1 and to attain a concentration of 25 nM siRNA/well (100 μl volume). Complexes (charge ratio = 4:1) were added to wells in triplicate. After cells had been incubated for 24 h with the polymer complexes, the media was removed and the cells were washed with PBS twice. The cells were then lysed with lysis buffer (100 μL/well, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM $Na_2EDTA$, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate) for 1 hour at 4 °C. After mixing by pipetting, 20 μL of the cell lysate was diluted 1:5 in PBS and quantified for lactate dehydrogenase (LDH) by mixing with 100 μL of the LDH substrate solution. After a 10–20 min incubation for color formation, the absorbance was measured at 490 nm with the reference set at 650 nm.

### Evaluation of GAPDH protein and gene knockdown by siRNA/polymer complexes

The efficacy of the series of polymers for siRNA delivery was screened using a GAPDH activity assay (Ambion). HeLas (12,000 cells/$cm^2$) were plated in 96-well plates. After 24 h, complexes (charge ratios = 4:1) were added to the cells at a final siRNA concentration of 25 nM in the presence of 10% serum (100 μl volume). The extent of siRNA-mediated GAPDH protein reduction was assessed 48 h post-transfection. As a positive control, parallel knockdown experiments were run using HiPerFect (Qiagen) following manufacturer's conditions. The remaining GAPDH activity was measured as described by the manufacturer using kinetic fluorescence measurements to analyze % remaining expression.

After the initial screen to identify the carrier that produced the most robust siRNA-mediated GAPDH knockdown, real time reverse transcription polymerase chain reaction (RT-PCR) was used to directly evaluate siRNA delivery. After 48 hours of incubation with complexes as formed above, cells were rinsed with PBS. Total RNA was isolated using Qiagen's Qiashredder and RNeasy mini kit. Any residual genomic DNA in the samples was digested (RNase-Free DNase Set, Qiagen) and RNA was quantified using absorbance at 260 nm.

Reverse transcription was performed using the Omniscript RT kit (Qiagen). A 25 ng total RNA sample was used for cDNA synthesis and PCR was conducted using the ABI Sequence Detection System 7000 using predesigned primer and probe sets (Assays on Demand, Applied Biosystems) for GAPDH and β-actin as the housekeeping gene. Comparative threshold cycle ($C_T$) analysis was used to quantify GAPDH, normalized to β-actin and relative to expression of untreated HeLas.

### Statistical methods

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

## Results and Discussion

### siRNA polymer carrier synthesis and characterization

Seven diblock copolymers were synthesized for use as gene carriers according to Scheme 1. The first block of these materials is composed of DMAEMA, which has been shown to efficiently condense nucleic acids at physiological pH [14–17]. The second ampholyte block contains DMAEMA, PAA, and hydrophobic BMA residues. This block was designed to enable endosomal escape of the bound cargo through a pH-induced conformation change. Under physiological conditions, this block is ampholytic in nature with both positive DMAEMA and negative PAA residues masking the hydrophobic BMA content [64–67]. However, upon uptake into endosomal compartments, this lower pH environment causes PAA carboxylate residues to become protonated. This protonation, along with a concurrent

increase in positive charge from DMAEMA residues that occurs at this pH, changes the polymer from a hydrophilic polyampholyte to a hydrophobic polycation that is capable of disrupting the endosomal membrane. Previous work has shown that the greater hydrophobicity of a polymer carrier increases efficacy of nucleic acid delivery [68–70]. However, a tradeoff exists with solubility and efficacy. While efficacy improves with increasing hydrophobicity, eventually phase-separation occurs, eliminating carrier usefulness. Therefore, we screened a series of polymers with BMA content increasing to the point of phase-separation to identify potential candidates for siRNA delivery.

The pDMAEMA was first prepared by polymerizing DMAEMA in the presence of the RAFT CTA and the radical initiator. The resultant pDMAEMA block (9,100 g/mol; DP 58) was used to prepare a series of diblock copolymers. The second blocks contain increasing BMA content as well as DMAEMA and PAA in approximately equimolar ratios. The feed and experimental polymer compositions for this series are shown in Table 1. Similar block sizes were observed for all seven diblocks giving the polymers an overall molecular weight of around 20,000 g/mol. Lower molecular weights were chosen to minimize polymer toxicity and to enable renal clearance for future *in vivo* testing. While all polymer compositions are relatively close to the feed composition, some deviation is observed likely due to differences in the monomer reactivity ratios.

