Biomacromolecules

August 2007 Published by the American Chemical Society Volume 8, Number 8

Communications

Facile Synthetic Procedure for ω, Primary Amine Functionalization Directly in Water for Subsequent Fluorescent Labeling and Potential Bioconjugation of RAFT-Synthesized (Co)Polymers†

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Received May 10, 2007; Revised Manuscript Received June 12, 2007

We describe a facile method to amine functionalize and subsequently fluorescently label polymethacrylamides synthesized via reversible addition−fragmentation chain transfer (RAFT) polymerization. RAFT-generated poly(*N*-(2-hydroxypropyl) methacrylamide-*b*-*N*-[3-(dimethylamino)propyl] methacrylamide) (poly(HPMA-*b*-DMAPMA)), a water soluble biocompatible polymer, is first converted to a polymeric thiol and functionalized with a primary amine through a disulfide exchange reaction with cystamine and subsequently reacted with the amine-functionalized fluorescent dye, 6-(fluorescein-5-carboxamido)hexanoic acid, succinimidyl ester (5-SFX). Poly(HPMA$_{258}$-*b*-DMAPMA$_{13}$) (M_n = 39 700 g/mol, M_w/M_n = 1.06), previously synthesized by RAFT polymerization, was used to demonstrate this facile labeling method. The problem with labeling the ω-terminal chain end of a RAFT-synthesized polymethacrylamide is that the reduced end yields a tertiary thiol with low reactivity. The key to labeling poly(HPMA-*b*-DMAPMA) is to first reduce the dithioester chain end with a strong reducing agent such as $NaBH_4$, and then functionalize the tertiary polymeric thiol with a primary amine through a disulfide exchange reaction with dihydrochloride cystamine. We show that the disulfide exchange reaction is efficient and that the amine-functionalized poly(HPMA-*b*-DMAPMA) can be easily labeled with the fluorescent dye, 5-SFX. This concept is proven by using a ninhydrin assay to detect primary amines and UV−vis spectroscopy to measure the degree of conjugation.

Introduction

Reversible addition-fragmentation chain transfer (RAFT) polymerization, since its discovery in 1998 by the CSIRO group,[1] has proven to be a facile method to synthesize polymers with controlled molecular weights that can provide precise polymer architectures with predetermined end group functionality. The end groups are determined by the choice of chain transfer agent (CTA) used in the polymerization. The CTA provides an ω-terminal chain end with a thiocarbonylthio moiety that can be utilized for post-polymerization modification. The thiocarbonylthio group is easily reduced to provide a polymeric thiol that can be further utilized for synthesizing bioconjugates, drug conjugates, or fluorescent labels. For example, our group first reported the formation of polymeric, sterically and electrosterically stabilized transition metal nanoparticles by in situ sodium borohydride ($NaBH_4$) reduction of dithioester end groups directly in water.[2] The control over a wide range of monomers using the RAFT process has been reported extensively,[1,3−8] but to date there are minimal reports on the post-polymerization modification of the thiocarbonylthio moiety.[2,5,7,9−11]

Many researchers have reported RAFT polymerizations of cationic,[12−15] anionic,[16−20] zwitterionic,[4,21,22] and nonionic

† Paper number 127 in a series entitled "Water Soluble Polymers".

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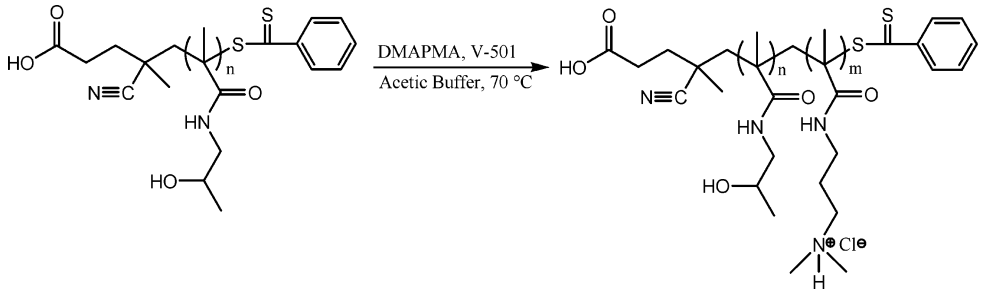
10.1021/bm700514q CCC: $37.00
Published on Web 07/24/2007

