Synthesis of Copolymers Containing an Active Ester of Methacrylic Acid by RAFT: Controlled Molecular Weight Scaffolds for Biofunctionalization

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We report the controlled radical copolymerization of *N*-(2-hydroxypropyl)methacrylamide (HPMA) with a monomer containing an active ester, *N*-methacryloyloxysuccinimide (NMS), by reversible addition fragmentation chain transfer (RAFT). The large difference in the reactivity ratios of HPMA and NMS resulted in significant variations in copolymer composition with increasing conversion during batch copolymerization. The use of a semi-batch copolymerization method, involving the gradual addition of the more reactive NMS, allowed uniformity of copolymer composition to be maintained during the polymerization. We synthesized polymers in a wide range of molecular weights (M_n = 3000−50 000 Da) with low polydispersities (1.1−1.3). The effect of the ratio of monomer to chain transfer agent (CTA) on the molecular weight of the polymer was investigated. Given the numerous applications of poly(HPMA)-based conjugates in designing polymeric therapeutics, these controlled molecular weight activated polymers represent attractive scaffolds for biofunctionalization. As a demonstration, we attached a peptide to the activated polymer backbone to synthesize a potent controlled molecular weight polyvalent inhibitor of anthrax toxin.

Introduction

Polymer therapeutics − polymers conjugated with drugs, proteins, or other ligands − have attracted enormous interest in the past two decades.[1−11] Polymer−drug conjugates offer several advantages over conventional chemotherapeutic agents, including lower nonspecific cytotoxicity, biocompatibility, longer circulation times, and increased solubility. We and others have also used polymeric scaffolds to design polyvalent inhibitors for pathogens and toxins.[4,12−29] Polyvalent molecules, which display multiple copies of a suitable ligand on a polymeric backbone, can have a much higher affinity for the target of interest than the corresponding monovalent ligand.

Poly(*N*-(2-hydroxypropyl)methacrylamide) (PHPMA) represents a particularly attractive scaffold for designing polymer therapeutics. PHPMA is a biocompatible, nonimmunogenic, and nontoxic polymer with a well-characterized pharamacokinetic profile.[3] It has been widely used for the tumor-specific delivery of oligonucleotides, targeted delivery to the gastrointestinal tract, and the delivery of anti-cancer drugs.[3] A copolymer of *N*-(2-hydroxypropyl)methacrylamide (HPMA) and a HPMA-doxorubicin conjugate, also known as PK1, entered phase I trials as an anti-cancer drug in 1994.[31] Since then, conjugates of PHPMA with chemotherapeutic drugs such as camptothecin, taxol, and platinates[32,33] have also progressed to clinical testing. Most of these conjugates are prepared by free radical copolymerization and therefore have a broad molecular weight distribution. The molecular weight distribution of the polymers can significantly influence a number of properties of the resulting conjugate, including pharmacokinetics, toxicity, and inhibitory potency.[3,30] Consequently, control over the chemical structure and molecular weight of the polymers is critical for designing effective polymer therapeutics and for elucidating structure−activity relationships. Thus, it would be useful to synthesize PHPMA conjugates of controlled molecular weight.

Several attempts have been made to synthesize PHPMA of controlled molecular weight. Godwin et al. polymerized *N*-methacryloyloxysuccinimide (NMS) by atom transfer radical polymerization (ATRP).[34,35] Poly(*N*-methacryloyloxysuccinimide) (PNMS) with number average molecular weight (M_n) as high as 40 000 Da was prepared with a polydispersity index of 1.2; subsequent reaction of the active ester groups with excess 1-amino-2-propanol yielded PHPMA.[34,35] Other authors have also reported the controlled polymerization of NMS by ATRP.[36−38] However, complete removal of the catalyst is difficult, which may limit the use of this approach for biomedical applications.[39]

