

# Corona-Stabilized Interpolyelectrolyte Complexes of siRNA with Nonimmunogenic, Hydrophilic/Cationic Block Copolymers Prepared by Aqueous RAFT Polymerization<sup>†</sup>

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**ABSTRACT:** The complexation of small interfering ribonucleic acid (siRNA) with a series of specifically designed block copolymers consisting of the hydrophilic, nonimmunogenic monomer *N*-(2-hydroxypropyl)methacrylamide (HPMA) and the cationic monomer *N*-[3-(dimethylamino)propyl]methacrylamide (DMAPMA) has been investigated for potential siRNA stabilization and delivery applications. Specific compositions of poly(HPMA-*b*-DMAPMA) copolymers were synthesized via aqueous reversible addition–fragmentation chain transfer (RAFT) polymerization and characterized using aqueous size exclusion chromatography with multiangle laser light scattering (SEC-MALLS) and <sup>1</sup>H NMR spectroscopy. The degree of soluble complex formation between a model siRNA and the polymers was determined by centrifugal membrane filtration experiments and quantitated by scintillation counting of <sup>32</sup>P ATP-labeled siRNA to determine complex solubility and to estimate the degree of complexation relative to cationic and neutral block lengths. Dynamic and static light scattering methods were employed to determine the hydrodynamic radii, molecular weights, and second virial coefficients of the complexes and to demonstrate their unimodal size distributions. In vitro enzymatic degradation studies of selected siRNA/block copolymer complexes were conducted to demonstrate the enhanced stability of the siRNA/poly(HPMA-*b*-DMAPMA) complexes. Furthermore, the siRNA/polymer complexes dissociate slowly under gel electrophoresis conditions. Therefore, the siRNA/polymer complexes demonstrate some highly desirable properties for potential applications in therapeutic siRNA stabilization and delivery.

## Introduction

The control of gene expression using specific nucleic acid sequences represents a significant step in the treatment of cancers, viral diseases, and genetic disorders. Recently, siRNAs (small interfering RNAs with 19–24 base-paired segments containing 2 nucleotide overhangs at the 3' end) have been discovered that turn off or silence specific genes, conclusively demonstrating their potential as the next generation of therapeutic agents.<sup>1,2</sup> Although preliminary results suggest that siRNA is a more potent inhibitor of gene expression and is less toxic to cells than other gene silencing agents (e.g., antisense oligodeoxyribonucleic acids (ODNs),<sup>3–7</sup> DNazymes,<sup>8,9</sup> or ribozymes<sup>9</sup>), delivery to the appropriate tissues and susceptibility to hydrolytic and enzymatic degradation in the bloodstream still pose significant challenges.<sup>10–12</sup>

Intracellular delivery of exogenous nucleic acids (DNA or RNA) can be achieved by a number of techniques, among which viral vector systems and nonviral transfection agents are commonly used. Viral systems<sup>13,14</sup> usually give high transfection efficiencies. However, safety concerns from potential mutation, recombination, and oncogenic effects greatly limit their therapeutic applications. In contrast, nonviral transfection agents, including cationic lipids and cationic polymers, are believed to cause less safety problems due to their relative simplicity. A number of cationic lipids can be formulated to assemble cationic

liposomes that either entrap or bind nucleic acids and internalize them into cells.<sup>15–17</sup> However, nonspecific cytotoxicity associated with cationic liposomes has been observed.<sup>18–19</sup>

One possibility for effective delivery and protection of siRNA in vivo involves stabilization with synthetic polycations or polycation-containing block copolymers to form specialized interpolyelectrolyte complexes (IPECs) or block ionomer complexes (BICs), respectively. Such systems with other polynucleic acids are well documented and variations of this concept continue to be employed in gene delivery today.<sup>20–30</sup> IPEC systems currently used in gene therapy are composed of complexed polycations (e.g., poly(vinyl pyridine), poly(L-lysine), and polyethylenimine (PEI)) and polynucleic acids (e.g., DNA or RNA). Strong electrostatic interactions between oppositely charged polyelectrolytes (e.g., polycations and polynucleic acids) allow for “self-assembly”, which can substantially hinder or prevent enzymatic degradation of the incorporated polynucleotide in the bloodstream.<sup>20,25,26</sup> The spontaneous formation of these complexes is largely driven by electrostatic interactions between the synthetic polycations and the “backbone” phosphate units of the polynucleotides. Furthermore, an overall gain in entropy due to the liberation of low-molecular-weight counterions and water during complexation increases the thermodynamic spontaneity of the process.<sup>26–30</sup> The structural characteristics and solubility of IPECs in aqueous conditions are governed by the polymeric cation/polynucleotide phosphate (*N/P*) ratio and are maintained by the formation of nonstoichiometric IPECs, where the *N/P* ratio  $\neq$  1. These imbalanced IPECs can form two types of structures: (1) positive IPECs that contain an excess of polycations and (2) negative IPECs that contain an excess of unpaired phosphates. While the preparation of these

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two electrostatically stabilized IPEC systems does eliminate solubility issues observed with stoichiometric IPECs, the negative IPECs are typically not effective transfection agents because of their inability to penetrate cell membranes. On the other hand, positive IPECs, due to their residual cationic nature, are often too interactive with a host of anionically charged small molecules and organelles<sup>26</sup> and can enter different cells non-selectively via adsorptive endocytosis.<sup>31,32</sup> Neutral IPEC systems formed by mixing stoichiometric polycations with polyanions could in principle avoid the aforementioned problems. However, such IPEC systems typically display low solubility in aqueous solutions due to their hydrophobic nature and lack of water-soluble, stabilizing moieties.

Recently, synthetic block copolymer-containing IPECs, usually referred to more specifically as block ionomer complexes (BICs), have been prepared that may effectively address circulation and solubility issues observed with conventional systems. Although multiple types of repeating units have been considered for the respective blocks, poly(ethylene oxide) (PEO)<sup>26,33–36</sup> and poly[(*N*-2-hydroxypropyl)methacrylamide] (PHPMA)<sup>37–40</sup> are often chosen as the hydrophilic, nonimmunogenic, neutral block and synthetic quaternary amine polymers or bioconjugates as the cationic segments for nucleic acid binding.

Synthetic procedures to form BICs with precise block lengths and desired charge distribution have been limited until recently. For example, conventional free radical polymerization, although useful for polymerizing a wide range of monomers directly in water, is not useful for preparing either narrow molecular weight distribution polymers or block copolymers. Classical anionic, some cationic, and group transfer reactions can produce both, but the procedures are not facile, often requiring inert atmospheres and postpolymerization transformations of protecting groups to attain appropriate functionality and water solubility. Finally, methods such as telechelic end group coupling (for example hydroxyl functional PEOs with carboxy-terminal polymers) are often inefficient due to adverse entropic effects, poor kinetics, and lack of stoichiometry.

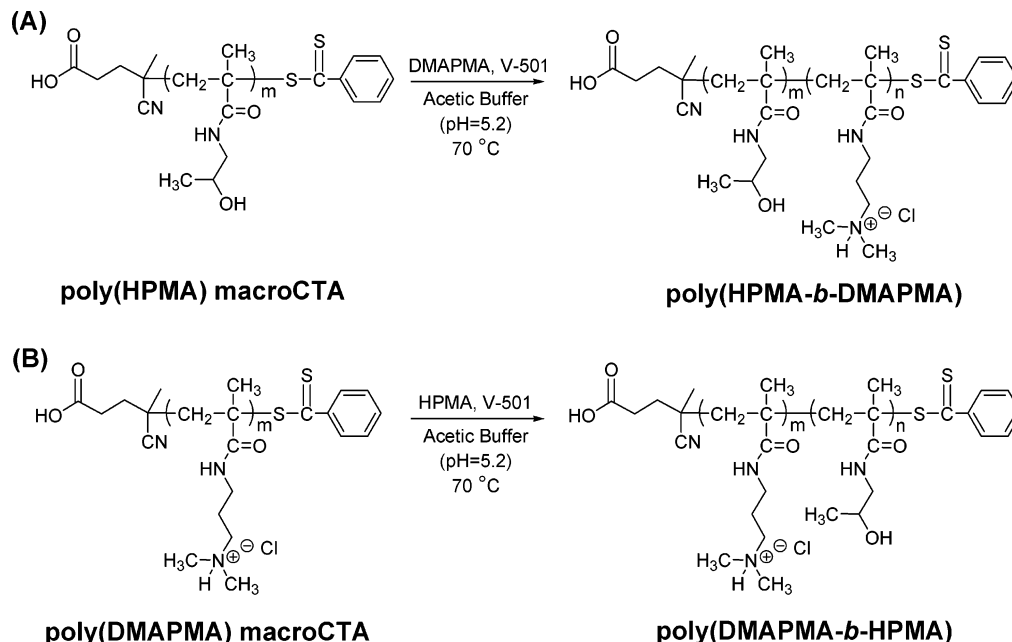
Fortunately, with rapid developments in controlled/"living" polymerization and specifically in controlled/"living" radical polymerization (CLRP) techniques,<sup>41–46</sup> it is now possible to prepare block copolymers from widely available monomers with desired functionality in a facile manner. Our laboratory, having a long-standing interest in the preparation of water-soluble, functional polymers, has recently focused efforts on the preparation of well-defined, near-monodisperse, water-soluble polymers via a controlled free radical polymerization technique known as reversible addition–fragmentation chain transfer (RAFT) polymerization.<sup>47</sup> Because RAFT allows for the direct synthesis of many different acrylamido and methacrylamido-monomers, as well as the preparation of functional, stimuli-responsive block copolymers, it has direct potential in the synthesis of precise block copolymer systems used to form stable, nonimmunogenic BICs. To date, the controlled RAFT polymerizations of anionic,<sup>47–50</sup> zwitterionic,<sup>47,51,52</sup> and neutral<sup>47,51–63</sup> acrylamido monomers have been reported in both organic and aqueous media employing a variety of chain transfer agents (CTAs) including xanthates,<sup>61</sup> dithiocarbamates,<sup>54,64</sup> trithiocarbonates,<sup>47,51,53,62</sup> and dithioesters.<sup>47–52,55–61,65–72</sup> Proper choice of monomer/CTA combination can afford control over the molecular weight and yield (co)polymers with low polydispersity indices (PDIs) under appropriate conditions. We have recently turned our attention to methacrylamido monomers and reported the first RAFT polymerization of a cationic methacrylamido