## siRNA/polymer complex characterization

The series of polymers and their respective siRNA-condensed particles were characterized for size and surface charge and the resulting data are shown in Table 2.

All polymers appeared unimeric (< 10 nm) in solution at concentrations from 0.1 mg/ml to 10 mg/ml at pH=7.4. Complexes formed from the polymer series and siRNA at theoretical charge ratios of 4:1 ranged in size from 85–236 nm. There was no definitive trend for the complex sizes with respect to BMA content, however, polymer 7 with 48% BMA content in the endosomolytic block exhibited the smallest particle size of 85 nm ± 18. The remainder of the particles had sizes from 144 to 236 nm, where the greatest sized particles were formed from polymer 6 which had 27% BMA content in the endosomolytic block. Polymer 7/siRNA particle sizes were further examined with charge ratios ranging from 1:1 to 8:1, and data are shown in Table 3. Polymer/siRNA particle sizes decrease dramatically as charge ratio increases with values of 643 nm ± 104 at 1:1 to 54 nm ± 8.4 at 8:1. As charge ratio approaches 1:1, the larger size could be due to formation of larger aggregates, especially considering that the carriers contain ~ 50% of the hydrophobic butyl methacrylate groups. As charge ratio (+/−) is increased, there is more electrostatic repulsion between complexes leading to aggregates of smaller sizes. Alternatively, as the particles approach charge neutrality, packing efficiency could be reduced due to lower local DMAEMA concentration, resulting in reduced electrostatic interactions and particle compactness.

The surface charge of siRNA/polymer complexes, based on ζ-potential measurements, was found to be similar and slightly positive for all polymers (~0.5 mV with a range of 0.13–1.1 mV). Moreover, complexes formed at +/− of 1:1, 2:1, 4:1, and 8:1 using polymer 7 showed no significant difference in surface charge, again with slightly positive values (0.18–0.99 mV). At 1:1 charge ratios, particles are expected to have very little surface charge, as the PAA and DMAEMA charges in the second block counterbalance each other. As the charge ratio increases to 2:1, 4:1, and 8:1, one would expect to see increases in positive surface charge, but interestingly that was not observed. It is clear from the sizing data that the particles change morphology with increasing amounts of polymer, becoming smaller. With increasing charge ratio, it is possible that the surface charge remains just slightly positive and is unaffected due to effective shielding of the DMAEMA positive charges, as many polymer chains and siRNA become packed within the core of the particles.

These alterations in particle size and surface charge are especially relevant design criteria for ensuring carrier uptake. For example, it is generally accepted that nanoparticles bearing a positive surface charge facilitate uptake by electrostatic interactions with negatively charged cell membranes [71]. However, the particles do not need to bear a large positive charge to mediate uptake. For instance, particles bearing a positive charge of only +2 mV was found to enable siRNA internalization in nearly 100% of HeLas over a 4 h period [72]. In separate work, particles formed with plasmid DNA at a charge ratio of +1.8 mV mediated the third highest uptake and best overall transfection of a luciferase plasmid in NIH 3T3s out of 140 distinct polymer compositions [73]. For particle size, 200 nm has been reported as the rough limit for cellular uptake by macropinocytosis [74], and nanoparticles in excess of this size may be excluded from cellular internalization altogether [75], resulting in the inability to mediate gene knockdown *in vitro* [71]. Indeed, utilizing a peptide-modified PEG carrier, Segura and Hubbell showed significant GAPDH knockdown (>50%) with particles ~200 nm [72]. However, using latex beads as a model, B16 cells internalized particles as large as 500 nm [76]. All of the siRNA/polymer nanoparticles formed in this work exhibit slight positive charges and are within the approximate size limits (Tables 2 and 3) for uptake, making them attractive candidates for intracellular delivery based on these criteria.

## Endosomolytic activity of carriers and siRNA/polymer complexes

Both polymer and siRNA/polymer complexes were evaluated for their ability to induce red blood cell hemolysis at pH values relevant to the endosomal/lysosomal trafficking pathway (Figure 1). No significant hemolysis was observed for polymers 1–3. Significant pH-dependent hemolytic activity was evident first with polymer 4, and enhanced activity was found as BMA content of the endosomolytic block increased (Figure 1A). Polymer 7 exhibited the greatest pH-dependent hemolysis with essentially no activity at pH=7.4, about 25% hemolysis at pH=6.6, and 85% hemolysis at pH=5.8. Polymers 5–7 were subsequently evaluated for hemolytic activity in their siRNA-complexed form, and the data are plotted in Figure 1B.