Reversible addition-fragmentation chain transfer (RAFT) polymerization has emerged as an alternative controlled radical polymerization technique.[40] Compatibility with a variety of monomers and the ability to carry out polymerization in a aqueous system are some of the salient features of RAFT. Recently, McCormick and co-workers reported the controlled polymerization of HPMA in aqueous media by RAFT.[42] PHPMA with M_n as high as 98 000 Da was reported with a narrow polydispersity index (1.08). Although this method is convenient for the synthesis of PHPMA, it is not amenable to the synthesis of activated polymers such as those containing active ester groups, due to their hydrolytic sensitivity. Activated polymers provide a versatile scaffold that can be conjugated with a wide variety of ligands via post-polymerization modifications, thereby providing a convenient method for synthesizing polymer−ligand conjugates.[43]

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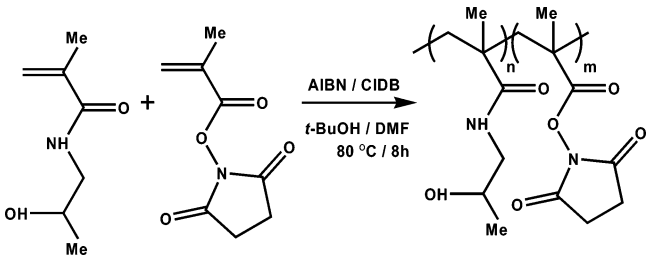
Scheme 1. Copolymerization of HPMA and NMS by RAFT[a]



[a] The product is a random copolymer.

Activated polymers based on NMS can be modified to form biocompatible PHPMA-based polymer conjugates. Schilli et al. attempted to polymerize NMS by RAFT in *N*,*N*-dimethylformamide (DMF), using 2-cyanoisopropyl dithiobenzoate as the CTA.[41] However, there was limited control over polymerization even at high concentrations of the CTA. The resultant PNMS had a polydispersity index of 1.46. Recently Savariar et al. reported the copolymerization of NMS with *N*-isopropylacrylamide by RAFT.[44] By analogy, we reasoned that the copolymerization of NMS and HPMA by RAFT (Scheme 1) would enable the synthesis of activated polymers of controlled molecular weight, and provide a convenient route to synthesize defined PHPMA-based polymer conjugates. To our knowledge, such controlled molecular weight activated copolymers have not been reported in the literature. We demonstrate the ability to synthesize these activated polymers in a range of molecular weights with low polydispersity indices. We have also demonstrated the utility of these activated polymers by synthesizing a defined polymer−peptide conjugate, which is a potent polyvalent inhibitor of anthrax toxin.

Experimental Section

Materials. HPMA (99.9%, Polysciences Inc., Warrington, PA) and *tert*-butyl alcohol (*t*-BuOH) (99.9%, Aldrich, Allentown, PA) were used as received. 1,3,5-Trioxane (99.5%), anhydrous diethyl ether (99.5%), and 1-amino-2-propanol (99.5%) were obtained from Acros (Somerville, NJ) and used as received. NMS[45] and 2-cyanoisopropyl dithiobenzoate (CIDB)[46] were prepared as described in the literature. DMF (99.5%, Acros) was dried over CaH_2 and distilled under reduced pressure. Acetone (99.5%, Acros) was dried over K_2CO_3. 2,2′-Azobis(isobutyronitrile) (AIBN) (97%, Aldrich, Allentown, PA) was purified by recrystallization from methanol. Peptide, Ac−HTSTYWWLDGAPK−Am, was purchased from Genemed Synthesis Inc. (South San Francisco, CA). Spectra/Por 3 (MWCO 3,500) membrane used for dialysis was obtained from Fisher Scientific (Rancho Domingez, CA).

^{1}H and ^{13}C NMR Spectroscopy. Quantitative ^{1}H NMR spectra of the copolymers were recorded on a Varian Unity 500 MHz spectrometer in DMSO-d_6 at room temperature. Approximately 200−300 scans were made (depending on the value of M_n). ^{13}C NMR spectra were also obtained on the same spectrometer in DMSO-d_6 at room temperature. Approximately 3000−4000 scans were made (depending on the value of M_n). No hydrolysis of active ester units was observed during ^{13}C NMR analysis.