species, namely *N*-[3-(dimethylamino)propyl]methacrylamide (DMAPMA), mediated by 4-cyanopentanoic acid dithiobenzoate (CTP) in aqueous media.<sup>73</sup> DMAPMA was polymerized with good control over the molecular weight and polydispersity in an acetic acid/sodium acetate buffer solution ( $M_n = 47\ 100$  g/mol,  $M_w/M_n = 1.08$ ), while the corresponding polymerization in water alone exhibited a loss of control due to thiocarbonylthio hydrolysis ( $M_n = 44\ 500$  g/mol,  $M_w/M_n = 1.62$ ). We also recently reported the first direct, controlled polymerization of *N*-(2-hydroxypropyl)methacrylamide (HPMA) under aqueous conditions at 70 °C employing CTP and V-501 as the RAFT CTA and initiating species, respectively.<sup>74</sup> As with the RAFT polymerizations of other  $\alpha$ -methyl-substituted methacrylamido monomers (i.e., DMAPMA and methacrylamide<sup>75</sup>), an acetic buffer was employed to minimize hydrolysis and maintain dithioester chain ends. To further demonstrate the retention of the dithioester chain ends and the overall "livingness" of the system, a macroCTA of HPMA was synthesized ( $M_n = 36\ 100$  g/mol,  $M_w/M_n = 1.05$ ) and subsequently self-extended to produce the corresponding poly(HPMA-*b*-HPMA) "homopolymer."

Poly[*N*-(2-hydroxypropyl)methacrylamide] (PHPMA) has been employed as a vehicle in drug and gene delivery over the past few decades.<sup>76–83</sup> Much of the work with this uncharged, hydrophilic, biocompatible polymer has focused on the delivery of anticancer drugs,<sup>79,80,82–83</sup> site-specific delivery in the GI tract,<sup>84,85</sup> tumor-specific delivery of antisense oligonucleotides to their target mRNAs,<sup>37,38</sup> and hydrogels.<sup>84,86–88</sup> As with other successful delivery vehicles, drug conjugates of PHPMA are well-suited for pharmaceutical applications because they exhibit the enhanced permeability and retention (EPR) effect, thus increasing the concentration of an active drug in tumor cells (i.e., 10–100 times higher than that of the free drug) and decreasing the dose limiting toxicity.<sup>89,90</sup> In a recent publication, Ulbrich et al. reported the chemical coating of polyelectrolyte-based DNA-containing nanoparticles with multifunctional and telechelic PHPMA to increase their *in vivo* residence times.<sup>39</sup> The nonimmunogenic properties of PHPMA and the facility of forming well-defined block copolymers with a variety of cationic monomers and controlled segmental lengths suggest the potential of this system for the delivery and stabilization of siRNA. The utility of our reported synthetic method, as compared to previous methods, lies in the ability to prepare both the macroCTA and block copolymer with specified segmental lengths directly in solution using unprotected monomers simply by controlling conversion. This will allow precise tailoring of the dimensions of the polycation/RNA core and the PHPMA corona for increased tumor cell uptake and retention.

Building on our earlier work and recent literature reports, we describe a series of novel, well-defined block copolymers of HPMA and DMAPMA with specifically chosen copolymer compositions. The complexation behavior of these copolymers with a model siRNA has been evaluated using centrifugal membrane filtration studies and light scattering experiments to determine complex stability, size, molecular weight, and degree of complexation. The stability of these complexes against degradation by a common RNA nuclease, ribonuclease A (RNase A), has been evaluated by both UV–vis spectroscopy and gel electrophoresis. Furthermore, slow siRNA release from its polymer complexes has been demonstrated by gel electrophoresis. The results suggest that HPMA/DMAPMA block copolymers may function as effective siRNA stabilizing and delivery agents with potential applications in siRNA therapeutics.

**Scheme 1. Synthetic Pathways for the Preparation of *N*-(2-hydroxypropyl)methacrylamide (HPMA)/*N*-[3-(dimethylamino)propyl]methacrylamide (DMAPMA) Block Copolymers by Aqueous Reversible Addition Fragmentation Chain Transfer (RAFT) Polymerization at 70 °C under Buffered Conditions (pH = 5.2)**



(A) Chain-extension of Poly(HPMA) Macro Chain Transfer Agent (macroCTA) with DMAPMA and (B) Chain-extension of Poly(DMAPMA) MacroCTA with HPMA

## Experimental Section

**Materials.** All chemicals were purchased from Aldrich at the highest available purity and used as received unless otherwise noted. 4,4'-Azobis(4-cyanopentanoic acid) (V-501, a gift from Wako Pure Chemicals Industries, Ltd.) was recrystallized from methanol. 4-Cyanopentanoic acid dithiobenzoate (CTP) was synthesized according to the literature procedure.<sup>91</sup> Deionized water (DI H<sub>2</sub>O) was obtained from a Barnstead NANO-Pure reverse osmosis/filtration unit (resistivity of 18.0 MΩ). Water used in complexation experiments was obtained from Sigma and shown to have no nuclease (DNase or RNase) activity. HPMA was synthesized according to a literature procedure reported by Kopeček et al.<sup>92,93</sup> DMAPMA was purified by vacuum distillation. RNase A was obtained from United States Biological.

**Synthesis of poly(HPMA) macroCTA.** Poly(HPMA) macroCTA was prepared by aqueous RAFT polymerization employing V-501 as the primary radical source and CTP as the RAFT CTA at 70 °C. The polymerization was performed directly in an aqueous acetic buffer (pH = 5.2, 0.27 mol/L acetic acid and 0.73 mol/L sodium acetate) with an initial monomer concentration ([M]<sub>0</sub>) of 1 M in a septum-sealed, round-bottom flask that was purged with nitrogen for 30 min prior to polymerization. Two separate macroCTAs were prepared with initial monomer to CTA ratios ([M]<sub>0</sub>/[CTA]<sub>0</sub>) of 200:1 and 800:1, while the CTA to initiator ratio ([CTA]<sub>0</sub>/[I]<sub>0</sub>) for both systems was kept at 5:1. The HPMA macroCTA synthesized with an initial [M]<sub>0</sub>/[CTA]<sub>0</sub> of 200:1, poly(HPMA<sub>70</sub>), was prepared by dissolving HPMA (11.5 g, 80 mmol), CTP (112 mg, 0.4 mmol), and V-501 (22.5 mg/0.08 mmol) in a 250 mL round-bottomed-flask and diluting the resulting solution to a final volume of 80 mL with aqueous acetic buffer. The resulting polymerization solution was reacted for 3 h at 70 °C and was terminated by immersion of the reaction flask in liquid nitrogen. The HPMA macroCTA synthesized at an initial [M]<sub>0</sub>/[CTA]<sub>0</sub> of 800:1, poly(HPMA<sub>258</sub>), was prepared by dissolving HPMA (28.64 g, 200 mmol), CTP (70.0 mg, 0.25 mmol), and V-501 (14.0 mg, 0.05 mmol) in 50 mL buffer (0.272 mol/L acetic acid and 0.728 mol/L sodium acetate, pH = 5.3) in a 500 mL round-bottomed flask and diluting the resulting solution to a final volume of 200 mL with aqueous acetic buffer. The resulting polymerization solution was reacted for 3 h at 70 °C and was terminated by

immersion of the reaction flask in liquid nitrogen. Both HPMA macroCTAs ( $M_n = 10\,000$  g/mol, PDI = 1.05 and  $M_n = 37\,000$  g/mol, PDI 1.15) were isolated by dialysis in acidic conditions (pH 3–4) at 4 °C followed by lyophilization.