Scheme 1. Synthetic Pathway for the Aqueous RAFT Block Polymerization of Poly(HPMA-*b*-DMAPMA)



monomers[23−31] in both aqueous and organic media with various CTAs that include xanthates,[32] dithiocarbamates,[30] trithiocarbonates,[15,24,33,34] and dithioesters.[5,7,12,13,16−18,21−23,27,29,35] Our research group has specifically focused on the RAFT synthesis of water-soluble responsive polymers in aqueous media. Recently we reported on the controlled polymerization of the methacrylamide monomer, *N*-(2-hydroxypropyl) methacrylamide (HPMA), via aqueous RAFT polymerization.[26] Poly(HPMA) is of primary interest in the drug delivery community because it provides a nonviral carrier that is biocompatible, water soluble, and nonimmunogenic. Poly(HPMA) has been studied by numerous groups for the delivery of anticancer drugs, most notably doxorubicin.[36−43] However, all of these studies use HPMA polymerized by conventional free-radical polymerizations that provide polydisperse nonviral polymeric carriers with poorly defined architectures. Two major properties that affect the efficacy of a polymeric carrier are the molecular weight and the molecular weight distribution. These factors affect the ability of the carrier to remain in circulation and ultimately the dose that is required for effective treatment. RAFT polymerization can provide polymers of predetermined molecular weights and architectures that can be tailored for specific drug or gene deliveries. Block copolymer architectures are of special interest because of their ability to form ordered structures (e.g., micelles, block ionomer complexes, and vesicles) in aqueous environments that have potential as nonviral drug/gene carriers.[44] Our group recently reported the aqueous RAFT polymerization of poly(HPMA-*b*-*N*-[3-(dimethylamino)propyl] methacrylamide (DMAPMA)) block copolymers through the chain extension of HPMA macro chain transfer agent (macroCTA) with the monomer DMAPMA (Scheme 1). These block polymers have shown promise in gene delivery through the formation of an electrostatic complex between the negatively charged backbone of polynucleotides and the positively charged DMAPMA block.[45] These blocks were able to form stable complexes with ribonucleic acids (RNA) and provide protection for the RNA from degradative enzymes.

