NANOSCALE RADIATION ENGINEERING OF ADVANCED MATERIALS FOR POTENTIAL BIOMEDICAL APPLICATIONS

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Summary

We are using RAFT polymerization to synthesize smart polymer nanocarriers for intracellular delivery of protein, peptide and nucleic acid drugs. In the coming program period we plan to synthesize these carriers **using radiation to initiate the RAFT polymerizations.** In this way we will avoid the need to add free radical initiators to initiate this polymerization, yielding a purer polymer-drug nanocarrier.

Achievements

Nanocarriers

Smart T- and pH-responsive polymer nanocarriers have been RAFT synthesized for enhanced intracellular delivery of biomolecular drugs such as peptides, proteins and nucleic acid drugs.

1. INTRODUCTION

The gene-knockdown activities of small, interfering RNA (siRNA) have led to their use as drug target validation tools in drug discovery, and also as potential therapeutics for a variety of diseases. The efficient intracellular delivery of these double-stranded RNA macromolecules has proven to be challenging, and achieving efficient and safe delivery of siRNA is a significant barrier 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 is often 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 incorporation in one block of cationic segments that complex nucleic acids and the incorporation of 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].

We have developed 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, which usually employs a free radical initiator such as AIBN (Azo-bis-IsoButyrl-Nitrile); we will replace such initiators with low dose rate irradiation in future collaborations within this CRP.

The diblock polymers 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 first block kept at constant MW of 9100, while in the second block, the ratio of DMAEMA and PAA to BMA was varied, along with the MW. As the percentage of BMA in the second block was systematically increased, these carriers became sharply hemolytic at endosomal pH regimes.

The diblock copolymers condensed siRNA into 80-250 nm particles with slight positive zeta potentials. The siRNA knockdown activities in HeLa cells generally followed the hemolytic activity trends, with the most hydrophobic second block (highest BMA mole fraction) exhibiting the best polyplex properties and knockdown activities. This pH-responsive ampholytic carrier designed to mediate endosomal release thus shows significant promise for the intracellular delivery of siRNA.

The ability of small interfering RNA (siRNA) to efficiently silence the expression of specific genes provides the basis for exciting new therapies based on RNA interference (RNAi). The efficient intracellular delivery of siRNA, starting with cell uptake, continuing through the endosomal trafficking pathway and eventually into the cytoplasm remains a significant challenge.

2. 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, ethane thiol (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 4-Cyano-4 (ethylsulfanylthiocarbonyl) sulfanylvpentanoic acid (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 ($[CTA]_0/[M]_0$ 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 v:v 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. (see Table 1 and Scheme 1 below) 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 v:v diethyl ether/pentane. The precipitated polymers were then redissolved in acetone and subsequently precipitated into pentane (x3) and dried overnight in vacuo. Gel permeation chromatography (GPC) was used to determine molecular weights and polydispersities (PDI, M_w/M_n) of both the poly(DMAEMA) 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 was used as the mobile phase).

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, Qiagen, Hs_GAPDH_3 HP, sense: 5'-GGUCGGAGUCAACGGAUUU-3', antisense: 5'AAAUCCGUUGACUCCGACC-3'). The theoretical charge ratios (+/-) are calculated using only the positively charged DMAEMA block and the negatively-charged siRNA, as the zwitterionic second block is approximately 50% protonated at pH=7.4 and the ratio of DMAEMA to PAA is generally within error of 1:1. 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 in PBS to 1x 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 of pH-dependent RBC membrane lysis.

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 polymer not complexed with FAM-labeled siRNA (Ambion Negative Control #1, FAM-labeled).

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 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 the kinetic fluorescence increase method over 5 min and was calculated according to the following equation: % remaining expression = $\triangle_{\text{fluorescence, GAPDH}}/\triangle_{\text{fluorescence, no treatment}}$, where $\triangle_{\text{fluorescence}} = \text{fluorescence}_{5\text{min}}\text{-fluoresecence}_{1\text{min}}$.

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 the RiboGreen assay (Molecular Probes) based on the manufacturer's instructions.
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. Reactions (20 μl total) consisted of 10 μL of 2X Taqman Universal PCR Mastermix, 1 μL of primer/probe, and 2 μL of cDNA, brought up to 20 μL with nuclease-free water (Ambion). The following PCR parameters were utilized: 95 ºC for 90 s followed by 45 cycles of 95 ºC for 30 s and 55 ºC for 60 s. 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.

3. KEY RESULTS AND DISCUSSION

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. 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 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, 72].