**Synthesis of poly(HPMA-*b*-DMAPMA) Block Copolymers.** As Scheme 1A illustrates, block copolymers of HPMA and DMAPMA were prepared directly in aqueous acetic buffer with both HPMA macroCTAs, poly(HPMA<sub>70</sub>) and poly(HPMA<sub>258</sub>), using V-501 as the primary radical source and a [M]<sub>0</sub> of 1.0 M DMAPMA. DMAPMA monomer stock solution, preneutralized with HCl, was added to each HPMA macroCTA to yield a [M]<sub>0</sub>/[CTA]<sub>0</sub> of 200:1 and a [CTA]<sub>0</sub>/[I]<sub>0</sub> of 5:1 for each system.

**Chain Extension of poly(HPMA<sub>70</sub>) with DMAPMA.** Block copolymers of poly(HPMA<sub>70</sub>) with DMAPMA were prepared by adding V-501 (22.5 mg, 0.08 mmol), poly(HPMA<sub>70</sub>) (4.0 g, 0.4 mmol), and 63.7 mL of an HCl-neutralized 1.25 mol·L<sup>-1</sup> DMAPMA stock solution (16.5 g, 80 mmol) to a 100 mL round-bottomed flask. The resulting polymerization solution was divided into four separate 25 mL round-bottomed flasks (i.e., 20 mL per flask), and each flask was subsequently sealed and purged with nitrogen for 30 min prior to being immersed in a 70 °C water bath. Each flask was removed from the water bath at different time intervals and quenched with liquid nitrogen, yielding copolymers of varying compositions.

**Chain Extension of poly(HPMA<sub>258</sub>) with DMAPMA.** Block copolymers of poly(HPMA<sub>258</sub>) with DMAPMA were prepared by adding 2 mL of a 7.1 mmol·L<sup>-1</sup> solution of V-501 (2 mg, 0.0071 mmol), poly(HPMA<sub>258</sub>) (1.85 g, 0.05 mmol), and 8 mL of an HCl-neutralized 1.25 mol·L<sup>-1</sup> DMAPMA stock solution (2.06 g, 10 mmol) to a scintillation vial. The resulting polymerization solution was divided into five separate 4 mL reaction vials (i.e., 2 mL in each vial), and each vial was subsequently sealed and purged with nitrogen for 30 min prior to being immersed in a 70 °C water bath. The reaction vials were removed from the water bath at different time intervals and quenched with liquid nitrogen, yielding copolymers of varying compositions.

**Synthesis of poly(DMAPMA) macroCTA.** Poly(DMAPMA) was prepared by aqueous RAFT polymerization employing V-501 as the primary radical source and CTP as the RAFT CTA at 70 °C. The polymerization was performed directly in an aqueous acetic

**Table 1. Conversion, Number-Average Molecular Weight ( $M_n$ ), and Polydispersity ( $M_w/M_n$ ) Data for the Preparation of a Series of *N*-(2-hydroxypropyl)methacrylamide (HPMA) and *N*-[3-(dimethylamino)propyl]methacrylamide (DMAPMA) Block (co)Polymers by Aqueous Reversible Addition Fragmentation Chain Transfer (RAFT) Polymerization**

sample	% conv. <sup>b</sup>	composition (theory)	composition (expt)	$M_n$ (theory) (g/mol)	$M_n^a$ (expt) (g/mol)	$M_w/M_n$
poly(HPMA <sub>70</sub> - <i>b</i> -DMPMA <sub>24</sub> )	12	74:26	73:27	15 000	15 400	1.08
poly(HPMA <sub>70</sub> - <i>b</i> -DMPMA <sub>49</sub> )	20	63:37	59:41	18 300	20 100	1.07
poly(HPMA <sub>70</sub> - <i>b</i> -DMPMA <sub>82</sub> )	34	51:49	46:54	24 000	27 000	1.1
poly(HPMA <sub>70</sub> - <i>b</i> -DMPMA <sub>105</sub> )	45	44:56	40:60	31 100	32 000	1.11
poly(HPMA <sub>258</sub> - <i>b</i> -DMPMA <sub>13</sub> )	9	93:7	95:5	40 700	39 700	1.06
poly(HPMA <sub>258</sub> - <i>b</i> -DMPMA <sub>23</sub> )	14	90:10	92:8	42 800	41 800	1.08
poly(HPMA <sub>258</sub> - <i>b</i> -DMPMA <sub>43</sub> )	22	15:85	86:14	46 100	45 900	1.08
poly(HPMA <sub>258</sub> - <i>b</i> -DMPMA <sub>53</sub> )	25	16:84	83:17	47 300	48 000	1.11
poly(DMAPMA <sub>77</sub> - <i>b</i> -HPMA <sub>115</sub> )	43	47:53	40:60	30 400	34 300	1.06

<sup>a</sup> As determined by <sup>1</sup>H NMR spectroscopy. <sup>b</sup> Conversions were determined by comparison of the UV signal at 274 nm of the monomer at  $t_0$  to that at  $t_x$ .

buffer (pH = 5.2, 0.27 mol/L acetic acid and 0.73 mol/L sodium acetate) with a  $[M]_0$  of 2 M in a septum-sealed, round-bottom flask that was purged with nitrogen for 30 min prior to polymerization. A single poly(DMAPMA) macroCTA was prepared with a  $[M]_0/[CTA]_0$  of 294:1, while the  $[CTA]_0/[I]_0$  was maintained at 8:1. Generally, DMAPMA (13.62 g, 80.0 mmol) was dissolved in 10 mL of acetic acid/sodium acetate buffer at 0 °C, and the pH of the solution was adjusted to 5 with HCl. CTP (76.0 mg, 0.272 mmol) and initiator (9.5 mg, 0.034 mmol) were added, and the resulting solution was diluted to 40 mL with additional buffer solution. The solution was degassed by purging with nitrogen for 30 min and allowed to react at 70 °C for 2 h. Poly(DMAPMA) macroCTA ( $M_n = 17\,000$  g/mol, PDI = 1.05) was isolated by dialysis in acidic conditions (pH 3–4) at 4 °C followed by lyophilization.

**Synthesis of poly(DMAPMA-*b*-HPMA) Block Copolymers.** Block copolymers of DMAPMA and HPMA were prepared directly in aqueous acetic buffer with poly(DMAPMA) macroCTA ( $M_n = 17\,800$  g/mol) using V-501 as the primary radical source and a  $[M]_0$  of 1.0 M HPMA (Scheme 1B). HPMA monomer was added to DMAPMA macroCTA to yield a  $[M]_0/[CTA]_0$  of 200:1 with a  $[CTA]_0/[I]_0$  of 5:1. Copolymers of DMAPMA and HPMA were prepared by adding 0.5 mL of a 4 mmol·L<sup>-1</sup> solution of V-501 (0.56, 0.002 mmol), DMAPMA macroCTA (170.0 mg, 0.01 mmol), HPMA (0.286 g, 2.0 mmol), and 1.5 mL of buffer to a septum-sealed 4 mL reaction vial. The resulting solution was purged with nitrogen for 30 min and the reaction flask immersed in a 70 °C water bath. Following sufficient polymerization time (6 h), the reaction flask was removed from the water bath and immersed in liquid nitrogen. The resulting copolymer was purified and isolated by dialysis against water and lyophilization, respectively.

**(Copolymer Characterization.** Both HPMA macroCTAs were characterized by aqueous size exclusion chromatography (ASEC-MALLS) employing an eluent of 20% acetonitrile/80% 0.05 M Na<sub>2</sub>SO<sub>4</sub> at a flow rate of 0.5 mL/min at 25 °C. Tosoh Biosciences TSK-gel columns (G3000 PW<sub>XL</sub> (<50 000 g/mol, 200 Å) and G4000 PW<sub>XL</sub> (2000–300 000 g/mol, 500 Å)) with a Polymer Labs LC 1200 UV-vis, Wyatt Optilab DSP interferometric refractometer, and a Wyatt DAWN-EOS multiangle laser light scattering (MALLS) detector ( $\lambda = 690$  nm). Block copolymers of HPMA and DMAPMA were analyzed by ASEC using an eluent of 1% acetic acid/0.10 M Na<sub>2</sub>SO<sub>4</sub> (aq) at a flow rate of 0.3 mL/min at 25 °C, SynChropak CATSEC columns (100, 300, and 1000 Å; Eichrom Technologies Inc.), a Polymer Labs LC1200 UV-Vis detector, a Knauer K-2301 RI detector ( $\lambda = 950$  nm), and a Wyatt DAWN-DSP multiangle laser light scattering detector ( $\lambda = 633$  nm). Conversions in each system were determined by comparing the area of the UV signal at 274 nm corresponding to monomer at  $t_0$  to the area at  $t_x$ . Absolute molecular weights and polydispersities were calculated using the Wyatt ASTRA SEC/LS software package. Copolymer compositions were determined with a Varian Mercury<sup>PLUS</sup> 300 MHz spectrometer with a delay time of 5 s. <sup>1</sup>H NMR was used to determine the copolymer composition of poly(HPMA-*b*-DMPMA) by integration of the relative intensities of the methyne-proton resonances at 3.78 ppm (macro-HPMA) and the dimethyl-proton resonances at 2.80 ppm (poly(DMAPMA)).

