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Facile Synthesis of Multivalent Folate-Block Copolymer Conjugates via Aqueous RAFT Polymerization: Targeted Delivery of siRNA and Subsequent Gene Suppression†

Adam W. York[†], Yilin Zhang[§], Andrew C. Holley[‡], Yanlin Guo[⊥], Faqing Huang^{*,§}, and Charles L. McCormick^{*,‡,§}

Department of Polymer Science, Department of Chemistry and Biochemistry and Department of Biological Sciences The University of Southern Mississippi, Hattiesburg, MS 39406

Abstract

Cell specific delivery of small interfering ribonucleic acid (siRNA) using well-defined multivalent folate-conjugated block copolymers is reported. Primary amine functional, biocompatible, hydrophilic-*block*-cationic copolymers were synthesized via aqueous reversible addition-fragmentation chain transfer (RAFT) polymerization. *N*-(2-hydroxypropyl)methacrylamide (HPMA), a permanently hydrophilic monomer, was copolymerized with a primary amine containing monomer, *N*-(3-aminopropyl)methacrylamide (APMA). Poly(HPMA) confers biocompatibility while APMA provides amine functionality allowing conjugation of folate derivatives. (HPMA-*stat*-APMA) was chain extended with a cationic block, poly(*N*-[3-(dimethylamino)propyl]methacrylamide) in order to promote electrostatic complexation between the copolymer and the negatively charged phosphate backbone of siRNA. Notably, poly(HPMA) stabilizes the neutral complexes in aqueous solution while APMA allows the conjugation of a targeting moiety, thus, dually circumventing problems associated with the delivery of genes via cationically charged complexes (universal transfection). Fluorescence microscopy and gene down-regulation studies indicate that these neutral complexes can be specifically delivered to cancer cells that over-express folate receptors.

Keywords

small interfering RNA; gene delivery; block copolymers; bioconjugation; chain transfer polymerization

Introduction

Since the discovery of the RNA interference (RNAi) pathway,¹ there has been extensive research on the delivery of small interfering RNA (siRNA), the effector molecules of the RNAi pathway, to specific cellular targets.² siRNA leads to reduced expression (gene “knockdown”) of an encoded protein by cleaving the targeted mRNA.^{2,3} siRNA is attractive for genetic based

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*To whom correspondence should be addressed. E-mail: Charles.McCormick@usm.edu or Faqing.Huang@usm.edu.

‡Department of Polymer Science

§Department of Chemistry and Biochemistry

⊥Department of Biological Sciences

Supporting Information Available: SEC chromatograms monitoring the UV-vis absorbance at 310 nm and real time-PCR traces are available free of charge via the Internet at <http://pubs.acs.org>.

therapeutics because it can reduce gene expression with limited dosage and less toxicity than other oligonucleotide treatments.^{2,4} However, the delivery of siRNA to specific tissues and its susceptibility to enzymatic degradation present significant challenges that must be addressed before effective delivery can be achieved.^{2,4,5}

Cationic polymers offer the potential to protect siRNA during delivery by forming electrostatic complexes through the interaction between the cationic polymer and the anionic phosphate backbone of oligo- and polynucleotides as demonstrated by various laboratories.⁶⁻¹⁴ These complexes, also known as interpolyelectrolyte complexes (IPECs),⁶ provide protection for bound oligo- or polynucleotides from degradative enzymes, while maintaining aqueous solubility. Both charged and neutral IPECs have been reported, although the majority of research involves cationically charged complexes.^{5,9} Though charged complexes remain water soluble, negative complexes are generally ineffective at transfection due to electrostatic repulsions between the complex and the cellular membrane, while positive complexes are effective at transfection, through adsorptive endocytosis, but lack cellular specificity.¹⁵⁻¹⁷ Additionally, cationically charged carriers can interact with oppositely charged biomolecules found in the blood stream. This leads to the formation of aggregates causing either rapid clearance of the aggregate or adverse side effects.^{18,19} In principle, neutral complexes can circumvent these problems, but often have low solubility leading to precipitation. The incorporation of a hydrophilic block can increase solubility, thus limiting precipitation. The hydrophilic block generally contains one of the well studied biocompatible polymers poly(*N*-(2-hydroxypropyl)methacrylamide) (PHPMA) or poly(ethylene oxide) (PEO).²⁰⁻²⁵

Generally, commercially available cationic polymers are used to protect and deliver oligo- and polynucleotides, but these polymers were not designed for gene delivery and hence have suboptimal properties including high cytotoxicity, inherently broad molecular weight distributions, and little structural uniformity.^{5,26,27} Furthermore, transfection relies on adsorptive endocytosis due to the cationically charged complex which leads to treatment of both healthy and unhealthy cells. Therefore, a significant need exists for the development of a neutral polymeric gene carrier with tunable properties that can be targeted to specific cellular receptors.