Complexes formed with polymers 5–7 at all charge ratios tested were found to be hemolytic in a pH-dependent fashion. The hemolysis exhibited by complexes was increased when compared with free polymer and was greater at a charge ratio of 4:1 versus 1:1. Polymer 7 showed the greatest hemolytic activity at a charge ratio of 4:1, with essentially no hemolysis at pH=7.4, 60% hemolysis at pH=6.8, and 100% hemolysis at pH=5.8. These data indicate that the pH-responsive hemolytic activity of these polymers is tightly linked to the incorporation of a hydrophobic moiety, butyl methacrylate. This finding corroborates previous reports on pH-responsive, membrane destabilizing polymers that have utilized incorporation of hydrophobic moieties such as alkyl amines or aromatic groups to enhance the pH-dependent hydrophobic transition of carboxylate functionalized polymers [42, 47, 77].

## Carrier-mediated siRNA uptake

Cellular internalization of siRNA complexes at 4:1 charge ratios was investigated using flow cytometry for polymers 4–7 based on their relevant pH-responsive endosomolytic characteristics (see Figure 1A). As expected, all polymer formulations showed much greater uptake (up to 25x) by cells than siRNA not complexed with a carrier (naked siRNA). Cellular uptake was also found to positively correlate with BMA content of the second block, with polymer 7 showing the highest level of uptake (23% siRNA positive cells, see Figure 2). Internalization of complexed siRNA by up to 23% of cells after only 4 h is a promising result, as the cumulative uptake is likely to be much higher after the full 48 h treatment. In addition, siRNA activity is considered to be catalytic; it can be recycled within the cytoplasm to destroy multiple mRNA transcripts, therefore having a long-term, multi-

generational effect [78]. The smaller size of the polymer 7 complexes could be a factor in the increased internalization, together with the enhanced endosomolytic effectiveness of the BMA-containing block to avoid recycling. The combination of increased uptake and endosomal release leads to strongly enhanced intracellular concentrations of siRNA with the polymer 7 complexes.

### siRNA/polymer complex cytotoxicity

The cytotoxicity of the polymer carriers was investigated by incubating HeLa cells in the presence of the complexes at charge ratios of 4:1 for 24 h. The resulting cell survival, as measured by intracellular lactate dehydrogenase activity versus untreated cells, is shown in Figure 3A. High relative survival was observed (>90% after 24 h) for all polymers tested. Synthetic polymers, in particular cationic polymers, are associated with appreciable cytotoxicity. For instance, PEI has been shown to trigger apoptosis and/or necrosis in a variety of cell lines [79]. This toxicity can be reduced by chemically modifying the polycation segment with hydrophilic segments [80], however, there is usually a tradeoff between efficacy and toxicity [81]. In this approach, the use of a near-neutral polyampholyte for the second block of the polymer delivery vehicle reduced the intrinsic cytoxicity of the polycation block with the cultured HeLas.

### Evaluation of GAPDH protein and gene knockdown by siRNA/polymer complexes

The ability of the carriers to effectively deliver siRNA was investigated in knockdown experiments against GAPDH with complexes formed from all polymers at theoretical charge ratios of 4:1. GAPDH protein levels were evaluated 48 h after treatment with the complexes, and data are shown relative to GAPDH protein levels of untreated cells (Figure 3B, black bars). Polymer carriers 1–3 were ineffectual at eliciting reduction of protein levels, likely due to their inability to mediate endosomal escape (Figure 1). However, GAPDH protein reduction became evident with the use of polymer 4 as a siRNA carrier. The knockdown of protein further increased as the BMA content of the carriers increased to 48% of the endosomolytic block (polymer 7). Polymer 7 showed the greatest ability to mediate siRNA knockdown of protein where GAPDH was reduced to 32% of control.

To further characterize carrier efficacy, polymers were analyzed for their ability to knockdown GAPDH mRNA levels. Similar to the protein measurements, polymers 1–3 elicited very little reduction of mRNA signal, as evaluated by RT-PCR (Figure 3B, white bars). Again, polymers 4–7 showed increased knockdown of GAPDH as the BMA content of the endosomolytic block increased. Specifically, GAPDH knockdown was reduced to 39%, 30%, 31%, and 21% of control at a charge ratio of 4:1, for polymers 4, 5, 6, and 7, respectively. Overall, our results are consistent with findings from other groups exploring delivery strategies for DNA which have found that the addition of hydrophobic domains, specifically N-oleyl moieties, phenylalanine resides, and butyl methacrylate, as utilized here, enhance delivery [68–70].