**Preparation of siRNA.** A 43-nucleotide model siRNA against human RNA polymerase II A was transcribed in vitro from synthetic DNA oligonucleotides by T7 RNA polymerase according to standard procedure.<sup>94</sup> Two DNA oligonucleotides, CGTAATAC-GACTCACTATTAGG and GGAGGAGATGGACAACAAGITTTG-TAACTTGTGTCCATCTCCTAATAGTGAGTCGTATTA, were annealed at an equal molar ratio to form a partially double-stranded DNA containing the T7  $\phi$ 2.5 promoter and the template DNA. Upon transcription, a siRNA in the form of a small hairpin, with the sequence of AGGAGAUGGACAACAAGUUACAACUUG-UUGUCAUCUCCUCC, was produced (the underlined nucleotides form base pairs). For <sup>32</sup>P-labeled siRNA preparation, 1  $\mu$ M of [ $\alpha$ -<sup>32</sup>P] ATP was included in the transcription solution. After transcription, the siRNA was purified by 8% denaturing polyacrylamide gel electrophoresis. siRNA was extracted from the gel and recovered by ethanol precipitation. The concentration of siRNA was determined by its absorbance at 260 nm, using an estimated molar extinction coefficient of 355,000 M<sup>-1</sup>·cm<sup>-1</sup>.

**Preparation of poly(HPMA-*b*-DMPMA)/siRNA Complexes.** All poly(HPMA-*b*-DMPMA)/siRNA complexes were prepared at *N/P* ratios of 1.0 at 25 °C. In a typical preparation, 4  $\mu$ L of 1  $\mu$ M <sup>32</sup>P ATP-labeled siRNA (4 pmol) were added to 10  $\mu$ L of phosphate-buffered saline solution (pH = 7.2, [NaCl] = 0.1 M) in a microcentrifuge tube. The resulting solution was further diluted with appropriate volumes of nuclease free water and (1  $\mu$ M) block polymer solution to give a final volume of 40  $\mu$ L and a total siRNA concentration of 50 nM. Complexation solutions were immediately vortexed following addition of polymer solution to allow for homogeneous mixing.

**Centrifugal Filtration Studies of siRNA/Block Copolymer Complexes.** Polymer/siRNA complex solutions were characterized by centrifugal filtration studies combined with scintillation counting measurements. In a typical experiment, a 40  $\mu$ L complex solution was centrifuged at 14 000 rpm for 10 min to remove any insoluble, precipitated siRNA/block copolymer complexes. The liquid portion of the centrifuged sample was placed in a fresh microcentrifuge tube, and both the dry tube with any precipitated complexes and the solution-filled tube with the remaining siRNA were analyzed by scintillation counting of <sup>32</sup>P ATP-labeled siRNA to obtain the percentages of precipitated and solubilized siRNA, respectively. The centrifuged complex solution was then placed in a siRNA-permeable, Millipore-Microcon YM-50 (regenerated cellulose 50 000 MWCO) centrifugal membrane filter and subsequently centrifuged at 14 000 rpm for 3 min. The resulting filtrate was analyzed by scintillation counting to quantitate the total amounts of bound/stabilized and unbound siRNA. The data listed in Table 2 show the experimental conditions employed in the preparation of each complex. Theoretical stoichiometric *N/P* ratios were obtained by preparing and mixing appropriate amounts of 1  $\mu$ M stock solutions of siRNA and block copolymer. Two separate control experiments, C<sub>1</sub> and C<sub>3</sub>, were employed in order to quantitate the amount of interaction of PHPMA with siRNA and to give a baseline for the amount of siRNA that is able to permeate the 50 000 MWCO membrane filter, respectively.

**Table 2. Experimental Conditions for Complexation of Small Interfering Ribonucleic Acid (siRNA) with Poly(*N*-(2-hydroxypropyl)methacrylamide)-*b*-*N*-[3-(dimethylamino)propyl]methacrylamide (HPMA-*b*-DMPMA) Block Copolymers (Theoretical *N/P* Ratio = 1.0) at 25 °C (pH = 7.2, 125 mM NaCl, 20 mM Phosphate Buffer)**

entry	(co)polymer	pmol RNA	pmol polymer	polymer/ RNA ratio	<i>N/P</i> ratio
C <sub>1</sub>	poly(HPMA <sub>70</sub> )	4	7	1.8	
C <sub>2</sub>	poly(DMAPMA <sub>77</sub> )	4	2.2	0.6	1
C <sub>3</sub>	siRNA only	4	0	0	
1	poly(HPMA <sub>70</sub> - <i>b</i> -DMPMA <sub>24</sub> )	4	7	1.8	1
2	poly(HPMA <sub>70</sub> - <i>b</i> -DMPMA <sub>49</sub> )	4	3.5	0.9	1
3	poly(HPMA <sub>70</sub> - <i>b</i> -DMPMA <sub>82</sub> )	4	2	0.5	1
4	poly(HPMA <sub>70</sub> - <i>b</i> -DMPMA <sub>105</sub> )	4	1.7	0.4	1
5	poly(HPMA <sub>258</sub> - <i>b</i> -DMPMA <sub>13</sub> )	4	13	3.3	1
6	poly(HPMA <sub>258</sub> - <i>b</i> -DMPMA <sub>23</sub> )	4	7.5	1.9	1
7	poly(HPMA <sub>258</sub> - <i>b</i> -DMPMA <sub>43</sub> )	4	4.0	1.0	1
8	poly(HPMA <sub>258</sub> - <i>b</i> -DMPMA <sub>53</sub> )	4	3.2	0.8	1

**Dynamic Light Scattering (DLS) Experiments.** The hydrodynamic diameters ( $D_H$ ) of siRNA/block copolymer complexes were obtained via dynamic light scattering experiments that employed a Malvern-Zetasizer Nano Series DLS detector with a 22 mW He-Ne laser operating at  $\lambda = 632.8$  nm, an avalanche photodiode detector with high quantum efficiency, and an ALV/LSE-5003 multiple  $\tau$  digital correlator electronics system. Samples were prepared at a total siRNA concentration of 3500 nM and contained a total mass per volume (i.e., block copolymer mass + siRNA mass per mL) of 0.5 mg/mL while maintaining a *N/P* ratio of 1.0. To remove dust, samples were centrifuged at 14 000 rpm for 10 min prior to characterization via DLS. All  $D_H$  measurements were performed in triplicate at 25 °C, and complex sizes were compared to those of the uncomplexed block copolymers and the pure siRNA.

**Static Light Scattering (SLS) Experiments.** Static light scattering measurements were performed on complexes of siRNA with poly(HPMA<sub>258</sub>-*b*-DMPMA<sub>13</sub>) using a Malvern Instruments Zetasizer at a constant scattering angle of 173°. The weight-average molecular weight,  $M_w$ , and second virial coefficient,  $A_2$ , of selected complexes were determined using the following equation:

$$\frac{K^*c}{R_\theta} = 2A_2c + \frac{1}{M}$$

where  $K^*$ ,  $C_p$ ,  $M_w$ ,  $R_\theta$ , and  $A_2$  are the optical constant, polymer concentration, molecular weight, Rayleigh ratio, and second virial coefficient, respectively. The specific refractive index increment,  $dn/dc$  for the complexes was obtained using the following equation:

$$\left(\frac{dn}{dc}\right)_{av} = w_A\left(\frac{dn}{dc}\right)_A + w_B\left(\frac{dn}{dc}\right)_B + w_C\left(\frac{dn}{dc}\right)_C$$

where  $w_A$ ,  $w_B$ , and  $w_C$  are the mass fractions of the pure polymers PHPMA, PDMAPMA, and the siRNA with  $dn/dc$  values of  $(dn/dc)_A$ ,  $(dn/dc)_B$ , and  $(dn/dc)_C$ , respectively. Because most of the mass in each complex was composed of PHPMA (i.e. polymer/siRNA = 3.3:1), the magnitude of  $w_A$  was assumed to be much greater than  $w_B$  and  $w_C$ ; therefore, the  $dn/dc$  value of 0.168 mL/g for PHPMA in phosphate-buffered saline solution (pH = 7.2, [NaCl] = 0.1 M) was employed to obtain the  $M_w$  for the complexes. LS samples were prepared using an initial stock solution of complexes with a total siRNA concentration of 7200 nM and a total mass per volume (i.e., block copolymer mass + siRNA mass per mL) of 1.0 mg/mL at an *N/P* ratio of 1.0. To remove dust, samples were centrifuged at 14 000 rpm for 10 min. The  $R_\theta$  at specific complex concentrations between 1.0 and 0.20 mg/mL were determined by LS and used to construct a Debye plot that yielded the  $M_w$  and  $A_2$  for the complexes. To prepare different complex concentrations, 20  $\mu$ L of the 1 mg/mL complex stock solution was placed in a 45  $\mu$ L LS cuvette and diluted with phosphate-buffered saline using a

P2 micropipet in 1–2  $\mu$ L increments. The  $R_\theta$  at each concentration was measured to obtain a linear Debye plot.