Typically, macromolecular species accumulate via the enhanced permeability and retention (EPR) effect in tumoral tissue because of poor lymphatic drainage.²⁸⁻³¹ These macromolecular carriers rely on adsorptive endocytosis for cellular internalization and in the case of cationic gene carriers this can result in various deleterious side effects as discussed above. However, it has been demonstrated that the conjugation of a targeting moiety, such as folic acid, to a non-viral carrier can increase cellular uptake as compared to carriers that solely rely on the EPR effect.³²⁻³⁴

Recent advances in controlled radical polymerization (CRP) have facilitated the synthesis of narrowly dispersed copolymers with controlled molecular weight, polymer architecture, and reactive structopendant or structoterminal functionality for bioconjugation.³⁵⁻⁴¹ Specifically, reversible addition-fragmentation chain transfer (RAFT) polymerization has proven to be one of the most versatile CRP techniques, allowing the polymerization of numerous monomer families under mild conditions.^{42,43} In addition, the development of aqueous RAFT polymerization⁴³⁻⁴⁵ now allows for the controlled polymerization of functional monomers, including primary amine containing monomers, without protecting group chemistry. This is a significant step towards the tailored construction of well defined copolymers for targeted gene delivery.

It has also been shown that the number and distribution of targeting moieties as well as choice of conjugation chemistries are important factors when designing non-viral polymeric carriers.

^{39,46} Therefore the synthesis of a polymeric carrier with well-defined architecture and multiple reactive pendant functionalities is required. Herein we report the facile synthesis of a novel, well-defined, multivalent terpolymer capable of forming neutral complexes with siRNA (less potential for adverse side effects) while providing multiple targeting moieties for cell specific delivery. The use of aqueous RAFT polymerization affords control over the block length and number of conjugation sites incorporated along the polymer backbone. The cationic block of the polymer forms an electrostatic complex with siRNA, while a statistical amine functional block confers water solubility and provides sites for conjugation of folic acid (FA). As demonstrated through zeta potential, confocal microscopy and gene knockdown studies, this tailored polymer allows the formation of neutral complexes which can be delivered specifically to cancer cells that over-express folate receptors.

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) and AIBN, a gift from Wako Pure Chemicals Industries, Ltd., was recrystallized from methanol. 4-Cyanopentanoic acid dithiobenzoate (CTP) was synthesized according to the literature procedure.⁴⁷ *N*-(3-Aminopropyl)methacrylamide hydrochloride (APMA) was purchased from PolySciences (Warrington, PA). *N*-(2-hydroxypropyl)methacrylamide (HPMA) was synthesized according to literature procedure.⁴⁸ *N*-[3-(Dimethylamino)propyl] methacrylamide (DMAPMA) was purified by vacuum distillation. Two strands of a 59-nucleotide (nt) model small interfering ribonucleic acid (siRNA) against human survivin were chemically synthesized and labeled with fluorescein (FAM) and cyanine-3 (Cy3) separately. Unlabeled and FAM-labeled sense strand, 5'-AGCCCUUUCUAAGGACCACCGCAUCU-3', was synthesized by Integrated DNA Technologies. Unlabeled and Cy3-labeled antisense strand, 3'-UUUCGGGAAAGAGUCCUGGUGGCGUAGAGGA-5' was synthesized by Biosynthesis Inc. A549 cells (human lung cancer cells) and KB cells (human epidermal cancer cells) were purchased from American Type Culture Collection (Manassas, VA).

Synthesis of (HPMA-*stat*-APMA) macroCTA

MacroCTAs were prepared employing V-501 as the primary radical source and CTP as the chain transfer agent at 70 °C. A typical reaction is as follows: HPMA (3.87 g, 27 mmol) and APMA (0.536 g, 3 mmol) were added to a 100 mL round-bottomed flask, dissolved in an aqueous acetic buffer (pH 5.2, 0.27 M acetic acid and 0.73 M sodium acetate) and diluted to a final volume of 30 mL ($[M]_0 = 1\text{ M}$). The initial feed composition was either 90 or 95 mol% HPMA and 5 or 10 mol% APMA. The round-bottomed flask was septum-sealed and purged with nitrogen for 45 min prior to polymerization. HPMA-*stat*-APMA macroCTAs were prepared with a $[M]_0/[CTA]_0$ ratio of 800/1, while the $[CTA]_0/[I]_0$ was kept at 5/1. (HPMA-*stat*-APMA) macroCTAs were isolated by dialysis (pH 3-4) at 4 °C followed by lyophilization.

Synthesis of (HPMA-*stat*-APMA)-*b*-DMAPMA Block Copolymers

HPMA-*stat*-APMA macroCTAs were chain extended with DMAPMA also using V-501 as the radical source at 70 °C. DMAPMA and HPMA-*stat*-APMA were added to a round-bottomed flask, dissolved in DI water, and subsequently titrated to a pH between 5-6 to give $[M]_0 = 1\text{ M}$. The round-bottomed flask was septum-sealed and subsequently purged with nitrogen for 45 min. Block copolymers were prepared with a $[M]_0/[CTA]_0 = 200$, while the $[CTA]_0/[I]_0$ was kept at 5/1. Each polymerization was terminated at predetermined time intervals by exposure to air and rapid cooling in liquid nitrogen. The (HPMA-*stat*-APMA)-*b*-DMAPMA copolymers were purified by dialysis (pH 3-4) at 4 °C and dried by lyophilization.

Thiocarbonylthio functionalities from (HPMA-*stat*-APMA)-*b*-DMPMA copolymers were removed following a literature procedure reported by Perrier and coworkers.⁴⁹ A typical reaction is as follows: (HPMA₃₁₅-*stat*-APMA₁₃)-*b*-DMPMA₂₃ (500 mg, 9.75 μ mol) was added to a 10 mL round-bottomed flask and dissolved with 4.9 mL of DMF. Azobisisobutyronitrile (AIBN) (32 mg, 0.195 mmol) was then added to the flask giving an AIBN:copolymer ratio of 20:1. The following solution was then septum-sealed and purged with nitrogen for 45 min and allowed to react at 70 °C for 4 h. The resulting copolymer was precipitated from DMF in cold anhydrous diethyl ether and washed repeatedly. This step was repeated three times and the copolymer was dried *in vacuo* overnight.