Aqueous Size Exclusion Chromatography (ASEC). The poly(HPMA-*co*-NMS) was converted to PHPMA by aminolysis of active ester units with 1-amino-2-propanol followed by dialysis and lyophilization. The molecular weight of PHPMA was analyzed by ASEC using a light scattering detector and a mixture of 80:20 v/v 0.05 M sodium sulfate and acetonitrile as the eluent. Analysis was carried out at 25 °C at a flow rate of 1.0 mL/min. Viscotek column (GMPWXL Mix Bed, dimensions: 7.8 mm × 30 cm) was used with a Waters 2487 Dual wavelength UV/vis absorbance detector, Waters 2414 refractive index detector, and a Viscotek 270 Trisec Dual Detector (λ = 670 nm) inline with the column. OmniSEC 4.0 software was used to determine the molecular weight. The d*n*/d*c* of PHPMA in the above eluent was 0.176 mL/g.[42]

Fourier Transform Infrared (FT-IR) Spectroscopy. The FT-IR spectrum of the poly(HPMA-*co*-NMS) (after removing end groups) was recorded on a BioRad Excalibur FTS3000 spectrometer from 400 to 4000 cm^{-1}.

Synthesis of Copolymer. The polymerizations were carried out under a dry nitrogen atmosphere using standard Schlenk techniques. A typical polymerization procedure (polymer 4, Table 1) is described below. A 20 mL Schlenk tube was charged with 0.32 g (2.2 mmol) of HPMA, 2.2 mL of *t*-BuOH (1M solution), 12.5 mg of 1,3,5-trioxane (1.4×10^{-4} mmol, internal standard), and 50 μL of a solution of CIDB (3.8 mg, 1.72×10^{-5} mol) in DMF. Approximately 50 μL of a solution of AIBN (1.5 mg, 8.6×10^{-6} mol) in DMF was transferred to the Schlenk tube. In another Schlenk tube, 0.1 g (0.54 mmol) of NMS was dissolved in 1.0 mL of anhydrous DMF (0.5 M solution). The molar feed ratio of HPMA and NMS was 80:20. The molar ratio of total monomer (HPMA and NMS) to CTA was 320. The contents of both the tubes were subjected to five freeze and thaw cycles. A small fraction (approximately 50 μL) of the sample was taken out from the *t*-BuOH solution for ^{1}H NMR analysis before heating the system at 80 °C. The NMS solution was taken in a 1.0 mL airtight syringe and continuously added to HPMA solution over a period of 6 h using a PHD-2000 model syringe pump (Harvard Apparatus, South Natick, MA). After the addition of NMS, the reaction was continued for an additional period of 2 h. Finally a small fraction of the sample (approximately 50 μL) was taken out and immediately analyzed by ^{1}H NMR spectroscopy (in $CDCl_3$) for monomer conversion (HPMA = 58%, NMS = 67%). The remaining polymer solution was precipitated with approximately 400 mL of a mixture of anhydrous diethyl ether and anhydrous acetone (1:1, v/v) and the copolymer was recovered by centrifugation. The copolymer was further washed several times with the anhydrous diethyl ether and anhydrous acetone mixture and immediately dried under vacuum for a period of 24 h (yield: 0.25 g,

Table 1. Copolymerization of HPMA/NMS by RAFT[a]

				conversion[b] (mol %)		copolymer composition[c] (mol %)			M_n ASEC results[e]		
polymer	[M]/[CTA]	NMS addition rate (μL/h)	polymerization time (h)	HPMA	NMS	HPMA	NMS	M_n theoretical[d] (kDa)	(kDa)	M_w (kDa)	PD
1	40	80	18.0	62.0	69.0	72.0	28.0	3.9	4.3	4.6	1.08
2	80	100	15.0	66.0	64.0	81.0	19.0	7.6	8.9	10.3	1.16
3	160	125	12.0	65.0	70.0	75.0	25.0	15.7	17.2	19.8	1.15
4	320	165	8.0	58.0	67.0	78.0	22.0	28.9	33.1	38.0	1.15
5	640	250	6.0	54.0	67.0	77.0	23.0	55.6	53.6	65.4	1.22