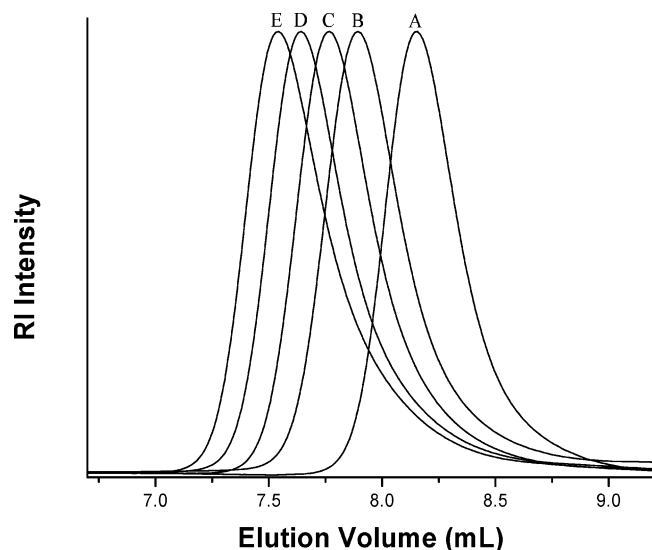
**Enzymatic Degradation Studies of siRNA/Copolymer Complexes.** The kinetics of degradation of free and complexed siRNA with RNase A were obtained using a JASCO V-530 spectrophotometer, monitoring at 260 nm (i.e., the  $\lambda_{max}$  for RNA) in kinetics/time course mode over a time interval of 25 min. Two block copolymer systems were employed in complexation with the siRNA, including poly(HPMA<sub>258</sub>-*b*-DMPMA<sub>13</sub>) and poly(HPMA<sub>258</sub>-*b*-DMPMA<sub>23</sub>). Complexation was conducted under phosphate-buffered conditions (20 mM, pH = 7.4) and employed a total siRNA concentration of 625 nM. As with the centrifugal filtration studies and LS measurements, the polymer stoichiometry was adjusted in each complexation to yield an *N/P* ratio of 1.0. The kinetics of degradation of siRNA without copolymer stabilization were also determined and employed as a control. In a typical kinetics measurement, 375 pmoles siRNA were combined with 120  $\mu$ L of phosphate buffer solution (pH = 7.4). The resulting solution was further diluted to 600  $\mu$ L with water and vortexed. Block copolymer solution (1  $\mu$ L) at the appropriate concentration was added to the siRNA solution, and the resulting complex solution was immediately vortexed to ensure homogeneous mixing. The complex solution was placed in a 1.4 mL quartz cuvette and the initial absorbance at 260 nm ( $A_{260}$ ) was recorded prior to the addition of 2  $\mu$ L of a 0.35 units/ $\mu$ L RNase A solution. Following rapid mixing, the resulting solution was placed in the spectrophotometer and the  $A_{260}$  was monitored over time.

To confirm polymer protection of siRNA against RNase A, analysis of siRNA/polymer complexes by agarose gel electrophoresis was performed. siRNA (1  $\mu$ g, 70 pmol) in 20 mM phosphate solution (pH 7.4) was mixed with 231 pmol of poly(HPMA<sub>258</sub>-*b*-DMPMA<sub>13</sub>) or 187 pmol of poly(HPMA<sub>258</sub>-*b*-DMPMA<sub>23</sub>) to maintain an *N/P* ratio of 1.0. RNase A (0.1 U) was then incubated with the siRNA/polymer complexes for 6 min at room temperature in a final volume of 4  $\mu$ L. Control reactions in the absence of the polymers were also performed. Gel-loading buffer containing 4  $\mu$ L of 0.2% bromophenol blue, 8 M urea, and 1X TBE buffer (Tris-borate-EDTA) was added to the reaction tube. The samples were immediately loaded onto a 1.5% agarose minigel (10 cm  $\times$  7 cm) and run for 10 min at 120 V. After staining by ethidium bromide, the agarose gel was imaged by a CCD camera.

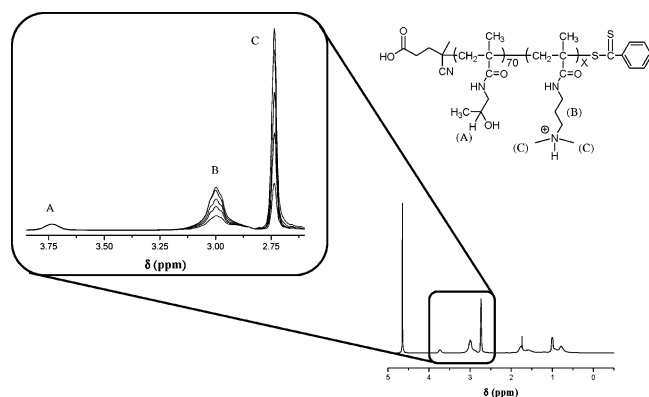
**siRNA Release from siRNA/Copolymer Complexes.** The demonstration of siRNA dissociation from the siRNA/polymer complexes was achieved by agarose gel electrophoresis. siRNA/poly(HPMA<sub>258</sub>-*b*-DMPMA<sub>13</sub>) complexes (10  $\mu$ L, containing 1  $\mu$ g siRNA) in a gel-loading buffer (*N/P* = 1.0, prepared as described above) were loaded at different times onto a 1.5% agarose minigel. The voltage was kept constant at 120 V during electrophoresis. A digital image of the gel was obtained by a CCD camera after ethidium bromide staining.

## Results and Discussion

**Synthesis of HPMA/DMPMA Block (Co)Polymers.** RAFT synthesis of block copolymers of HPMA and DMPMA (Scheme 1A) employed two HPMA macroCTAs, namely poly(HPMA<sub>70</sub>) and poly(HPMA<sub>258</sub>). Figure 1 shows aqueous, cationic SEC-MALLS traces for the controlled chain extension of poly(HPMA<sub>70</sub>) with DMPMA under aqueous conditions at 70 °C via RAFT, while the analogous SEC traces for the controlled chain extension of poly(HPMA<sub>258</sub>) with DMPMA can be found in the Supporting Information. The unimodal nature of the chromatograms along with the shift to lower elution volumes is qualitatively indicative of high blocking efficiency. Table 1 lists the conversion, molecular weight, and polydispersity data for each block copolymer system, indicating that the chain extensions of both HPMA macroCTAs with DMPMA proceed in a controlled fashion, producing block copolymers with low  $M_w/M_n$  and reasonable agreement between experi-



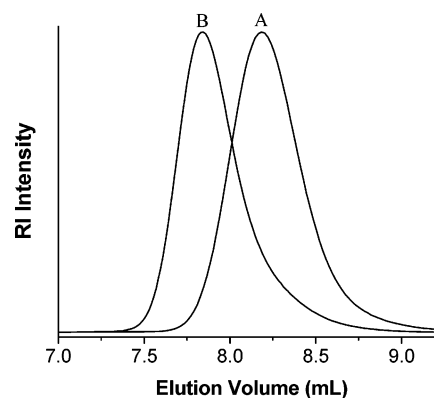
**Figure 1.** Aqueous, cationic size exclusion chromatography multiangle laser light scattering (SEC-MALLS) traces demonstrating the efficient blocking of poly(*N*-(2-hydroxypropyl)methacrylamide<sub>70</sub>) (HPMA) macro chain transfer agent (macroCTA) with *N*-[3-(dimethylamino)propyl]methacrylamide (DMAPMA), (A) poly(HPMA) macroCTA, (B) poly(HPMA<sub>70</sub>-*b*-DMAPMA<sub>24</sub>), (C) poly(HPMA<sub>70</sub>-*b*-DMAPMA<sub>49</sub>), (D) poly(HPMA<sub>70</sub>-*b*-DMAPMA<sub>82</sub>), and (E) poly(HPMA<sub>70</sub>-*b*-DMAPMA<sub>105</sub>).