Synthesis of DiNHS-Folate

The carboxylic groups of folic acid (FA) were activated using standard carbodiimide chemistry. First 1 g (2.26 mmol) of FA was dissolved in 15 mL of DMSO in a 50 mL round bottomed flask equipped with a stir bar. Once the FA was completely dissolved 4.67 g (22.6 mmol) of DCC and 1.30 g (11.3 mmol) of NHS were added to the solution to synthesize mono-NHS FA while 4.67 g (22.6 mmol) of DCC and 1.3 g (11.3 mmol) of NHS were added to the FA solution to synthesize diNHS-FA. Each solution was allowed to react at 21 °C for 40 h in the dark. After the reaction was complete, a white precipitate was filtered from the solution and the activated FA was precipitated and washed repeatedly in cold anhydrous diethyl ether. The product was then dried *in vacuo* overnight to yield 1.35 g of product (94%) and used without further purification. ¹H NMR (DMSO-*d*₆, ppm): 8.65 (s, PtC₇H, 1H), 8.59-8.57 (d, -CONHCHCO-OSu, 1H, *J* = 7.2 Hz), 7.70-7.67 (d, Ph-C₂H and Ph-C₆H, 2H, *J* = 8.7 Hz), 6.90 (br s, NH₂, 2H), 6.68-6.65 (d, Ph-C₃H and Ph-C₅H, 2H, *J* = 8.7 Hz), 4.91-4.84 (m, -CONHCHCO-OSu, 1H), 4.51-4.49 (d, PtC₆-CH₂NH-Ph, 2H, *J* = 5.7 Hz), 2.96-2.91 (t, -CH₂CH₂CO-OSu, 2H, *J* = 6.9 Hz), 2.82 (s, -NHCHCO-OSuC₃H₂ and -NHCHCO-OSuC₄H₂, 4H), 2.82 (s, -CH₂CH₂CO-OSuC₃H₂ and -CH₂CH₂CO-OSuC₄H₂, 4H), 2.31-2.26 (m, -CHCH₂CH₂CO-OSu, 2H).

DiNHS-Folate Conjugation to (HPMA-*stat*-APMA)-*b*-DMPMA Block Copolymers

Following isolation of the chain terminated block copolymer the pendant functional primary amine groups from the incorporated APMA were labeled with NHS-activated FA. A typical reaction is as follows: 30 mg (0.585 μ mol) of (HPMA₃₁₅-*stat*-APMA₁₃)-*b*-DMPMA₂₃ copolymer was dissolved in 200 μ L to give a final concentration of 2.92 mM. 130 times excess diNHS-FA (58 mg) was then dissolved in 350 μ L of DMSO and (HPMA₃₁₅-*stat*-APMA₁₃)-*b*-DMPMA₂₃ copolymer solution was added to the activated FA solution at a rate of 20 μ L every 20 minutes. Triethylamine (TEA) was added to a final concentration of 39 mM to serve as a catalyst. The resulting solution was allowed to react for 48 h at room temperature. Following reaction, excess ammonium hydroxide (100% by volume) was added to quench the remaining activated esters of the activated FA. Quenching was carried out for 24 h. The resulting reaction mixture was then directly placed in 12-14K MWCO dialysis tubing and was first dialyzed against 0.6 M NaCl solution followed by dialysis against DI water for 3 days followed by lyophilization.

Copolymer Characterization

All polymers were characterized by aqueous size exclusion chromatography (ASEC) utilizing an eluent of 1 wt% acetic acid/0.10 M Na₂SO₄ (aq) at a flow rate of 0.25 mL/min at 25 °C, Eprogen, Inc. CATSEC columns (100, 300, and 1000 Å), a Polymer Labs LC1200 UV/Vis detector, a Wyatt Optilab DSP interferometric refractometer (λ = 690 nm), and a Wyatt DAWN-DSP multiangle laser light scattering (MALLS) detector (λ = 633 nm). Absolute molecular weights and polydispersities were calculated using the Wyatt OmniSEC software. ASEC chromatograms for HPMA₃₂₀-*stat*-APMA₃₃ macroCTA and (HPMA₃₂₀-*stat*-APMA₃₃)-*b*-

DMPMA₃₄ block copolymer are shown in Figure 1. dn/dc measurements for HPMA-*stat*-APMA macroCTAs were performed with a Wyatt Optilab DSP interferometric refractometer ($\lambda = 690$ nm) at 35 °C and determined using OmniSEC software. Conversions for the macroCTA and chain extension with DMAPMA were determined by comparing the area of the monomeric UV signal detected at 274 nm at t_0 to the area at t_x using a Polymer Labs LC1200 UV/Vis detector. Removal of the thiocarbonylthio functionality from (HPMA-*stat*-APMA)-*b*-DMAPMA copolymers was confirmed by monitoring the UV signal at 310 nm before and after removal (Supporting Information).