[a] Polymerization conditions: HPMA = 0.32 g (2.2 mmol) in 2.2 mL of *t*-BuOH, 1.0 M solution, NMS = 0.1 g (0.54 mmol) in 1.0 mL of DMF, HPMA:NMS = 80:20 mol/mol, temp. = 80 °C, [CTA]/[AIBN] = 2.0, For polymer 5, the [CTA]/[AIBN] = 1.5. [b] HPMA conversion determined by comparing the initial and final concentrations from ^{1}H NMR, however, the initial concentration of NMS was calculated theoretically. [c] In mol %, determined by ^{1}H NMR. [d] Theoretical M_n based on [M]/[CTA] ratio at given conversion after aminolysis. [e] Determined by ASEC after aminolysis.

60%). The absence of residual monomers in the copolymer was confirmed by ^{1}H NMR (in DMSO-d_6). The copolymer composition was determined by ^{1}H NMR spectroscopy (78 and 22 mol % for HPMA and NMS respectively). ^{1}H NMR (DMSO-d_6): δ [ppm] = 7.78−7.83 (dd), 7.1−7.6 (br), 4.5−4.8 (br), 3.6−3.9 (br), 2.6−3.1 (br), 0.9−1.5 (br).

Kinetics. The kinetics of the copolymerization was followed by withdrawing samples from the polymerization mixture at different intervals of time and analyzing the residual monomer concentrations by ^{1}H NMR in $CDCl_3$. Monomer concentrations were calibrated by comparing the integration of the peaks due to the vinyl protons of the monomers with those of an inert internal standard; 1,3,5-trioxane.[48]

Removal of End Groups. The dithiobenzoate end group was removed according to the procedure reported by Perrier et al.[49] Typically 0.2 g of poly(HPMA-*co*-NMS), (M_n = 4.5 kDa, 28 mol % NMS, polymer **1**, Table 1) and 150 mg of AIBN (20 times higher than copolymer, mol/mol) were dissolved in 3 mL of anhydrous DMF. The solution was heated at 80 °C for 90 min. Finally the copolymer was precipitated in 400 mL of a mixture of anhydrous diethyl ether and acetone (1:1 v/v) and collected by centrifugation. The copolymer was dried under vacuum for a period of 24 h (yield: 0.19 g, 95%). The absence of the dithiobenzoate end group was confirmed by ^{1}H NMR spectroscopy. ^{1}H NMR (DMSO-d_6): δ [ppm] = 7.1−7.6 (br), 4.5−4.8 (br), 3.6−3.9 (br), 2.6−3.1 (br), 0.9−1.5 (br). ^{13}C NMR (DMSO-d_6, δ): 175.5, 173.1, 170.2, 68.5, 64.5, 55.8, 47.5, 44.7, 39.5, 29.7, 25.8, 21.6, 18.0. FT-IR (KBr pellet): 3440−3412 (−OH, −NH), 1738 (−N−C═O of NHS), 1665 (C═O of amide).

Biofunctionalization. A total of 1.5 mg of poly(HPMA-*co*-NMS), (21 mol % NMS, M_n = 33 kDa, polymer **4**, Table 1) was dissolved in anhydrous dimethyl sulfoxide (DMSO) (100 mg/mL). Peptide Ac−HTSTYWWLDGAPK−Am (2 mg) predissolved in DMSO was added to the polymer solution followed by the addition of excess triethylamine. The reaction was carried out at 50 °C for 24 h followed by quenching with 1-amino-2-propanol. The polymer solution was dialyzed against MilliQ water for 48 h to remove unreacted peptide and other organic reagents. The extent of peptide attached was determined by ^{1}H NMR spectroscopy (3.0 mol % conjugation) with a yield of 14%. ^{1}H NMR (D_2O): δ [ppm] = 7.8−8.4 (br), 6.6−7.6 (br), 4.1−4.6 (br), 3.7−4.0 (br), 3.4−3.6 (br), 2.8−3.4 (br), 2.3−2.8 (br), 1.5 − 2.3 (br), 0.85−1.5 (br), 0.55−0.85 (br). The yield of conjugation was observed to be dependent on the ligand. We obtained a yield of 80% for the attachment of biotin-(2-amino-ethyl)-amide to poly(HPMA-*co*-NMS).