**Figure 2.** Relevant chemical shift assignments for <sup>1</sup>H NMR spectra of *N*-(2-hydroxypropyl)methacrylamide/*N*-[3-(dimethylamino)propyl]methacrylamide block copolymers at different compositions.

mental and theoretical molecular weights. Figure 2 shows the <sup>1</sup>H NMR spectra for the series of block copolymers listed in Table 1. Copolymer compositions and, therefore, experimental molecular weights were determined by integration of the relative normalized resonances of the methyne-proton peaks at 3.7 ppm (poly(HPMA)) and the dimethyl-proton peaks at 2.7 ppm (poly(DMAPMA)). The copolymer composition data in Table 1 show a small “overshoot” in the experimental molecular weights with respect to theoretical values that is attributed to the formation of homopolymer impurity during the RAFT process.

Block copolymers of HPMA and DMAPMA were also prepared by chain-extension of poly(DMAPMA<sub>77</sub>) macroCTA with HPMA (Scheme 1B) under aqueous conditions at 70 °C. Figure 3 shows the aqueous, cationic SEC traces of poly(DMAPMA<sub>77</sub>) macroCTA and the resulting poly(DMAPMA-*b*-HPMA) block copolymer. The shift of the MWD to lower elution volume with a low amount of “dead” polymer chains being formed is again indicative of efficient blocking and confirms that there is no significant block order dependence when preparing block copolymers of HPMA and DMAPMA via RAFT. As with the chain extension of HPMA macroCTA with DMAPMA, blocking of DMAPMA macroCTA with



**Figure 3.** Aqueous, cationic size exclusion chromatography multiangle laser light scattering (SEC-MALLS) chromatograms demonstrating the efficient blocking of *N*-[3-(dimethylamino)propyl]methacrylamide (DMAPMA) macro chain transfer agent (macroCTA) with *N*-(2-hydroxypropyl)methacrylamide (HPMA), (A) poly(DMAPMA<sub>74</sub>) macroCTA, (B) poly(DMAPMA<sub>74</sub>-*b*-HPMA<sub>115</sub>).

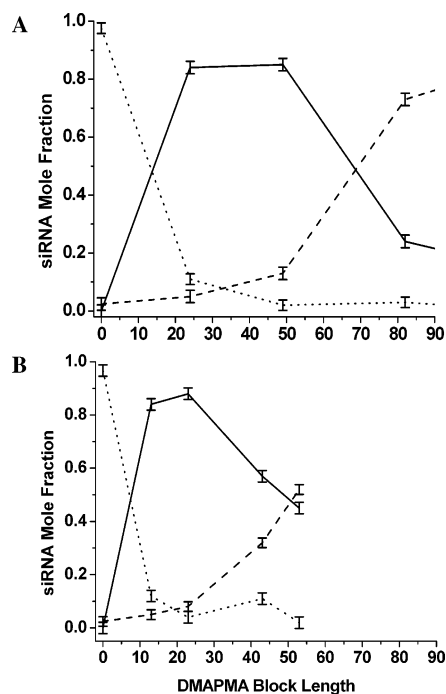
**Table 3.** Scintillation Counting Results for Centrifugation and Filtration Studies Following Small Interfering Ribonucleic Acid (siRNA) Complexation with Poly(*N*-(2-hydroxypropyl)methacrylamide)-*b*-*N*-[3-(dimethylamino)propyl]methacrylamide (HPMA-*b*-DMAPMA) Copolymers (Theoretical *N/P* Ratio = 1)

entry	% unbound siRNA	% bound siRNA	% precipitated siRNA
C <sub>1</sub>	97.6	0.0	2.4
C <sub>2</sub>	1.0	23.0	76.0
C <sub>3</sub>	97.0	0.0	3.0
1	11.0	84.0	5.0
2	2.0	85.0	13.0
3	3.0	24.0	73.0
4	1.0	17.0	82.0
5	12.0	84.0	4.0
6	4.0	88.0	8.0
7	11.0	57.0	32.0
8	2.0	45.0	53.0

HPMA also produces well-defined block copolymers with low  $M_w/M_n$  values and reasonable agreement between experimental and theoretical molecular weights.

**Synthesis and Characterization of siRNA/Block (Co)polymer Complexes.** Because the minimization of residual cationic or anionic charge in the formation BIC systems for gene delivery applications is essential to successful tumor-cell transfection, all complexes formed with HPMA/DMAPMA block copolymers and siRNA were prepared at stoichiometric *N/P* ratios. Table 2 shows the conditions employed in the complexation experiments with a fixed quantity of siRNA (4 pmol) and appropriate amounts of eight separate HPMA/DMAPMA block copolymer samples. For the eight samples (e.g., entries 1–8), the length of the cationic DMAPMA block was varied while the neutral HPMA block was maintained at DP values of 70 (entries 1–4) or 258 (entries 5–8). Additionally, three control conditions (e.g., entries C<sub>1</sub>–C<sub>3</sub>) were employed, where C<sub>1</sub> and C<sub>2</sub> involve complexation of PHPMA and PDMAPMA homopolymers with siRNA, respectively, and C<sub>3</sub> represents the behavior of siRNA in the absence of polymers.

Centrifugal filtration studies were conducted (Experimental Section) in order to determine the extent of polymer/siRNA complexation, specifically the efficiency of each copolymer in forming corona-stabilized IPECs that would remain in solution. The data from the centrifugal filtration experiments are given in Table 3. Poly(HPMA<sub>70</sub>) (entry C<sub>1</sub>) showed minimal interaction with siRNA, as evidenced by the fact that nearly 100% of the RNA passed through the centrifugal filter. Homo-



**Figure 4.** Mole fractions of free (···), bound/stabilized (—), and precipitated (---) small interfering ribonucleic acid following complexation with (A) entries 1–4 and (B) entries 5–7 in Table 2, obtained by centrifugal filtration and scintillation counting as a function of *N*-[3-(dimethylamino)propyl]methacrylamide (DMAPMA) block length.

poly(DMAPMA<sub>77</sub>) complexed strongly with siRNA (entry C<sub>2</sub>) and demonstrated that the PHPMA block was necessary in order to stabilize the siRNA/copolymer complexes. The third control experiment consisted of the centrifugal filtration of the siRNA in the absence of polymer (entry C<sub>3</sub>) and was conducted in order to account for the portion of siRNA that is retained by adsorption on the centrifugal membrane filter.

It is evident from entries 1–8 in Table 3 that there is a relationship between the DMAPMA block length and effective stabilization of the resulting siRNA/copolymer complexes. Specifically, when shorter DMAPMA block lengths are employed (i.e., entries 1, 2, 5, and 6), high quantities of soluble siRNA/copolymer complexes are formed (e.g., 84–88%) with minimal amounts (e.g., 4–13%) of aggregated complexes, which can be removed by centrifugation. Furthermore, solutions of 1, 2, 5, and 6 also contain only small amounts (2–12%) of unbound siRNA. Conversely, when longer DMAPMA block lengths are employed (i.e., entries 3, 4, 7, and 8), significantly lower amounts of solubilized siRNA/copolymer complexes are formed and the percentage of aggregated complexes is greatly increased.

The data listed in Table 3 are shown graphically in Figure 4A and B for copolymers prepared with respective HPMA block lengths of 70 (e.g., entries 1–4) and 258 (e.g., entries 5–8). The mole fractions of siRNA solubilized in siRNA/copolymer complexes (solid line), precipitated siRNA/copolymer complexes (dashed line), and uncomplexed or free siRNA (dotted line) are plotted as a function of DMAPMA block length. It is evident that, for both neutral HPMA block lengths, there exists an optimal range of cationic DMAPMA block lengths yielding the highest fraction of stabilized siRNA/copolymer complexes. For the copolymer series prepared with poly(HPMA<sub>70</sub>) macroCTA (i.e., entries 1–4), DMAPMA block lengths between 25 and 50 units allow for efficient complexation of siRNA (little free RNA) with minimal complex precipitation, while the series prepared with poly(HPMA<sub>258</sub>) (i.e., entries 5–8) requires shorter

**Table 4. Independent Hydrodynamic Diameters of Small Interfering Ribonucleic Acid (siRNA), Poly(*N*-(2-hydroxypropyl)methacrylamide<sub>258</sub>-*b*-*N*-[3-(dimethylamino)propyl]methacrylamide<sub>13</sub>) (HPMA<sub>258</sub>-*b*-DMAPMA<sub>13</sub>), and the siRNA/Poly(HPMA<sub>258</sub>-*b*-DMAPMA<sub>13</sub>) Complex Obtained by Dynamic Light Scattering**

sample	concn (mg/mL)	$D_H$ (nm)	standard deviation (nm)
siRNA	0.50	2.95	± 0.341
poly(HPMA <sub>258</sub> - <i>b</i> -DMAPMA <sub>13</sub> )	2.00	9.84	± 0.317
siRNA/poly(HPMA <sub>258</sub> - <i>b</i> -DMAPMA <sub>13</sub> ) complex	0.50	11.25	± 0.471

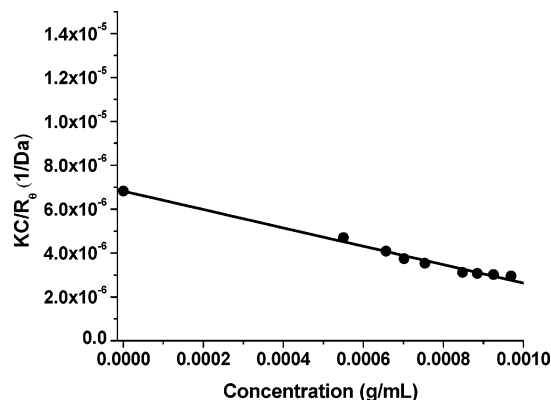
sample	$M_w$ (theory) (kDa)	$M_w$ (expt) (kDa)	$A_2$ (mL mol/g <sup>2</sup> )
Poly(HPMA <sub>258</sub> - <i>b</i> -DMAPMA <sub>13</sub> )	145.2	146.7	$-2.1 \times 10^{-3}$

DMAPMA block lengths, between 13 and 23 units, to obtain a comparable effect.