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 2 and Table 2). 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) during this timeframe (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 [73]. 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. We have shown recently that PAA-containing protein conjugates exhibit reduced extracellular recycling and increased accumulation of protein within the cell [74] compared to conjugates made with the analogous non-destabilizing monomers. 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, showed that high relative survival was observed (>90% after 24 h) for all polymers tested. Synthetic polymers, in particular cationic polymers, can be associated with appreciable cytotoxicity. For instance, PEI has been shown to trigger apoptosis and/or necrosis in a variety of cell lines [75]. This toxicity can be reduced by chemically modifying the polycation segment with hydrophilic segments [76], however, there is usually a tradeoff between efficacy and toxicity [77].

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 2, black bars). Polymer carriers 1-3 were ineffectual at eliciting reduction of protein levels, likely due to their inability to mediate endosomal escape. 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 (Fig.2). 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 transfection [64-66].
Because the polymer 7, 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. Alteration of theoretical charge ratios was found to strongly affect gene knockdown. 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. Our DLS experiments indicated that increasing copolymer content in the complexes resulted in more condensed particles 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 [78, 79]. We also completed a dose-response study using P7 at a charge ratio of 4:1. 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 compared to HiPerFect.

We have recently developed a new generation of siRNA delivery polymers that exhibit enhanced transfection efficiency and low cytotoxicity. This design incorporates a longer endosomolytic, second block with increased hydrophobic content to induce micelle formation. These polymers

spontaneously form spherical micelles in the size range of 40 nm with CMC (critical micelle concentration) values of approximately 2 μg/ml based on dynamic light scattering (DLS), ^{1}H-NMR, electron microscopy, and selective partitioning of the small molecule pyrene into the hydrophobic micelle core. The siRNA binding to the cationic shell block did not perturb micelle stability or significantly increase particle size. The self-assembly of the diblock copolymers into particles was shown to provide a significant enhancement in mRNA knockdown at siRNA concentrations as low as 12.5 nM. Under these conditions the micelle--based systems showed an 89 % reduction in GAPDH mRNA levels as compared to only 23 % (10 nM siRNA) for the non-micelle system. The reduction in mRNA levels becomes nearly quantitative as the siRNA concentration is increased to 25 nM and higher. Flow cytometry analysis of fluorescent-labeled siRNA showed uptake in 90% of the cells and a 3-fold increase in siRNA per cell compared to a "gold standard" lipid transfection agent. These results demonstrate the potential utility of this carrier design for siRNA drug delivery. See Fig. 4 below from ref. 80.

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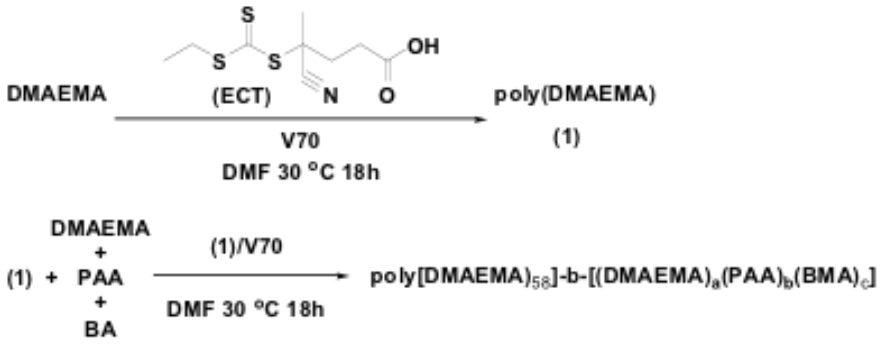
Table 1. Molecular weights, polydispersities, and monomer compositions for the poly(DMAEMA) macroCTA, the resultant diblock copolymers, and their corresponding nomenclature.

Polymer	M_n[a] 1st block (g/mol)	M_n[a] 2nd block (g/mol)	PDI[a]	Theoretical % BMA 2nd block	Theoretical % PAA 2nd block	Theoretical % 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 detrmined by ^{1}H NMR spectroscopy (3 wt % in $CDCl_3$; Bruker DRX 499)



Scheme 1. RAFT mediated synthesis of diblock copolymers consisting of a cationic poly(DMAEMA) block and an endosomolytic polyampholyte block.

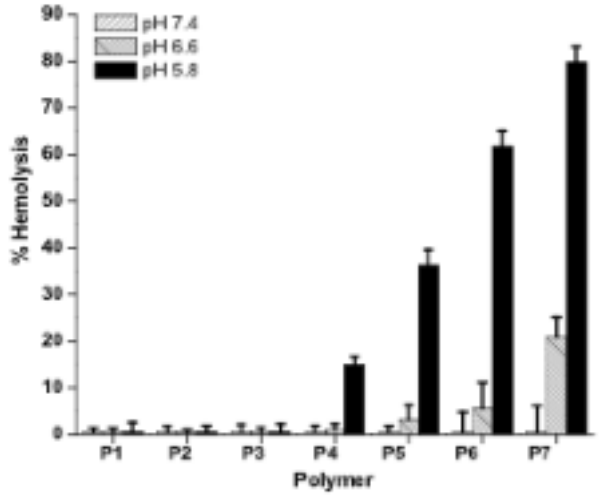

pH 7.4
pH 6.6
pH 5.8
% Hemolysis
90
80
70
60
50
40
30
20
10
0
P1
P2
P3
P4
P5
P6
P7
Polymer

FIG. 1.