Cytotoxicity Assay. The efficacy of the PHPMA-based polyvalent inhibitor was evaluated by measuring the inhibition of anthrax toxin-mediated cytotoxicity in a mouse macrophage cell line, RAW264.7. A mixture of PA (10^{-9} M), LF (3×10^{-10} M), and various concentrations of the polyvalent inhibitor (on a per-peptide basis) was added to RAW264.7 cells coated in a 96-well plate. After 4 h, 20 μL of a mixture of MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and phenazine methosulfate (PMS) was added to each well and incubated for 1 h. Live cells reduce MTS into a soluble formazan product which absorbs at 490 nm. Absorbance at 490 nm thus gives an estimate of cell viability. The half-maximum inhibitory concentration (IC_{50}) of the polyvalent inhibitor was evaluated by measuring cell viability at different inhibitor concentrations.

Results and Discussion

RAFT involves a reversible addition-fragmentation chain transfer between an active and a dormant species, and the polymerization is performed in the presence of a suitable chain transfer agent (CTA)[40,50] (Scheme 2). It has been demonstrated that successful implementation of the RAFT process requires careful selection of the CTA and reaction conditions, depending upon the monomer.[51−55] The Z group in CTA stabilizes the intermediate radical and promotes addition to the C═S bond, whereas the R moiety should be a good homolytic leaving group

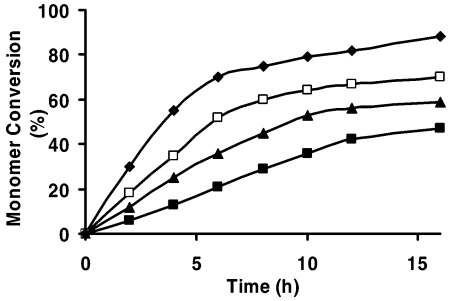
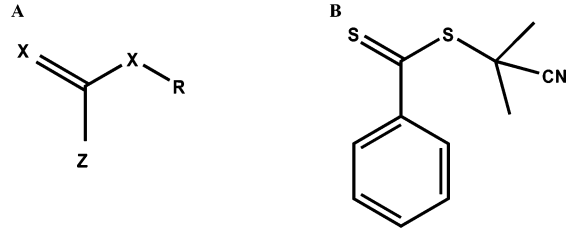


Figure 1. Kinetics of HPMA homopolymerization at [M]/[CTA] ratios of 40 (■), 80 (▲), 160 (□), and 320 (◆). [M] = 1.0, [CTA]/[AIBN] = 2.0.

Scheme 2. Structures of (A) a Typical CTA and (B) the CTA Used in This Work, CIDB



(Scheme 2A), and the formed R* radical should be able to reinitiate the polymerization. Here we selected 2-cyanoisopropyl dithiobenzoate (CIDB; Scheme 2B) as the CTA as it is a highly active chain transfer agent, and the leaving group radical (R*) is capable of reinitiating the polymerization. We used AIBN as the initiator.

Preliminary copolymerizations with various HPMA/NMS feed ratios in DMF under batch conditions at 80 °C revealed that NMS is significantly more reactive than HPMA. The reactivity ratios for HPMA and NMS were estimated to be 0.12 and 3.46 respectively, based on the extended Kelen−Tudos method.[56] Copolymerization of monomers with such a large difference in reactivity ratios results in the preferential incorporation of the more reactive monomer within the copolymer chain. The resulting change in the monomer ratio in the feed results in significant drifts in the copolymer composition with conversion. These compositional drifts may have a significant influence on the properties of the polymer, especially when low incorporation of NMS units is desired.