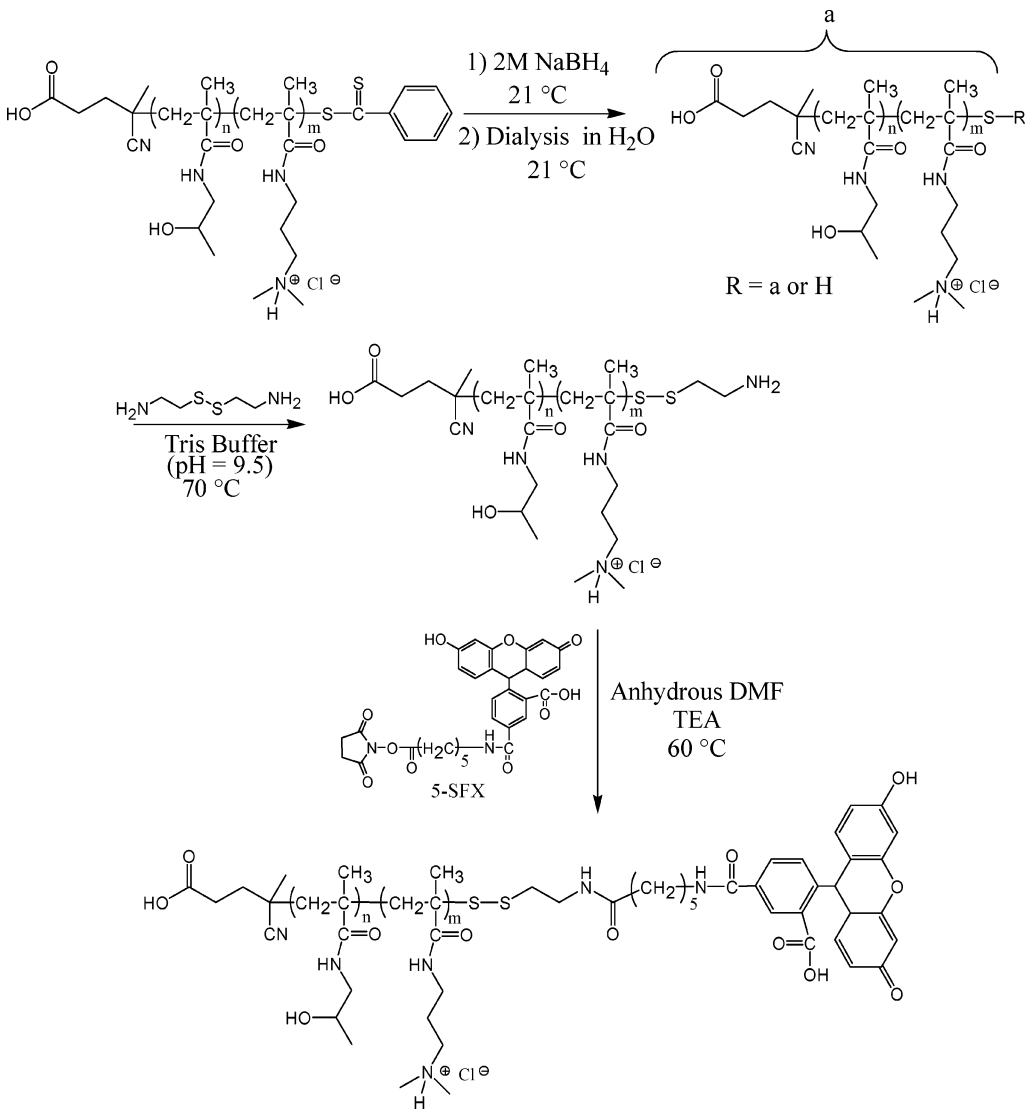
RAFT polymerization yields functionality that can be transformed in a facile manner to reactive telechelic endgroups for conjugation to drugs, peptides, proteins, targeting moieties, nanoparticles, or fluorescent dyes.[2,9,10,46−50] The facile reduction of the ω-terminal thiocarbonylthio group with a reducing agent, such as amines[50−52] or $NaBH_4$,[2,9,10,53] provides a polymeric thiol. Thiol functional groups are extensively used throughout the biosciences for peptide or protein conjugation, drug conjugation, and dye labeling.[54−57] Thiols are reactive toward functionalities such as other thiols, maleimides, and iodoacetamides. Polymer dye and bioconjugates have also been synthesized via thiol chemistry. For example, Ruffner et al. reported on the delivery of antisense oligonucleotides using HPMA polymer that contained active thiol groups for conjugation.[58] The authors demonstrated that a thiolated oligonucleotide could be rapidly conjugated to the activated thiol, thus forming a disulfide linkage between the polymer and the oligonucleotide. The conjugated oligonucleotide was efficiently taken up into cultured mammalian cells, and the oligonucleotide could be readily cleaved through treatment with dithiothreitol. Hubbell and co-workers reported on the use of poly(ethylene glycol-*b*-propylene sulfide-*b*-peptide) (poly(PEG-PSS-Pep)) triblock copolymer for the nonviral delivery of small interfering RNA (siRNA).[59] Cationic peptides were linked to the diblock copolymer, poly(PEG-PSS), by the formation of a disulfide bond between the diblock copolymer chain end and a cysteine residue of the peptide. The cationic peptide allowed for the electrostatic interaction between the polymer and the siRNA. The triblock copolymers demonstrated their ability to successfully transport siRNA into the cell and suppress the targeted gene. Recently our laboratory reported on labeling the ω-chain end of poly(*N*-isopropyl acrylamide) (PNIPAM) through the reaction of the polymeric thiol with *N*-(1-pyrene)maleimide.[9] Reduced PNIPAM yields a secondary thiol, which is less reactive than a primary thiol, and, because of this, a large excess of *N*-(1-pyrene)maleimide to polymeric thiol (150:1) and a long reaction time were needed in order to achieve high conversion. In addition, it has been shown that conjugates linked through maleimides can undergo hydrolysis in aqueous environments, and, in the case of dye labeling, this hydrolysis can affect the overall fluorescence of the dye conjugates.[54,55] Upon reduction of RAFT polymers, a secondary polymeric thiol forms for non-α-methyl-substituted monomers, and a tertiary polymeric thiol forms for α-methyl-substituted monomers. Transformation of secondary and tertiary thiols to primary amines allows shorter reaction times and lower molar quantities for conjugation to expensive peptides, proteins, and fluorescent dyes. Herein we report a new synthetic pathway that allows the less reactive tertiary polymeric thiol of poly(HPMA-*b*-DMAPMA) to be fluorescently labeled by 6-(fluorescein-5-carboxamido)hexanoic acid, succinimidyl ester (5-SFX). This is achieved through a disulfide exchange reaction between the tertiary polymeric thiol and cystamine, yielding a primary amine at the ω-terminal chain end and subsequently reacting the amine-activated polymer with the fluorescent dye 5-SFX.