Copolymer compositions were determined with a Varian Mercury^{PLUS} 300 MHz spectrometer in D₂O with a delay time of 2 s. ¹H NMR was used to determine the copolymer composition of HPMA-*stat*-APMA copolymers by integration of the relative intensities of the methyne-proton resonances of HPMA at 3.75 ppm and the methylene resonances of APMA between 2.80 to 3.20 ppm. DMAPMA block compositions of (HPMA-*stat*-APMA)-*b*-DMAPMA copolymers were also determined by ¹H NMR by integration of the relative intensities of the methyne-proton resonances of HPMA at 3.75 ppm and the dimethyl proton resonances of DMAPMA at 2.72 ppm.

Characterization of Folate-Conjugated Polymers

Conjugation of FA to (HPMA-*stat*-APMA)-*b*-DMAPMA copolymers was verified by ¹H NMR and UV-vis spectroscopy. Due to difficulty in characterizing FA conjugated polymers via ASEC-MALLS, these spectroscopic techniques were used to show the copolymers before and after FA conjugation. ¹H NMR was performed on a Varian Mercury^{PLUS} 300 MHz spectrometer in DMSO-*d*₆ with delay times of 2s. The amount of FA conjugated to the polymer backbone of (HPMA₃₁₅-*stat*-APMA₁₃)-*b*-DMAPMA₂₃ was estimated by integration of the methyne-proton resonance of HPMA at 3.75 ppm and the proton resonance of FA at 8.64 ppm (s, PtC₇H, 1H). These values were estimated employing a Lorentzian/Gaussian line fit using MestReC NMR data processing software version 4.7.

UV-vis spectroscopy was carried out using a JASCO V-530 spectrophotometer for FA conjugated (HPMA-*stat*-APMA)-*b*-DMAPMA block copolymers. An average extinction coefficient of 8000 M⁻¹cm⁻¹ for free FA in phosphate buffer solution (20 mM Pi / 0.1 M NaCl pH 7.4) was used to determine the number of FA units for (HPMA-*stat*-APMA)-*b*-DMAPMA copolymers.

Dynamic Light Scattering (DLS) and Zeta Potential

Dynamic light scattering (DLS) and zeta potential measurements of block copolymer/siRNA complexes under aqueous conditions were performed using 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 τ digital correlator electronics system. DLS measurements were carried out at a FA-block copolymer or complex concentration (siRNA + FA-block copolymer) of 0.1 mg mL⁻¹ in phosphate buffer saline solution (20 mM Pi / 0.1 M NaCl pH 7.4). Data analysis of DLS measurements was performed using the CONTIN method. Zeta potential measurements were carried out at a complex concentration of 0.8 mg mL⁻¹ in 20 mM NaCl solution at pH 7.4 in a zeta folded capillary cells purchased from Malvern Instruments. A higher concentration for zeta potential measurements was required because 0.1 mg mL⁻¹ concentration was too dilute for instrument detection. Samples were vortexed immediately to ensure homogenous mixing and equilibrated for 10 min at 25 °C. To remove dust, samples were centrifuged at 14,000 RPM for 10 minutes prior to characterization *via* DLS and zeta potential. Both DLS and zeta potential measurements were performed in triplicate.

Preparation of block copolymer/siRNA complexes for fluorescence microscopy

FA labeled (HPMA-*stat*-APMA)-*b*-DMPMA/siRNA complexes were prepared with a nitrogen/phosphate (*N/P*) ratio equal to 1. A typical preparation is as follows: 0.6 μL of 10 μM dual-labeled (Cy3-siRNA-FAM) or unlabeled siRNA was mixed with 1.7 μL or 1.1 μL of 10 μM FA conjugated (HPMA₃₁₅-*stat*-APMA₁₃)-*b*-DMPMA₂₃ or un-conjugated (HPMA₃₁₅-*stat*-APMA₁₃)-*b*-DMPMA₂₃, respectively. All samples were immediately vortexed to ensure homogenous mixing. Block copolymer/siRNA complexes were equilibrated at room temperature for 10min before adding to a 12-well plate where each well contained 600 μL of cell media.

Cell culture

KB and A549 cells were maintained and proliferated in folate free RPMI 1640 (Gibco, #27016) cell media supplemented with 10% fetal calf serum (FCS), 100units mL^{-1} penicillin, 100 $\mu\text{g mL}^{-1}$ streptomycin at 37 °C in 95% air humidified atmosphere and 5% CO_2 .

Cell treatment with block copolymer/siRNA complexes and lipofectamine for fluorescence microscopy

Prior to treatment, KB and A549 cells were seeded on cover-glasses in a 12-well plate with 600 μL of folate-free RPMI1640 cell media supplemented with 10% FCS and were cultured for 24 h. Block copolymer/siRNA complexes were then added to the cell media and incubated for 1 h. After treatment the cells were fixed with 4% paraformaldehyde and washed with phosphate buffered saline (PBS) prior to imaging. The cells were then stained with 4',6-diamidino-2-phenylindole (DAPI) in 12 μL of mounting gel. The cover glasses were then placed on precleaned microscope slides for analysis. For lipofectamine 2000 (Invitrogen) control group, the transfection was carried out according to manufacturer recommendations. 50 μL of Opti buffer was mixed with 1 μL lipofectamine and added to 50 μL Opti buffer with 0.6 μL of 10 μM Cy3-siRNA-FAM and mixed for 20min. The resulting solution was then added to 500 μL of media (total 600 μL) and the cells were treated for 3 hours. After treatment the cells were fixed with 4% paraformaldehyde and washed with PBS and then stained with DAPI in 12 μL of mounting gel. The cover glass was then placed on precleaned microscope slides for analysis.