We reasoned that a semi-batch copolymerization method, in which the more reactive NMS is slowly added to a solution initially containing only HPMA, would allow us to compensate for the huge difference in reactivity ratios of these monomers.[57−64] We selected *t*-BuOH as the solvent for HPMA as alcohols are known to be good solvents for polymerization of HPMA, whereas DMF was used to dissolve NMS. Savariar et al.[44] recently reported the copolymerization of NMS with *N*-isopropylacrylamide using CIDB as the CTA and *t*-BuOH as a solvent. No hydrolysis of either CTA or NMS was observed in *t*-BuOH. Maintaining the molar ratios of monomers in the reaction mixture constant during a semi-batch copolymerization requires continuous feeding of the more reactive monomer at an appropriate rate. To find the proper feeding rate for NMS, we first studied the kinetics of homopolymerization of HPMA under similar conditions.

Kinetics of Homopolymerization of HPMA. Polymerization kinetics of HPMA was studied at 1.0 M concentration using four different monomer to CTA ratios ([M]/[CTA]). As shown in Figure 1, a linear consumption of HPMA was observed for conversions up to 40−50% for [M]/[CTA] ratios between 40

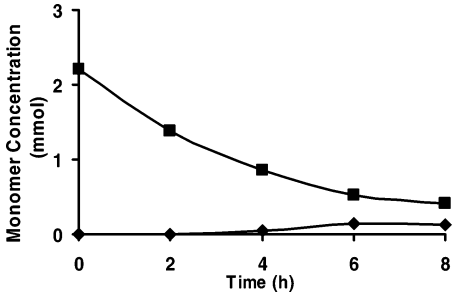


Figure 2. Kinetics of semi-batch copolymerization of HPMA and NMS ([M]/[CTA] = 320). NMS was continuously added over a period of 6 h. The concentrations of HPMA (■) and NMS (◆) in the Schlenk tube were monitored over a period of 8 h.

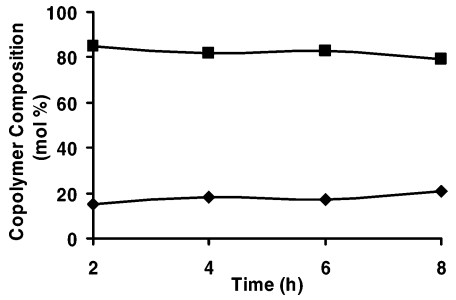


Figure 3. Variation in copolymer composition with time. The mol % of HPMA (■) and NMS (◆) was determined at periodic intervals.

and 320. The rate of polymerization decreased with a decrease in [M]/[CTA] ratio; such a "retardation effect" is often observed in polymerization by RAFT.[65−70] The CTA to initiator ratio ([CTA]/[AIBN]) was maintained at 2.0. Controlled polymerization of HPMA was confirmed by a monotonic increase in M_n with time as well as low polydispersity indices. Knowledge of the kinetic profile of HPMA was used to determine the rate of addition of NMS for semi-batch copolymerization.

Kinetics of Semi-Batch Copolymerization. Copolymerization kinetics was studied by gradual feeding of 1.0 mL of NMS solution in DMF (0.5 M) to the reaction system containing 2.2 mL of HPMA in *t*-BuOH (1.0 M) at a [M]/[CTA] ratio of 320 ([M] = [HPMA] + [NMS]). NMS was added over a period of 6 h and the polymerization was allowed to continue for an additional 2 h. Samples were taken out periodically every 2 h, and a small fraction of the sample was utilized for the analysis of monomer concentrations by ^{1}H NMR spectroscopy. The major fraction of the samples was precipitated and utilized for the analysis of copolymer composition by ^{1}H NMR spectroscopy (before aminolysis) and to determine the M_n by ASEC after aminolysis. The concentrations of HPMA and NMS were determined by comparing the integration of the vinyl proton peaks with those of the internal standard.