The data in Table 3 and Figure 4 provide the basis for rational structural design despite the lack of precise knowledge of the details of intermolecular complexation of the cationic units of the block copolymer and the more conformationally restricted nucleotide units of siRNA. A minimal but sufficient number of DMAPMA repeating units should be present to complex essentially all siRNA. The neutral, hydrophilic block needs to be sufficiently long to sterically stabilize the complexed segments as determined by the control experiment (C3, Table 3). It should be possible to manipulate these block lengths for a specified interpolymer complex core and a hydrophilic, stabilizing HPMA corona, the latter capable of being “tuned” for specified hydrodynamic dimensions.

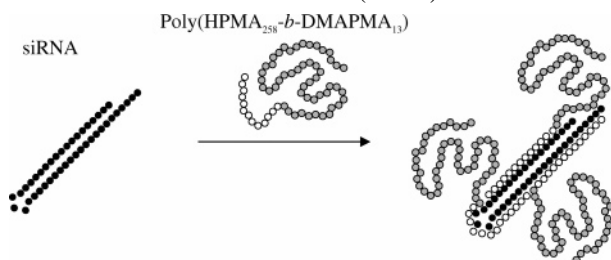
**Dynamic Light Scattering of siRNA/Block (Co)polymer Complexes** DLS measurements were conducted for the complex formed with poly(HPMA<sub>258</sub>-*b*-DMAPMA<sub>13</sub>) (entry 5, Table 2). This specific block copolymer was chosen because it possesses desirable complexation behavior (Table 3 and Figure 4), i.e., it forms soluble complexes with most of the siRNA with a minimal amount of cationic charge per polymer chain. Although comparable complexation results were obtained with other block copolymers prepared with poly(HPMA<sub>70</sub>) macroCTA (e.g., entries 1 and 2), entry 5 was selected because its longer HPMA block should have a larger hydrodynamic volume and possibly better EPR properties than those formed with shorter HPMA blocks. Finally, the higher-molecular-weight complexes were predicted to have a greater scattering intensity than the analogous lower-molecular-weight structures, allowing for greater signal-to-noise in DLS measurements.

Because the complex concentrations employed in the centrifugation studies were too low for accurate DLS measurements, higher concentrations with identical ratios of siRNA (3.5 μM) and block copolymer (11.55 μM) were employed for light scattering studies. For this reason, the complexing behavior at these higher concentrations was again followed by scintillation counting studies and found to be identical to that of entry 5 in Figure 4. (i.e., around 85% of the siRNA was complexed and solubilized). Values of hydrodynamic diameters ( $D_H$ ) of poly(HPMA<sub>258</sub>-*b*-DMAPMA<sub>13</sub>), siRNA, and the siRNA/poly(HPMA<sub>258</sub>-*b*-DMAPMA<sub>13</sub>) complex, respectively, were calculated from the diffusional data assuming spherical geometries and are listed in Table 4. Given the relative hydrodynamic diameters of the siRNA and poly(HPMA<sub>258</sub>-*b*-DMAPMA<sub>13</sub>) of 3.0 and 9.85, respectively, the  $D_H$  value of 11.25 nm obtained for the siRNA/poly(HPMA<sub>258</sub>-*b*-DMAPMA<sub>13</sub>) complex appears to indicate quite effective charge neutralization and thus



**Figure 5.** Debye plot for complexes of small interfering ribonucleic acid formed with poly(*N*-(2-hydroxypropyl)methacrylamide<sub>258</sub>-*b*-*N*-[3-(dimethylamino)propyl]methacrylamide<sub>13</sub>) (HPMA-*b*-DMPMA) and the resulting weight-average molecular weight ( $M_w$ ) and second virial coefficient ( $A_2$ ) values.

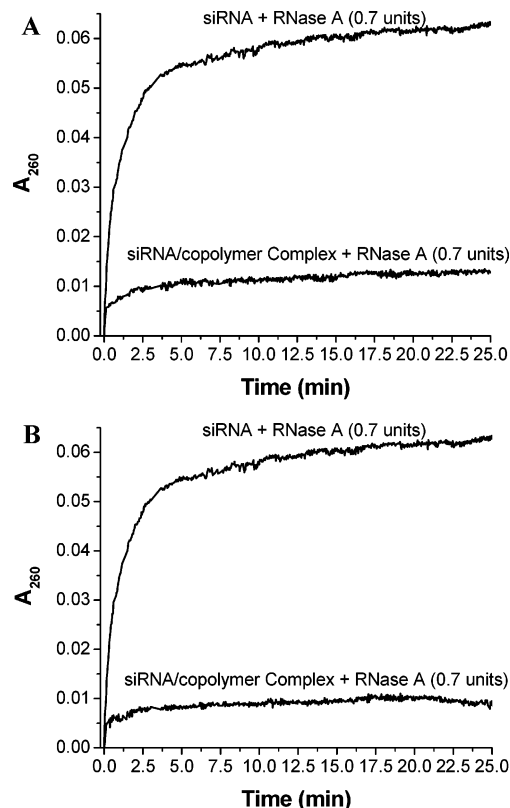
**Scheme 2. Idealized Representation of the Complexation of Poly(*N*-(2-hydroxypropyl)methacrylamide<sub>258</sub>-*b*-*N*-[3-(dimethylamino)propyl]methacrylamide<sub>13</sub>) (HPMA<sub>258</sub>-*b*-DMPMA<sub>13</sub>) with Small Interfering Ribonucleic Acid (siRNA)**



compaction of the complexed segments constituting the inner core. Interestingly, while this manuscript was in preparation, Aoyama et al. reported similar  $D_H$  values for complexes formed with a comparable 42-nucleotide siRNA and a hydrophobically modified macrocyclic octamine surfactant.<sup>95</sup>

**Static Light Scattering (SLS) of siRNA/Block (Co)polymer Complexes.** SLS measurements were also conducted for complexes formed with poly(HPMA<sub>258</sub>-*b*-DMPMA<sub>13</sub>) and siRNA (entry 5, Table 2). The weight-average  $M_w$  of this complex was determined to be 146.7 kDa (Figure 5) and its compact nature is evidenced by the negative  $A_2$  value of  $-2.1 \times 10^{-3} \text{ mL mol/g}^2$  obtained from the slope of the Debye plot. Total compensation for anionic charges along a single siRNA requires a cationic/anionic molar ratio of 1.0 or a ratio of 3.3/1 block copolymer/siRNA on average. Assuming single siRNA complexes with 3–4 copolymers/complex (Scheme 2), the theoretical  $M_w$  of the complexes should be between 134 and 173 kDa, bracketing our experimentally determined value.

**Enzymatic Degradation Assay of siRNA/Copolymer Complexes.** The in vitro degradation rate of siRNA by RNase A in the presence and absence of HPMA/DMPMA copolymers was studied in order to evaluate the enhanced stability of siRNA gained by complexation. Two separate block copolymer entries from Table 2, poly(HPMA<sub>258</sub>-*b*-DMPMA<sub>13</sub>) and poly(HPMA<sub>258</sub>-*b*-DMPMA<sub>23</sub>), were chosen for this assay due to their lower cationic content per polymer chain and superior complexation performance, as observed by <sup>1</sup>H NMR and centrifugal filtration experiments, respectively. Figure 6 shows conclusive evidence concerning the stabilization of siRNA against enzymatic degradation when protected by HPMA/DMPMA block ionomers. Because RNase A cleaves at multiple sites on the siRNA, specifically after each C and U in the sequence, an increase in

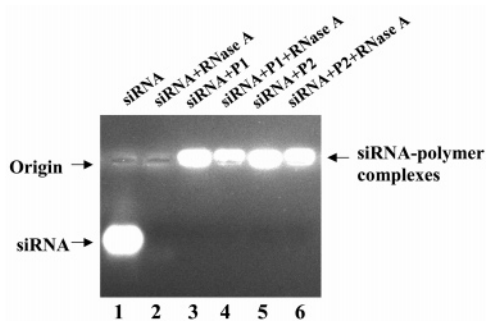


**Figure 6.** Enzymatic degradation of small interfering ribonucleic acid with RNase A in the presence and absence of (A) poly(*N*-(2-hydroxypropyl)methacrylamide<sub>258</sub>-*b*-*N*-[3-(dimethylamino)propyl]methacrylamide<sub>13</sub>) (HPMA<sub>258</sub>-*b*-DMPMA<sub>13</sub>) and (B) poly(HPMA<sub>258</sub>-*b*-DMPMA<sub>23</sub>).