Fluorescence Microscopy

Fluorescence cell images were taken using a Nikon fluorescence microscope (Eclipse 80i, Plan Fluor 40X/0.75 DIC M/N2 lens). The images were processed utilizing Image-Pro Plus software. Multiple fields were examined for each sample to ensure uniform distribution of block copolymer/siRNA and lipofectamine/siRNA complexes throughout. Each cell treatment was repeated in triplicate to show reproducibility of the observed results.

Cell treatment with block copolymer/siRNA complexes for gene down-regulation

KB cells and A549 cells (3×10^4 cells per well) were seeded in 24-well plates (Flow Laboratories, Inc. McLean, VA) in folate free RPMI 1640 (Gibco, Carlsbad, CA) supplemented with 10% FCS (HyClone, Logan, UT) one day before experiments. Cells were treated with either free siRNA or block copolymer/siRNA complexes. In addition, KB cells were treated block copolymer/siRNA complexes in the presence of free folic acid (1mM). All cell experiments contained 200 nM anti-survivin siRNA and were treated for 4 hours, followed by replacement with fresh media. After 2 days, total RNA was extracted with TriZol (Invitrogen) following the manufacture's protocol. Reverse transcription was performed with MMLV transcriptase (Promega, Madison, WI). Finally, real time PCR (RT-PCR) was carried out with SYBR (Sigma-Aldrich, St. Louis, MO) on MX3000P PCR system (Stratagene). The primer pairs for detecting the survivin gene were 5'-AGCCCTTTCTCAAGGACCAC and 5'-

TCCTCTATGGGGTCGTCATC. PCR primers for beta-actin gene were 5'-CATGTACGTTGCTATCCAGGC and 5'-CTCCTTAATGTCACGCACGAT. Gene down-regulation experiments were repeated in triplicate to ensure accuracy of results. The passage numbers of KB and A549 cells are 12 and 4, respectively. RT-PCR traces for KB and A549 cells treated with free siRNA, folate conjugated block copolymer/siRNA complexes, and folate conjugated block copolymer/siRNA complexes in the presence of free folic acid (1mM) can be seen in the Supporting Information.

Results and Discussion

Synthesis of (HPMA-*stat*-APMA)-*b*-DMPMA Block Copolymers

HPMA-*stat*-APMA macro chain transfer agents (macroCTAs) were prepared via aqueous RAFT polymerization (Scheme 1). These reactions were carried out in acetate buffer (pH = 5.2) at 70 °C utilizing 4-cyanopentanoic acid dithiobenzoate (CTP) and 4,4'-azobis(4-cyanopentanoic acid) (V-501) as the CTA and initiator, respectively. Two HPMA-*stat*-APMA macroCTAs were synthesized containing either 5 or 10 mol% APMA in the monomer feed. The resulting HPMA-*stat*-APMA macroCTAs were successfully chain extended with DMAPMA under similar conditions (Scheme 1) yielding the statistical block copolymers (HPMA₃₁₅-*stat*-APMA₁₃)-*b*-DMPMA₂₃ and (HPMA₃₂₀-*stat*-APMA₃₃)-*b*-DMPMA₃₄. Both polymers displayed polydispersities (PDIs) below 1.15 as determined by aqueous size exclusion chromatography (ASEC). ASEC chromatograms are shown in Figure 1 before and after chain extension with DMAPMA. The unimodal nature of the chromatograms in combination with the shift to lower elution volume indicates efficient chain extension. Low molecular weight tailing is apparent in the GPC chromatograms which is a typical observation in RAFT polymerizations and has been attributed to premature termination and/or loss of the CTA thiocarbonylthio moiety.^{10,42} However, GPC chromatograms indicate that the presence of low molecular weight species is minimal suggesting that the majority of chain ends retain the CTA functionality allowing for effective chain extension.

Copolymer molecular weights, PDIs, compositions and dn/dc measurements are shown in Table 1. ¹H NMR (Figure 2) was utilized to determine copolymer composition through the integration of the relative intensities of the methyne-proton resonances of HPMA at 3.75 ppm to the methylene resonances of APMA between 2.80 to 3.20 ppm for HPMA-*stat*-APMA macroCTAs (Figure 2A) or the DMAPMA methylene-resonance at 2.70 ppm for (HPMA-*stat*-APMA)-*b*-DMPMA copolymers (Figure 2B). MacroCTA copolymer compositions are in agreement with theoretical values, Table 1. As indicated in Table 1, the experimental molecular weights ($M_{n, \text{exp}}$) are in close agreement with the theoretical molecular weights ($M_{n, \text{th}}$). A positive deviation was observed in the $M_{n, \text{exp}}$ with respect to $M_{n, \text{th}}$, which has been previously observed in our laboratories as well as others.⁵⁰⁻⁵⁵ This “overshoot” in the molecular weight of the (HPMA-*stat*-APMA) macroCTAs and in (HPMA-*stat*-APMA)-*b*-DMPMA copolymers may be attributed to a loss of CTA (e.g. irreversible coupling reactions, aminolysis or hydrolysis) in the early stages or during the polymerization.^{10,51,56,57} Nevertheless, narrowly dispersed copolymers with precise architecture and reactive pendant functionality were obtained allowing for FA conjugation, siRNA complexation and subsequent delivery.