As seen in Figure 2, a significant decrease in concentration of HPMA was observed during the initial 4 h accompanied by the incorporation of almost all of the NMS added to the reaction system. However, the reactivity of both monomers decreased after 4 h, presumably due to a decrease in the rate of polymerization at higher conversions of monomers. Approximately 65−70% of both monomers was consumed by 8 h.

We determined the composition of the copolymers at different intervals of time by ^{1}H NMR spectroscopy (Figure 3). As seen in Figure 3, the use of a semi-batch method enabled the composition of the copolymer to be maintained constant during the polymerization. The observed increase in M_n with time (Figure 4) confirmed the controlled nature of the copolymerization. The polydispersity remained low throughout the polymerization (Figure 4A).

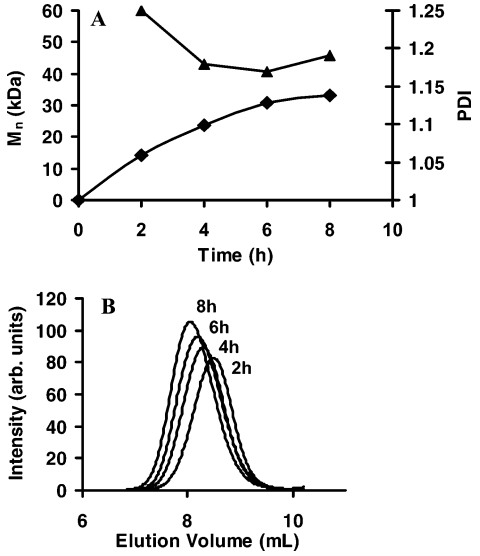


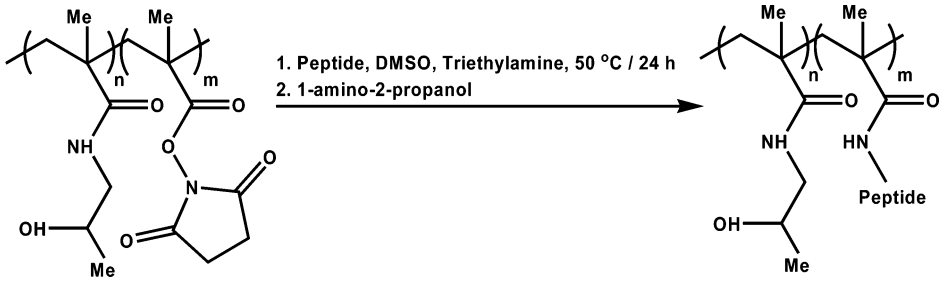
Figure 4. Characterization of polymer at various time intervals during a semi-batch RAFT copolymerization. Samples were removed at periodic intervals, converted to PHPMA by aminolysis, and characterized by ASEC. (A) Variation of M_n (◆) and PDI (▲) with time. (B) ASEC traces of PHPMA at 2, 4, 6, and 8 h.

Effect of [M]/[CTA] on M_n. Next, we studied the effect of [M]/[CTA] ratio on M_n. The results obtained are summarized in Table 1. Copolymers with M_n varying from 5000−50 000 Da were prepared with 20−25 mol % incorporation of NMS. The M_n values calculated by ASEC were found to be close to theoretical predictions with low polydispersity indices.

Removal of End Groups. Thio-acylation of primary or secondary amines with dithioesters is well documented in the literature.[71,72] In general, polymers produced by RAFT retain the thio-carbonyl end group which imparts pink color to an otherwise colorless polymer.[40,73] The thio-carbonyl end groups in copolymers can competitively react with amine-terminated ligands and can also form disulfide linkages[74] during the aminolysis of copolymers with 1-amino-2-propanol. To avoid these side reactions, we removed the thio-carbonyl end group. There are various methods reported in the literature namely hydrolysis,[75,76] pyrolysis,[77] aminolysis,[73,78−80] metal assisted elimination,[81] peroxide treatment,[82] reduction,[83] and radical addition-fragmentation[49] for elimination of thio-carbonyl end groups in polymers synthesized by RAFT. To avoid the use of any organic reagents, which react with active ester groups, we followed the radical addition-fragmentation method as it does not require any organic reagents other than AIBN. The complete elimination of thio-carbonyl end groups was confirmed by ^{1}H NMR spectroscopy. We also observed the disappearance of the pink color from poly(HPMA-*co*-NMS) after removing the end group.