Experimental Section

Materials. All reagents were purchased from Aldrich and used without further purification unless otherwise stated. The RAFT polymer poly($HPMA_{258}$-*b*-$DMAPMA_{13}$) was synthesized as previously reported.[45] 5-SFX was purchased as a single isomer from Invitrogen Molecular Probes.

ω-Chain End Amine Functionalization of Poly($HPMA_{258}$-*b*-$DMAPMA_{13}$). Poly($HPMA_{258}$-*b*-$DMAPMA_{13}$) ω-end groups were amine functionalized with cystamine. $HPMA_{258}$-*b*-$DMAPMA_{13}$ (40.6 mg) was added to a 1.85 mL vial equipped with a micro stir bar. The polymer was diluted with 183 μL of 2 M $NaBH_4$ and allowed to react for 2 h, yielding the reduced polymer. The reduced polymer was then dialyzed against deionized (DI) water for 3 days and subsequently

Scheme 2. Synthetic Pathway for the Amine Functionalization and Fluorescent Labeling with 5-SFX for Poly(HPMA-*b*-DMAPMA)



lyophilized. The reduced polymer was then functionalized with a primary amine through a reaction with excess dihydrochloride cystamine. Reduced poly(HPMA$_{258}$-*b*-DMAPMA$_{13}$) (22.4 mg) was dissolved in 100 μL of 4 M cystamine in tris buffer (pH 9.5) to give a final polymer concentration of 5.6 mM. This reaction was carried out at 70 °C for 92 h. The solution was then dialyzed against DI water for 3 days followed by lyophilization. The amine content of the resulting polymer was determined by ninhydrin assay, and the polymer was again dialyzed for 3 more days against DI water and subsequently lyophilized. A second ninhydrin assay indicated a constant amine concentration.

Fluorescent Labeling with 5-SFX. Amine-functionalized polymer was fluorescently labeled with 5-SFX. Amine-functionalized poly(HPMA$_{258}$-*b*-DMAPMA$_{13}$) (11.5 mg (0.29 μmol)) was added to a 1.5 mL microcentrifuge tube and diluted with 97 μL of a 15 mM solution of 5-SFX in anhydrous dimethylformamide to give a final polymer concentration of 3 mM, or a 5-SFX to polymer ratio of 5:1. A catalytic amount of triethylamine was also added to the solution. The reaction solution was allowed to react in the dark at 60 °C for 15 h. The solution was then dialyzed in the dark against DI water for 3 days and subsequently lyophilized. The dried, fluorescently labeled polymer was then analyzed via UV−vis spectrometry, which indicated that free fluorophore was still present. The labeled polymer was then dissolved in 20 mM phosphate/0.1 M NaCl buffer (pH 7.4) and purified using 10 000 molecular weight cut-off Microcon centrifuge tubes in order to remove free fluorophore. This was repeated until minimal absorbance was observed. The sample was then washed repeatedly with DI water to remove the buffer solution. The retentate was diluted with DI water and lyophilized. The percent conjugation was then determined using UV−vis spectroscopy.