The novelty of these copolymers lies in the incorporation of three monomer components that address various issues related to the effective delivery of siRNA. The packaging and protection of the siRNA is accomplished by the incorporation of a cationic block that can form an electrostatic complex with siRNA, while the aqueous stability and targeted delivery of siRNA is accomplished by the incorporation of a statistical block that contains a hydrophilic monomer and a primary amine monomer providing conjugation sites. RAFT polymerization allows

control over the number of conjugation sites and the block lengths for construction of narrowly dispersed polymers designed specifically for siRNA delivery.

Folate Conjugation to (HPMA-*stat*-APMA)-*b*-DMPMA Block Copolymers

In order to prevent unwanted side reactions during the conjugation of FA-derivatives, the thiocarbonylthio groups at the ω -terminus of all polymers were removed through a standard literature procedure involving the reaction of the block copolymers with a large excess of azobisisobutyronitrile (AIBN) (Scheme 1).⁴⁹ The removal of the thiocarbonylthio functionalities was confirmed by monitoring the thiocarbonylthio absorbance at 310 nm with a UV-vis detector in-line with our ASEC instrumentation (Supporting Information, Figure S1).

The carboxylic groups of FA were neutralized to prevent intrapolymer complexation with the cationic block of the copolymer. The carboxylic groups were first activated with *N*-hydroxysuccinimide (NHS) yielding a diNHS-FA. DiNHS-FA was successfully conjugated to the (HPMA-*stat*-APMA)-*b*-DMPMA copolymers (Scheme 1) via the reaction of the APMA primary amine functionality with a large excess of diNHS-FA. Following diNHS-FA conjugation, excess ammonia was added to quench the remaining activated esters to prevent intrapolymer electrostatic interactions. Successful conjugation of FA to the polymer was confirmed by UV-vis and ¹H NMR spectroscopy.

UV-vis spectroscopy was used to confirm the presence of FA. The absorbances at 350 nm and 280 nm confirm that FA was indeed conjugated to the backbone of the polymer. As shown in Figure 3, the absorbance spectrum for free FA is almost identical to the absorbance spectrum for FA conjugated (HPMA-*stat*-APMA)-*b*-DMPMA suggesting successful conjugation of diNHS-FA to the polymer backbone. Based on an average extinction coefficient for free FA at pH = 7, UV-vis spectroscopy indicate conjugation of 11 FA units out of 13 possible for (HPMA₃₁₅-*stat*-APMA₁₃)-*b*-DMPMA₂₃ and 23 FA units out of 33 for (HPMA₃₂₀-*stat*-APMA₃₃)-*b*-DMPMA₃₄. Due to the similarity of UV-vis spectra between the synthesized copolymers, only (HPMA₃₁₅-*stat*-APMA₁₃)-*b*-DMPMA₂₃ is illustrated in Figure 3.

¹H NMR spectroscopy further proved the conjugation of folate along the backbone of the (HPMA-*stat*-APMA)-*b*-DMPMA copolymers. Figure 4 shows the spectra of free FA (Figure 4A), the block copolymer (HPMA₃₁₅-*stat*-APMA₁₃)-*b*-DMPMA₂₃ prior to conjugation (Figure 4B) and the FA conjugated copolymer after neutralization of the folic acid with ammonium hydroxide (Figure 4C). Chemical shifts from folic acid 8.64 ppm (s, PtC₇H, 1H), 7.66-7.63 (d, Ph-C₂H and Ph-C₆H, 2H, *J* = 8.4 Hz) and 6.64-6.61 (d, Ph-C₃H and Ph-C₅H, 2H, *J* = 8.4 Hz) are visible in the ¹H NMR spectrum shown in Figure 4C suggesting the successful conjugation of FA to the polymer backbone. The amount of FA conjugated to the polymer backbone of (HPMA₃₁₅-*stat*-APMA₁₃)-*b*-DMPMA₂₃ was estimated by integration of the methyne-proton resonance of HPMA at 3.75 ppm and the proton resonance of FA at 8.64 ppm (s, PtC₇H, 1H) and was found to be approximately 12-13 FA units per chain. Due to the amount of sample synthesized, the ¹H NMR spectrum could only be obtained for FA conjugated (HPMA₃₁₅-*stat*-APMA₁₃)-*b*-DMPMA₂₃.

Given the higher percentage of FA conjugation to (HPMA₃₁₅-*stat*-APMA₁₃)-*b*-DMPMA₂₃, as indicated by the spectroscopic techniques utilized, only (HPMA₃₁₅-*stat*-APMA₁₃)-*b*-DMPMA₂₃ was studied for complexation with siRNA and subsequent cellular treatment for fluorescence microscopy and gene suppression experiments.