Because the polymer with the greatest butyl methacrylate content in the endosomolytic block showed the most promise as a siRNA carrier, a further investigation into its ability to mediate gene knockdown was performed with respect to charge ratio and siRNA dose (Figure 4). Alteration of theoretical charge ratios was found to strongly affect gene knockdown (Figure 4A). GAPDH was reduced to 51%, 42%, 21%, and 14% of control levels with charge ratios of 1:1, 2:1, 4:1, and 8:1, respectively. Particularly at charge ratios of 4:1 and 8:1, gene knockdown was similar to the commercially available carrier HiPerFect, where GAPDH levels were reduced by over 80%. Importantly, the effects on GAPDH levels are specific to the siRNA that is delivered, as when a control siRNA is utilized at a charge ratio of 8:1, there is no significant effect on GAPDH levels. Altering the

charge ratio may have resulted in differing levels of condensation of the siRNA within the nanoparticles. The DLS experiments indicated that increasing copolymer content in the complexes resulted in more condensed particles (Table 3), and these functional studies suggest that more compact particles can be internalized more efficiently or with increased siRNA bioavailability. These findings are consistent with previous reports indicating that more compact DNA/polyethyleneimine and DNA/polylysine complexes internalize at higher rates and achieve higher transfection efficiencies [82, 83].

A dose-response study was completed using P7 at a charge ratio of 4:1 (Figure 4B). Although there was little response in GAPDH gene expression at 1 nM or 5 nM siRNA, expression was reduced to 77%, 21%, and 12% of control when 10 nM, 25 nM, or 50 nM of siRNA was delivered using polymer 7. This level of knockdown approaches that seen using 50 nM HiPerFect, a commercially available positive control. However, all the diblock copolymers demonstrated enhanced biocompatibility, as measured by cytotoxicity assays (Figure 3A and Table S1, Supplementary Information) compared to HiPerFect.

## Conclusions

siRNA-based therapies have a great deal of potential for the treatment of debilitating diseases such as cancer. Here, we have reported the development of a new modular diblock carrier that combines siRNA condensing activity and a new pH-responsive endosomolytic block that is active at near charge neutrality. Using the RAFT controlled polymerization technique, a series of diblock copolymers were synthesized that incorporate the siRNA-condensing, cationic block formed from DMAEMA and a second ampholytic block, formed from DMAEMA, BMA, and PPA, to enhance endosomal escape. siRNA/polymer particles formulated with the tercopolymer block at 48% BMA, 29% PAA, and 23% DMAEMA, exhibited favorable size and charge (85 nm at 4:1 charge ratios and slightly positive), the greatest pH-dependent hemolysis, and the most efficient uptake of siRNA over all other polymer carriers examined. Moreover, siRNA delivery using polymer 7 elicited the greatest GAPDH mRNA and protein reduction, which was found to occur in a siRNA concentration and charge ratio-dependent manner. These findings indicate that there is an intimate link between butyl methacrylate content with effective intracellular siRNA delivery. The effectiveness and low cytotoxicity of this new carrier make it a promising candidate for future siRNA delivery studies in disease models.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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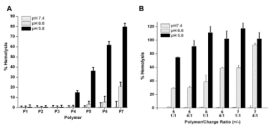


**Figure 1.**

Hemolysis of (A) polymers as a function of pH at a concentration of 10 μg/ml and (B) polymer/siRNA complexes of polymers 5–7 at theoretical charge ratios of 1:1 and 4:1 (25 nM siRNA dose which corresponds to polymer concentrations of 0.67 and 2.64 μg/ml, respectively). 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 ± standard deviation.