Determination of Amine Functionalization. Amine functionalization of poly(HPMA$_{258}$-*b*-DMAPMA$_{13}$) was determined using a ninhydrin assay. Ninhydrin assay solution was prepared by dissolving 200 mg of ninhydrin in 5 mL of ethylene glycol and 8 mg of $SnCl_2 \cdot H_20$ into 5 mL of 0.2 M citrate buffer (pH 5) and mixing the resulting two solutions. Amine-functionalized polymer (3.2 mg) was diluted with 29 μL of DI water to give a 3 mM polymer solution. The solution (6 μL of 3 mM polymer) was then diluted with 200 μL of ninhydrin assay solution and was vortexed to ensure homogeneous mixing. The resulting solution was then heated to 100 °C for 20 min. The solution was then diluted to 600 μL using DI water, and the absorbance at 570 nm was measured using a JASCO V-530 spectrophotometer. The amine-functionalized polymer was analyzed after 3 and 6 days of dialysis against DI water to ensure the complete removal of excess cystamine. To determine amine functionalization, a calibration curve was constructed using hexylamine. Four different aliquots of 3 mM hexylamine were diluted with 200 μL of ninhydrin assay solution and heated to 100 °C for 20 min. Each solution was diluted to 600 μL using DI water, and the absorbance at 570 nm was measured to create a calibration curve.

Determination of Fluorescent Labeling. Determination of percent conjugation of 5-SFX to amine-functionalized poly(HPMA$_{258}$-*b*-DMAPMA$_{13}$) was also determined using a JASCO V-530 spectropho-

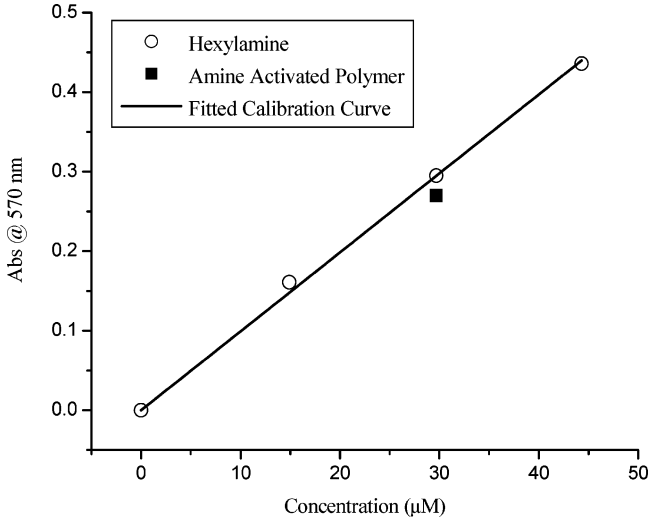


Figure 1. Absorbance of ninhydrin at 570 nm versus amine concentration for hexylamine (○) and amine-activated polymer (29.7 μM) (■), and the fitted calibration curve (—).

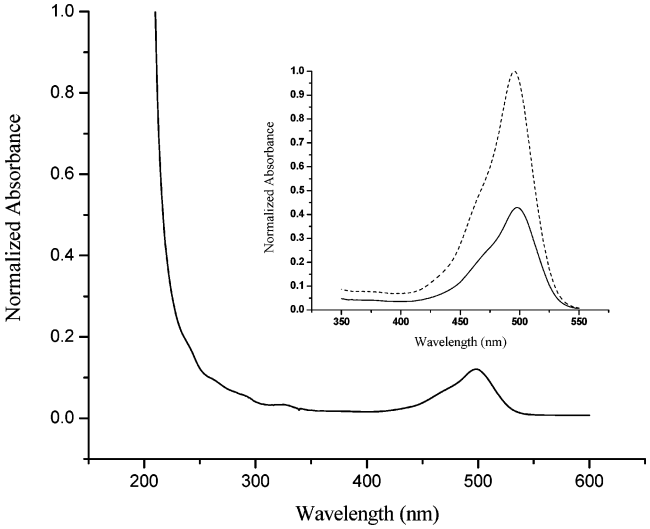


Figure 2. Absorbance spectrum of fluorescently labeled poly(HPMA$_{258}$-*b*-DMAPMA$_{13}$). Inset shows magnified region of absorbance due to 5-SFX before membrane filtration (----) and after membrane filtration (—).

tometer. The fluorescently labeled polymer was dissolved in 209 μL of DI water to yield a polymer concentration of 0.5 mM. A 5 μL portion of the 0.5 M solution was then diluted with 600 μL of 20 mM phosphate/0.1 M NaCl buffer (pH 7.4). The absorbance of the polymer conjugate at 494 nm was then measured, and the concentration of 5-SFX was calculated using the reported extinction coefficient of 68 000 M^{-1} cm^{-1}.[60,61] This measurement was performed in quadruplet to ensure accuracy.