Dynamic Light Scattering and Zeta Potential Experiments

Neutral FA conjugated block copolymer/siRNA complexes were prepared according to a method previously reported by our laboratories which showed siRNA stabilization and

protection from enzymatic degradation.¹⁰ The complexes used in these studies were characterized via dynamic light scattering (DLS) and zeta potential at 25 °C. Prior to siRNA complexation, the D_h of the FA conjugated block copolymer was 10.8 ± 0.3 nm and the zeta potential was $+25.4 \pm 0.7$ mV. Given the required need of siRNA for subsequent cellular experiments and the concentration and volume required for an accurate measurement of the D_h of free siRNA, DLS experiments were not performed. However, as reported previously by our laboratories the D_h of a siRNA containing 49 nt was determined via DLS and was found to be 2.95 ± 0.34 nm.¹⁰ It is practical to assume that a slightly larger but similar D_h would be expected for the siRNA used in these studies which contains 59 nt. The hydrodynamic diameter (D_h) of the complex as determined by DLS was 15.2 ± 2.4 nm and the zeta potential of the complex was -3.88 ± 0.21 mV. Zeta potential measurements and DLS indicate that the complexes are near neutral but remain sterically stable due to the presence of the hydrophilic block.

Cellular Delivery of Multivalent Folate-Block Copolymer/siRNA Complexes

siRNA delivery to cancer cells using FA conjugated (HPMA-*stat*-APMA)-*b*-DMPMA copolymers was followed by fluorescence microscopy (Figure 5). A dual, fluorescently-labeled (Cy3 and FAM) anti-human survivin siRNA was mixed and vortexed with block copolymers before cell treatment. Human survivin is a protein that regulates the cell cycle and is commonly over-expressed in cancer cell lines. Cells treated with copolymer/siRNA complexes were washed with PBS after 1 hour to remove free complexes prior to imaging. KB cells, which are known to over-express folate receptors, were used to show efficient, cell specific delivery of neutral multivalent FA-complexes (Figure 5). For direct comparison, KB cells in Figure 5C were treated with block copolymer/siRNA complexes prior to FA conjugation while cells in Figure 5E were treated with FA-conjugated copolymer. The strong Cy3 fluorescence in Figure 5E indicates successful folate receptor binding, demonstrating the viability of this terpolymer system to effectively deliver siRNA to folate-receptor expressing cell lines.

To further prove cell specific delivery, the human lung cancer cell line A549, which has low folate receptor expression,⁵⁸ was treated with both un-conjugated (Figure 5D) and FA conjugated complexes (Figure 5F). Neither image showed Cy3 fluorescence providing evidence of cell specificity. The low folate receptor expression in the A549 cell line was confirmed by running RT-PCR experiments and comparing the folate receptor expression levels of the A549 versus the KB cell line (Supporting Information, Figure S3). For positive controls both cell lines were treated with lipofectamine/siRNA complexes (Figure 5A and 5B), where lipofectamine is a nonspecific transfection agent. The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) for nuclei location (blue fluorescence).

Gene Down-Regulation

To demonstrate that these neutral carriers can lead to cell specific gene down-regulation, KB cells and A549 cells were treated with FA conjugated (HPMA₃₁₅-*stat*-APMA₁₃)-*b*-DMPMA₂₃/siRNA complexes (siRNA concentration = 200 nM) for 4 hours, followed by 48 hours incubation. Additionally, KB cells were treated with FA conjugated (HPMA₃₁₅-*stat*-APMA₁₃)-*b*-DMPMA₂₃/siRNA complexes in the presence of free FA (1 mM) to show inhibition of cellular uptake due to the competition of folate receptor binding between free FA and FA conjugated copolymer/siRNA complexes. Survivin mRNA was then quantified by RT-PCR analysis (Figure 6). As shown in Figure 6, approximately 60% mRNA down-regulation was observed in the KB cell line, while no mRNA down-regulation was in the A549 cell line, which has low folate receptor expression. The presence of free FA led to negligible mRNA down-regulation indicating minimal cellular uptake of FA conjugated (HPMA₃₁₅-*stat*-APMA₁₃)-*b*-DMPMA₂₃/siRNA complexes. These results indicate that FA conjugated (HPMA₃₁₅-*stat*-APMA₁₃)-*b*-DMPMA₂₃/siRNA complexes can deliver siRNA through

folate receptor-mediated endocytosis leading to the down-regulation of human survivin in KB cells.

Conclusions

The facile synthesis of a novel, well-defined, multivalent terpolymer capable of forming neutral complexes with siRNA and providing multiple targeting moieties for cell specific siRNA delivery has been demonstrated. These well-defined primary amine-containing copolymers provide a platform for preparing multivalent polymeric bioconjugates suitable for targeted drug/gene delivery. To demonstrate the use of these terpolymers for effective siRNA delivery, folic acid was conjugated to multiple sites along the terpolymer backbone followed by electrostatic complexation with siRNA. These multivalent FA-block copolymer/siRNA complexes proved to be particularly effective for cell-specific siRNA delivery and successful gene down-regulation as evidenced by reduced mRNA levels of survivin in KB cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Scheme 1.

Reaction pathway for the synthesis of (*N*-(2-hydroxypropyl)methacrylamide-*stat*-*N*-(3-aminopropyl)methacrylamide)-*b*-*N*-(3-dimethylaminopropyl)methacrylamide, (HPMA-*stat*-APMA)-*b*-DMAPMA copolymers and subsequent conjugation of folic acid.

Figure 1.

Aqueous, cationic SEC-MALLS traces demonstrating blocking of *N*-(2-hydroxypropyl) methacrylamide-*stat*-*N*-(3-aminopropyl)methacrylamide (HPMA-*stat*-APMA) macro chain transfer agents with *N*-(3-dimethylaminopropyl)methacrylamide (DMAPMA). A) (—) (HPMA₃₁₅-*stat*-APMA₁₃) macroCTA and B) (---) (HPMA₃₁₅-*stat*-APMA₁₃) -*b*-DMAPMA₂₃ and A) (—) (HPMA₃₂₀-*stat*-APMA₃₃) macroCTA and B) (---) (HPMA₃₂₀-*stat*-APMA₃₃) -*b*-DMAPMA₃₄.