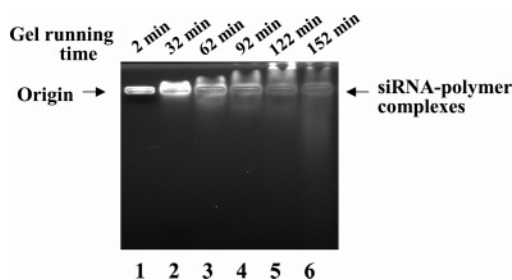
$A_{260}$  or hyperchromic effect is observed when the siRNA is degraded (a result of cleavage-induced unfolding of the hairpin as well as subsequent cleavage events of smaller RNA fragments). For both assays shown in Figure 6, most of the siRNA is degraded in the control experiments (i.e., where free siRNA is combined with RNase A) in less than 5 min. However, when siRNA is stabilized with either poly(HPMA<sub>258</sub>-*b*-DMPMA<sub>13</sub>) or poly(HPMA<sub>258</sub>-*b*-DMPMA<sub>23</sub>) prior to exposure to RNase A, minimal degradation is observed. For both copolymer systems, there is a small amount of degradation observed initially, as evidenced by a small increase in  $A_{260}$ . However, this immediately subsides, indicating no additional protection against degradation by RNase A. The initial degradation of siRNA for both complex systems can be attributed to the fact that small amounts of free siRNA are present prior to addition of RNase A. This is supported by the centrifugal filtration studies, where poly(HPMA<sub>258</sub>-*b*-DMPMA<sub>13</sub>) and poly(HPMA<sub>258</sub>-*b*-DMPMA<sub>23</sub>) both yield small amounts of free siRNA around 12 and 4%, respectively. Furthermore, comparison of the degradation curves of the two polymers shows a lower amount of siRNA degradation for the poly(HPMA<sub>258</sub>-*b*-DMPMA<sub>23</sub>) complex system, as would be expected based on its lower amount of free siRNA.

The above conclusion was further corroborated by agarose gel electrophoresis. As can be seen from Figure 7, unprotected siRNA was completely hydrolyzed within 6 min by 0.1 U RNase A (lane 2 as compared with lane 1). On the other hand, both poly(HPMA<sub>258</sub>-*b*-DMPMA<sub>13</sub>) and poly(HPMA<sub>258</sub>-*b*-DMPMA<sub>23</sub>) can effectively protect the siRNA from RNase A degradation (lanes 4 and 6 as compared with lanes 3 and 5). As expected, the neutral siRNA/polymer complexes ( $N/P = 1.0$ )





**Figure 7.** Polymer protection of small interfering ribonucleic acid (siRNA) against RNase A degradation. P1 and P2 represent poly(*N*-(2-hydroxypropyl)methacrylamide<sub>258</sub>-*b*-*N*-[3-(dimethylamino)propyl]methacrylamide<sub>13</sub>) (HPMA<sub>258</sub>-*b*-DMAPMA<sub>13</sub>) and poly(HPMA<sub>258</sub>-*b*-DMAPMA<sub>23</sub>), respectively. Each lane had 1  $\mu$ g siRNA and lanes 2, 4, and 6 contained 0.1 U RNase A. The *N/P* ratio was kept at 1.0 for lanes 3–6.



**Figure 8.** Dissociation of small interfering ribonucleic acid (siRNA)/poly(*N*-(2-hydroxypropyl)methacrylamide<sub>258</sub>-*b*-*N*-[3-(dimethylamino)propyl]methacrylamide<sub>13</sub>) complexes (*N/P* = 1.0) under the conditions of agarose gel electrophoresis. Each lane was loaded at different times 10  $\mu$ L siRNA–polymer complexes containing 1  $\mu$ g siRNA.

do not migrate in an electric field, in contrast to naked siRNA (lane 1).

#### siRNA Dissociation from siRNA/Copolymer Complexes.

For applications as siRNA stabilization/delivery agents, it is essential that siRNA not only forms stable complexes (for siRNA protection) with the polymers but are also slowly dissociable to make the siRNA available for the formation of an active RISC (RNA-induced silencing complex) that degrades target mRNA. As a preliminary study, gel electrophoresis is particularly suitable for demonstrating siRNA dissociation. Neutral siRNA/polymer complexes (*N/P* = 1.0) do not migrate in an electric field (Figure 7), but dissociated forms of siRNA possess either positive or negative charges and would migrate toward either cathode or anode depending on the sign of net charges. Figure 8 indicates that siRNA is indeed able to dissociate slowly from the siRNA/polymer complexes. As siRNA was slowly released from its complexes during gel electrophoresis, the nonmigrating neutral complexes decreased their intensity at the location of the loading origin (from lane 1 to lane 6, electrophoresized from 2 to 152 min). At the same time, dissociated forms of siRNA entered the gel and distributed as a continuous broad band. The migration distance and direction from the origin were determined by the time of siRNA dissociation, the form and the effective charge of the respective dissociated siRNA complexes. The gel indicates the slow dissociation of siRNA/polymer complexes can produce both positive and negative siRNA migrating complexes. As expected, longer gel-running time led to a broader band of siRNA distribution.

#### Conclusions

A series of well-defined, near-monodisperse, HPMA/DMAPMA block copolymers has been prepared by aqueous

RAFT polymerization in acetic buffer at 70 °C. The chain extension of DMAPMA macroCTA with HPMA produces block copolymers with low polydispersities and minimal homopolymer. The facility by which block copolymers of HPMA and DMAPMA can be prepared in either blocking direction demonstrates the usefulness of RAFT polymerization in synthesizing block copolymers for corona-stabilized interpolyelectrolyte complexes. The resulting HPMA/DMAPMA block copolymers were employed in the successful complexation and ultimate solution stabilization of a model 43-nucleotide siRNA under simulated physiological conditions. This specific siRNA is of interest because, if effectively delivered to a target cancer cell, it could potentially silence the gene that codes for the synthesis of human RNA polymerase II A. Studies probing the binding affinity and stabilization effects of these copolymers with siRNA as a function of “complexing” DMAPMA and “stabilizing” HPMA block lengths suggest an optimum range for the former; as the length of the DMAPMA block increases further the likelihood of interpolymer bridging between siRNA entities and thus formation of aggregates increases. Our ability to synthesize independently HPMA and DMAPMA blocks of predefined lengths with narrow dispersities by RAFT provides an excellent means of tuning HPMA/DMAPMA block copolymers for optimal siRNA stabilization, *in vivo* delivery and circulation time, and intracellular release.

Poly(HPMA<sub>258</sub>-*b*-DMAPMA<sub>13</sub>) was chosen for dynamic light scattering experiments. The relative sizes of the siRNA, block copolymer, and siRNA/block copolymer complex were calculated from experimental data, assuming a spherical shape for simplicity. The collapsed complexes are quite small ( $\sim 11.3$  nm), indicating a compact structure. A Debye plot was constructed from static light scattering, yielding a complex molecular weight of approximately 146.7 kDa. A negative  $A_2$  value was also determined for this complex, indicating that this interpolyelectrolyte complex is extremely collapsed, yet stable in solution. Assuming a model in which 3–4 block copolymer chains of entry 5 are complexed to one siRNA, experimental molecular weight is within the calculated range of theoretical values.

The ability of these block copolymer systems to protect and stabilize siRNA from enzymatic degradation has also been evaluated. Kinetic profiles were thus measured for siRNA in the presence and absence of copolymer. Two HPMA/DMAPMA block copolymer systems that showed promising results in the centrifugal filtration studies were employed, namely poly(HPMA<sub>258</sub>-*b*-DMAPMA<sub>13</sub>) and poly(HPMA<sub>258</sub>-*b*-DMAPMA<sub>23</sub>). Both block copolymer systems were effective in preventing enzymatic degradation of the siRNA by ribonuclease A. In addition, siRNA can slowly dissociate from its copolymer complexes. Although these initial results are very promising, further studies will be necessary to assess delivery/release/activity *in vivo*.

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**Supporting Information Available:** Aqueous, cationic SEC-MALLS traces demonstrating the efficient blocking of (A) poly(HPMA<sub>258</sub>) with DMAPMA, resulting in (B) poly(HPMA<sub>258</sub>-*b*-DMAPMA<sub>23</sub>) and (C) poly(HPMA<sub>258</sub>-*b*-DMAPMA<sub>53</sub>). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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