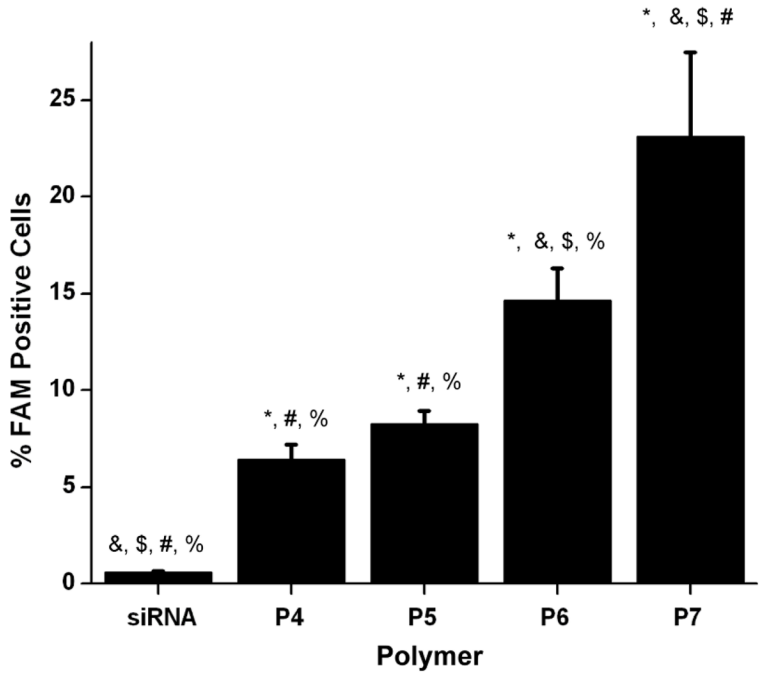



**Figure 2.**

HeLa cell internalization of FAM-labeled siRNA and polymer/siRNA complexes formed with polymers 4–7 at theoretical charge ratios of 4:1 (after 4 h). Data are from three independent experiments conducted in triplicate, with error bars representing standard error of the mean. Statistical significance was evaluated at a level of $p < 0.05$ with the following symbols indicating significance versus naked siRNA and P4, P5, P6, and P7 carriers, respectively: *, &, $, #, %.

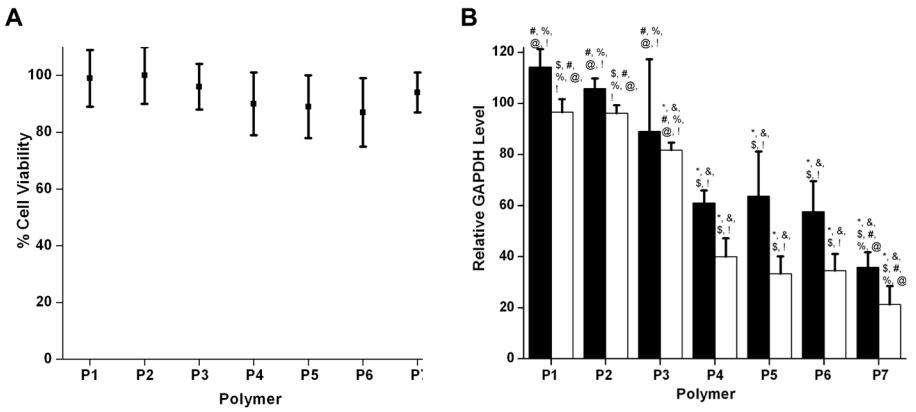



**Figure 3.**

HeLa cytotoxicity (A) and GAPDH knockdown (B) as a function of siRNA polymer carrier. HeLa cells were transfected with siRNA against GAPDH at 25 nM using polymer/siRNA complexes formulated at theoretical charge ratios of 4:1. (A) After 24 h, cell lysate was collected and assayed for lactate dehydrogenase, a measure of cell viability, and data is shown relative to untreated cells. (B) After 48 h, both protein (black) and mRNA levels (white) were examined using a GAPDH enzyme activity assay and RT-PCR, respectively; the data is shown relative to cells receiving no treatment. Data are from three independent experiments conducted in triplicate with error bars representing standard deviation. Statistical significance was evaluated at a level of $p < 0.05$ with the following symbols indicating significance versus P1, P2, P3, P4, P5, P6, and P7 carriers, respectively: *, &, $, #, %, @, !.

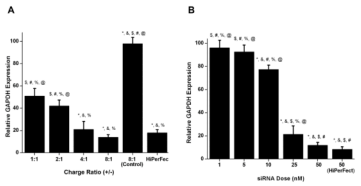
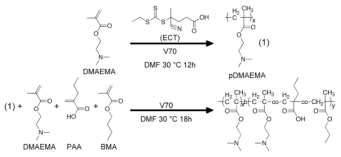



**Figure 4.**

GAPDH knockdown in HeLa cells was measured using real time RT-PCR 48 h after treatment with complexes as a function of charge ratio (1:1–8:1) (A) and siRNA dose (1–50 nM) (B) with polymer 7 as the carrier at 4:1 charge ratios. Negative control siRNA #1 (Ambion) and a commercially available transfection reagent, HiPerFect (Qiagen), were used as negative and positive controls, respectively. For A, statistical significance was evaluated at a level of $p < 0.05$ with the following symbols indicating significance versus 1:1, 2:1, 4:1, 8:1, 8:1 (Control), and HiPerFect treatments, respectively: *, &, $, #, %. For B, statistical significance was evaluated at a level of $p < 0.05$ with the following symbols indicating significance versus 1 nM, 5 nM, 10 nM, 25 nM, 50 nM, and 50 nM (HiPerFect) treatments, respectively: *, &, $, #, %, @.