Figure 2. Chemical shift assignments for ^1H NMR spectra for A) (*N*-(2-hydroxypropyl)methacrylamide₃₁₅-*stat*-*N*-(3-aminopropyl)methacrylamide₁₃) and B) (*N*-(2-hydroxypropyl)methacrylamide₃₁₅-*stat*-*N*-(3-aminopropyl)methacrylamide₁₃)-*b*-*N*-(3-dimethylaminopropyl)methacrylamide₂₃) copolymers.

Figure 3. UV-vis spectra for A) free folic acid (FA) and B) FA conjugated (*N*-(2-hydroxypropyl)methacrylamide₃₁₅-*stat*-*N*-(3-aminopropyl)methacrylamide₁₃)-*b*-*N*-(3-dimethylaminopropyl)methacrylamide₂₃) measured in phosphate buffer pH 7.4.

Figure 4. ^1H NMR spectra carried out in $\text{d}_6\text{-DMSO}$ for A) free folic acid (FA), B) (*N*-(2-hydroxypropyl) methacrylamide₃₁₅-*stat*-*N*-(3-aminopropyl) methacrylamide₁₃)-*b*-*N*-(3-dimethylaminopropyl) methacrylamide₂₃) ((HPMA₃₁₅-*stat*-APMA₁₃)-*b*-DMPMA₂₃) block copolymer, and C) FA conjugated (HPMA₃₁₅-*stat*-APMA₁₃)-*b*-DMPMA₂₃.

Figure 5.

Fluorescent microscope images of small interfering RNA (siRNA) (Cyanine-3 and fluorescein (FAM) labeled) delivery to KB cells (A, C, E) and A549 cells (B, D, F). Lipofectamine (A, B) (+ control), un-conjugated (*N*-(2-hydroxypropyl)methacrylamide₃₁₅-*stat*-*N*-(3-aminopropyl)methacrylamide₁₃)-*b*-*N*-(3-dimethylaminopropyl)methacrylamide₂₃, (HPMA₃₁₅-*stat*-APMA₁₃)-*b*-DMPMA₂₃ (C, D) (- control), and folic acid conjugated (HPMA₃₁₅-*stat*-APMA₁₃)-*b*-DMPMA₂₃ (E, F). Nuclei were stained with 4',6-diamidino-2-phenylindole (blue). For clarity FAM fluorescence is not shown. Scale bars = 50 μm.

Figure 6.

Quantitative real time-PCR analysis of cell specific down-regulation of human survivin messenger RNA (mRNA) by anti-survivin small interfering RNA (siRNA). The control contained either KB cells/siRNA or A549 cells/siRNA. The other three experiments (marked KB, KB/Free FA and A549) were treated with folate conjugated (*N*-(2-hydroxypropyl)methacrylamide₃₁₅-*stat-N*-(3-aminopropyl)methacrylamide₁₃)-*b-N*-(3-dimethylaminopropyl)methacrylamide₂₃/siRNA complexes in the presence (KB/Free FA) or absence (KB, A549) of 1 mM free folic acid (FA). All experiments were done in triplicate and error bars represent $\pm$ S.D.

Table 1

Conversion, composition, molecular weight (M_n), polydispersity (PDI), and dn/dc values for the preparation of (*N*-(2-hydroxypropyl) methacrylamide-*s*-*N*-(3-aminopropyl)methacrylamide)-*b*-*N*-(3-dimethylaminopropyl)methacrylamide) (HPMA-*stat*-APMA)-*b*-DMAPMA copolymers by aqueous RAFT polymerization.

Sample	%conv. ^a	Composition (Theory)	Composition (exp) ^b	$M_{n, th}$ (g/mol) ^c	$M_{n, exp}$ (g/mol)	PDI ^c	dn/dc ^e
HPMA ₃₁₅ - <i>stat</i> -APMA ₁₃	38	95 : 5	96 : 4	44,300	47,400 ^d	1.10	0.167
HPMA ₃₂₀ - <i>stat</i> -APMA ₃₃	40	90 : 10	90 : 10	46,700	51,800 ^d	1.13	0.169
(HPMA ₃₁₅ - <i>stat</i> -APMA ₁₃)- <i>b</i> -DMAPMA ₂₃	6	----	90 : 4 : 6	49,400	51,300 ^b	1.14	----
(HPMA ₃₂₀ - <i>stat</i> -APMA ₃₃)- <i>b</i> -DMAPMA ₃₄	9	----	83 : 8 : 9	54,800	57,600 ^b	1.13	----

^aConversions were determined by comparison of the UV signal at 274 nm of the monomer at t₀ to that at t_x.

^b A_s determined by ¹H NMR.

^cTheoretical M_n ($M_{n, th}$) Calculated from conversion (ρ) using $M_{n, th} = ([M]_0/[CTA]_0) \cdot MW_{monomer} \cdot \rho + M_{w, CTA}$.

^d A_s determined by aqueous, cationic SEC-MALLS.

^eDetermined by Wyatt Optilab DSP interferometric refractometer ($\lambda = 690$ nm).