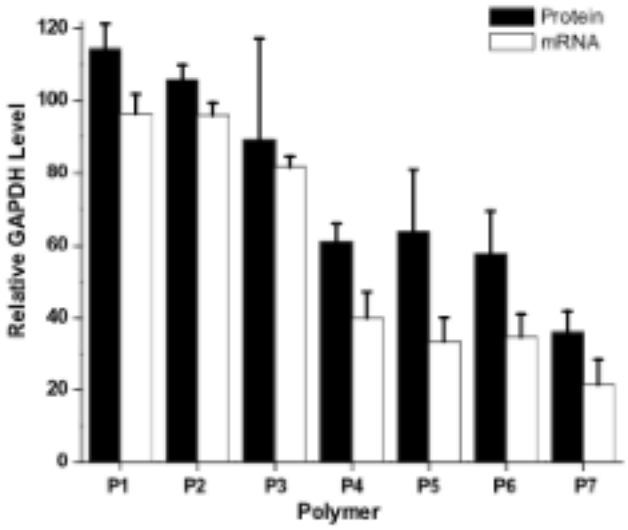

Protein
mRNA
Relative GAPDH Level
120
100
80
60
40
20
0
P1
P2
P3
P4
P5
P6
P7
Polymer

FIG. 2.

Table 2. Size and ζ-potential measurements of particles formulated with siRNA at a theoretical charge ratio of 4:1 as a function of butyl methacrylate composition.

Polymer #	Diameter (nm)	PDI	Zeta Potential (mV)	Standard Error
P1	166	0.14	1.1	1.32
P2	189	0.09	0.13	0.69
P3	197	0.06	0.47	0.59
P4	144	0.11	0.41	1.2
P5	193	0.32	0.52	0.77
P6	236	0.06	0.67	0.95
P7	85	0.20	0.18	1.0

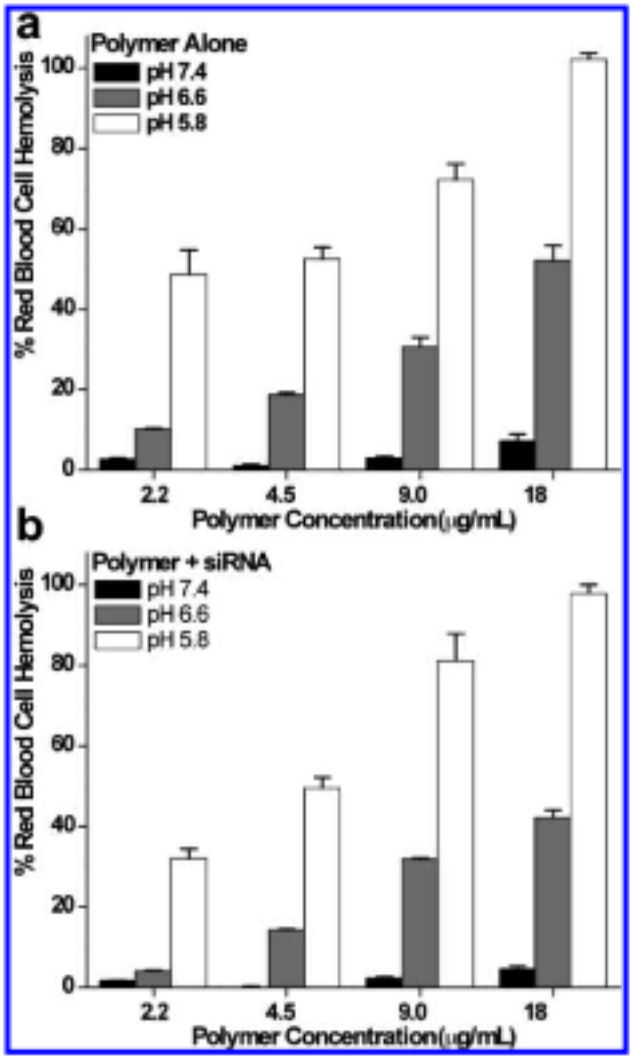


Figure 4. Hemolysis of the (a) diblock copolymer as a function of pH at concentrations of 2.2, 4.5, 9.0, and 18 μg/mL and (b) diblock copolymer/siRNA complexes at theoretical charge ratios of 1:1, 2:1, 4:1, and 8:1 (25 nM siRNA). Hemolytic activity is normalized relative to a positive control, 1% v/v Triton X-100, and the data represent a single experiment conducted in triplicate ± standard deviation.