**Scheme 1.**

RAFT-mediated synthesis of diblock copolymers consisting of a cationic poly(dimethylaminoethyl methacrylate) (DMAEMA, x=58) block and an endosomolytic polyampholyte block incorporating DMAEMA and propylacrylic acid (PAA) in equimolar ratios, and butyl methacrylate (BMA) (y~70).

**Table 1**

Molecular weights, polydispersities, and monomer compositions for the poly(DMAEMA) macroCTA, the resultant diblock copolymers, and their corresponding nomenclature.

| Polymer | $M_n$[a] 1st block (g/mol) | $M_n$[a] 2nd block (g/mol) | PDI[a] | Feed% BMA 2nd block | Feed% PAA 2nd block | Feed% DMAEMA 2nd block | Experimental[b]% BMA 2nd block | Experimental[b]% PAA 2nd block | Experimental[b]% DMAEMA 2nd block |
|---|---|---|---|---|---|---|---|---|---|
| mCTA | 9 100 | - | 1.16 | - | - | - | - | - | - |
| P1 | 9 100 | 6 900 | 1.58 | 0 | 50 | 50 | - | 47 | 53 |
| P2 | 9 100 | 8 900 | 1.56 | 5 | 47.5 | 47.5 | 1 | 48 | 51 |
| P3 | 9 100 | 8 300 | 1.54 | 10 | 45 | 45 | 12 | 40 | 48 |
| P4 | 9 100 | 9 300 | 1.46 | 15 | 42.5 | 42.5 | 19 | 44 | 37 |
| P5 | 9 100 | 10 100 | 1.51 | 20 | 40 | 40 | 24 | 40 | 36 |
| P6 | 9 100 | 10 000 | 1.48 | 30 | 35 | 35 | 27 | 37 | 36 |
| P7 | 9 100 | 11 300 | 1.45 | 40 | 30 | 30 | 48 | 29 | 23 |

[a] As determined by SEC Tosoh TSK-GEL R-3000 and R-4000 columns (Tosoh Bioscience, Montgomeryville, PA) connected in series to a Viscotek GPCmax VE2001 and refractometer VE3580 (Viscotek, Houston, TX). HPLC-grade DMF containing 0.1 wt % LiBr was used as the mobile phase. The molecular weights of the synthesized copolymers were determined using a series of poly(methyl methacrylate) standards.

[b] As determined by $^1$H NMR spectroscopy (3 wt % in $D_2O$ or $CDCl_3$; Bruker DRX 499)

**Table 2**

Size and ζ-potential measurements of particles of varying butyl methacrylate content formulated with siRNA at a theoretical charge ratio of 4:1 (+/−). Data is compiled from two independent experiments ± standard error of the mean.

| Polymer | Diameter (nm) | Zeta Potential (mV) |
|---|---|---|
| P1 | 166 ± 31 | 1.1 ± 1.3 |
| P2 | 189 ± 28 | 0.13 ± 0.7 |
| P3 | 197 ± 24 | 0.47 ± 0.6 |
| P4 | 144 ± 24 | 0.41 ± 1.2 |
| P5 | 193 ± 37 | 0.52 ± 0.8 |
| P6 | 236 ± 30 | 0.67 ± 0.9 |
| P7 | 85 ± 19 | 0.18 ± 1.0 |

## Table 3

Size and ζ-potential measurements of particles formulated with polymer 7, the composition with the greatest butyl methacrylate content, and siRNA as a function of charge ratio. Data is compiled from two independent experiments ± standard error of the mean.

| Theoretical Charge Ratio (+/−) | Diameter (nm) | Zeta Potential (mV) |
|---|---|---|
| 1:1 | 643 ± 104 | 0.27 ± 1.1 |
| 2:1 | 530 ± 80 | 0.99 ± 0.9 |
| 4:1 | 85 ± 15 | 0.18 ± 1.0 |
| 8:1 | 54 ± 8.4 | 0.41 ± 0.8 |