Biofunctionalization. We demonstrated the utility of these well-defined biocompatible activated polymers by conjugating them to a peptide that has previously been shown to inhibit the assembly of anthrax toxin.[16] Anthrax toxin is comprised of three proteins: a cellular receptor-binding protein (protective antigen, PA) and two enzymes (Lethal factor (LF) and edema factor (EF)). Once PA binds to the cell surface receptors, it gets proteolytically cleaved and forms heptamers ($[PA_{63}]_7$). The enzymatic components of the toxin bind to $[PA_{63}]_7$ and after a series of events, are translocated into the cytoplasm. A peptide HTSTYWWLDGAP, identified by Mourez et al, prevents toxin

Scheme 3. Synthesis of PHPMA-based Polyvalent Inhibitor[a]



[a] The peptide (Ac−HTSTYWWLDGAPK−Am) has its N-terminus acetylated and C-terminus amidated. The ϵ-amino group of the terminal lysine residue of the peptide reacts with the active ester group of the copolymer.

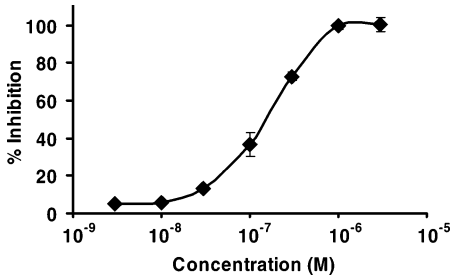


Figure 5. Inhibitory activity of PHPMA-based polyvalent inhibitor of anthrax toxin. Inhibition of anthrax toxin mediated cytotoxicity on RAW264.7 cell line was evaluated at different concentrations (on a per-peptide basis) of the polyvalent inhibitor.

assembly: the binding of LF to the heptamer.[16] A polyvalent inhibitor, obtained by attaching multiple copies of this peptide to a polyacrylamide backbone, was significantly more potent than the corresponding monovalent inhibitor.[16] We synthesized a biocompatible polyvalent anthrax toxin inhibitor by conjugating the peptide to poly(HPMA-*co*-NMS) (polymer **4**, Table 1, average number of repeat units = 231). The conjugation was carried out in anhydrous DMSO at 50 °C, followed by quenching the unreacted active ester groups with 1-amino-2-propanol to form a PHPMA-based polyvalent inhibitor (Scheme 3).

These biocompatible polyvalent inhibitors were tested for their efficacy in neutralizing anthrax toxin in vitro. RAW264.7 cells were exposed to a combination of protective antigen and lethal factor in the presence of various concentrations of the inhibitor. The polyvalent inhibitor had a half-maximum inhibitory concentration (IC_{50}) of 150 nM (Figure 5); in contrast, the monovalent peptide does not inhibit cytotoxicity at concentrations as high as 250 μM. Thus, the polyvalent inhibitor was at least 3 orders of magnitude more potent than the corresponding monovalent peptide.

Conclusions

We have used RAFT to synthesize random copolymers of HPMA and NMS with M_n as high as 50 000 Da and low polydispersity indices. The use of semi-batch copolymerization enabled the synthesis of copolymers with homogeneous composition along the chain. Biofunctionalization of the random copolymer was demonstrated by conjugating the copolymer with a peptide resulting in a biocompatible polyvalent inhibitor of anthrax toxin with nanomolar potency. These defined activated polymers could be useful for synthesizing conjugates of PHPMA with a variety of ligands and drugs for applications ranging from the design of well-defined potent polyvalent inhibitors for pathogens and toxins, to drug delivery, gene delivery, and in vivo imaging.

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