Results and Discussion

The synthetic pathway for the amine functionalization and the fluorescent labeling with 5-SFX for poly(HPMA$_{258}$-*b*-DMAPMA$_{13}$) is outlined in Scheme 2. After reduction of the dithioester end group with $NaBH_4$ and subsequent purification, the reduced polymer contains free polymeric thiols and coupled polymer chains through disulfide linkages. The addition of excess cystamine to this mixture at basic pH allows a disulfide exchange to occur between the polymer chains and the cystamine in solution, yielding a primary amine at the ω-chain end of the polymer. Primary amines are key to transformation in the biosciences, allowing facile reactions with activated carboxylic acids as well as other functional groups. The concentration of free amines can be determined via a ninhydrin assay by observing the absorbance at 570 nm. Figure 1 shows the absorbance of the amine-activated poly(HPMA$_{258}$-*b*-DMAPMA$_{13}$) at 570 nm along with a calibration curve for the model amine compound, hexylamine. The percent of reduced poly(HPMA$_{258}$-*b*-DMAPMA$_{13}$) reacted with cystamine was found to be 91.7 $\pm$ 0.3%. The presence of a free amine at the RAFT polymer chain ends allows for easy conjugation between the polymer and the activated fluorophore 5-SFX. The primary amine, a more reactive group than a tertiary thiol, allows smaller quantities of fluorescent dyes to be used. For example, the ratio of fluorescent dye to polymer in this report was 5:1, while, in our group's previous study,[9] a molar ratio of 150:1 was needed for sufficient reaction between a secondary thiol and a maleimide. After conjugation of 5-SFX to the polymer, excess fluorescent dye was evident even after dialysis against DI water. This was attributed to the fact that the block copolymer contains a cationic block and 5-SFX is anionically charged at neutral pH, causing the free fluorophore to be bound to the cationic groups of the DMAPMA block. In order to remove the excess 5-SFX, the polymer was dissolved in 20 mM phosphate/0.1 M NaCl buffer (pH 7.4) to promote ion exchange and ultimately release the bound 5-SFX. The free 5-SFX was then removed using a centrifugal membrane filtration device. Figure 2 shows the absorbance of the fluorescently labeled polymer before and after centrifugal membrane filtration. The decrease in maximum absorbance is clearly observed by comparing the two absorbance spectra. The degree of 5-SFX conjugation to poly(HPMA$_{258}$-*b*-DMAPMA$_{13}$) was obtained by comparing the concentration of 5-SFX, determined by measuring the maximum absorbance at 494 nm (Figure 2), to the known concentration of the polymer. The degree of conjugation was found to be 80.1 $\pm$ 2.6%.

Conclusions

A facile method to functionalize the RAFT polymer poly(HPMA$_{258}$-*b*-DMAPMA$_{13}$) with a primary amine and subsequently label the ω-chain end with an amine reactive dye has been reported. The reduction of the dithioester chain end, using $NaBH_4$, yields a tertiary polymeric thiol with a low reactivity. To circumvent this problem, the reduced polymer was functionalized with a primary amine via a disulfide exchange with dihydrochloride cystamine. The degree of functionalization was measured using a ninhydrin assay by measuring the maximum absorbance at 570 nm. The degree of functionalization was found to be 91.7 $\pm$ 0.3%. The primary amine at the ω-chain end of the RAFT polymer, which is more reactive than a tertiary thiol, allows for facile conjugation between poly(HPMA$_{258}$-*b*-DMAPMA$_{13}$) and the fluorescent dye, 5-SFX. The degree of conjugation was found using a UV−vis spectrometer by measuring the maximum absorbance at 494 nm, giving a degree of conjugation of 80.1 $\pm$ 2.6%. The facility by which RAFT-generated polymers can be functionalized as described in this work points to future utility in drug delivery, gene delivery, biodiagnostics, as well as other areas of biotechnology.

Acknowledgment. The authors would like to thank and acknowledge the Department of Energy (DE-FC26-01BC15317), the National Science Foundation (DMR-0213883), and the Robert M. Hearin Foundation for financial support.

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BM700514Q