ACS Macro Letters

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Stimuli-Responsive Polymer-Antibody Conjugates via RAFT and Tetrafluorophenyl Active Ester Chemistry

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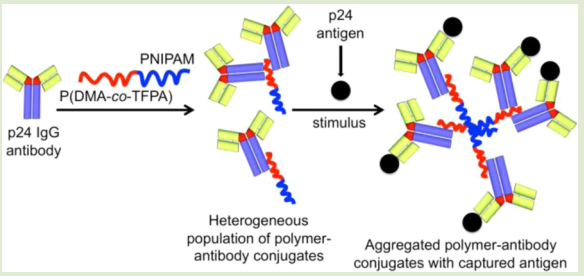
Supporting Information

ABSTRACT: Highly efficient polymer-antibody conjugations were demonstrated via a tetrafluorophenyl active ester. A well-defined diblock copolymer was synthesized by reversible addition−fragmentation chain transfer (RAFT) polymerization with a temperature-responsive block, poly(*N*-isopropylacrylamide), and a block of *N*,*N*-dimethylacrylamide and 2,3,5,6-tetrafluorophenyl acrylate active ester. The polymer was conjugated to anti-p24 IgG antibody with about 100% efficiency in as little as 2 h at room temperature in a pH 10.8 buffer. The temperature-responsiveness of the polymer was conferred to the polymer−antibody conjugates after conjugation. The conjugates bound p24 antigen specifically and with binding efficiency comparable to native antibodies. Thus, the active ester diblock copolymer can facilitate the synthesis of temperature-responsive bioconjugates, which may be promising reagents for immunoassays, bioseparations, and specimen-enrichment applications.



Utilizing stimuli-responsive polymers for immunoassays, drug delivery, bioseparations, switchable enzyme activity, and affinity precipitation has been explored for more than two decades.[1−6] Stimuli-responsive polymer−protein conjugates are particularly attractive reagents for bioseparations because recognition events occur at the molecular scale and appropriate stimuli rapidly aggregate the conjugates to enable effective isolation for downstream analysis. Protein conjugation to synthetic polymers can be achieved by modifying polymer chain ends with reactive chemistries (i.e., *N*-hydroxysuccinimide (NHS) esters, maleimides, etc.),[7−11] also called a "grafting-to" approach. Controlled radical polymerization (CRP) techniques such as reversible addition−fragmentation chain transfer (RAFT) polymerization have enabled an interesting alternative for polymer−protein conjugates in which proteins are used as chain transfer agents (CTAs) from which to synthesize a polymer.[12−15] This is referred to as a "grafting-from" approach and has been demonstrated with a number of proteins.[16]

Perhaps the most attractive approach to synthesize polymers for conjugations remains the direct polymerization of active ester monomers. This approach results in multiple functional groups for direct biomolecule conjugation without postsynthesis modification of the polymer.[17] For example, acryl and methacryl esters of NHS have been successfully used to synthesize poly(acrylamides) for biomolecule conjugation.[18−20] Fluorine-containing active esters such as pentafluorophenyl (meth)acrylates have emerged as a potential alternative to NHS-esters due to their better resistance to hydrolysis and higher solubility in a wide range of organic solvents.[21] Recently, CRP has been utilized for the polymerization of fluorine-containing active ester monomers to produce polymers with predetermined molecular weights, narrow molecular weight distributions, and a high degree of chain-end functionalization.[22,23] Gan et al. demonstrated the successful synthesis of well-defined poly(2,3,5,6-tetrafluorophenyl methacrylate) via atom transfer radical polymerization (ATRP).[24] Theato et al. reported the first synthesis of pentafluorophenyl methacrylates (PFPMA) via RAFT polymerization.[25] Barz et al. also utilized RAFT polymerization to synthesize poly(PFPMA) that was further employed to obtain poly(*N*-2-hydroxypropyl methacrylamide)−folate conjugates.[26,27] In a recent study, Klock et al. modified poly(PFPMA) via post-RAFT functionalization with various amines to assemble a library of water-soluble polymers.[28] Boyer and Davis synthesized glycopolymers via RAFT polymerization of pentafluorophenyl acrylates and subsequent modification of the poly(pentafluorophenyl acrylate) with amine-functional sugars.[29]

Most of the previous studies focused on reaction between fluorophenyl active ester and small molecule amines.[21,25,27,28] However, conjugations of large biomolecules (i.e., antibody) with well-controlled, stimuli-responsive block copolymers of fluorophenyl active esters have not been reported. Herein, we demonstrate facile antibody conjugation directly to the backbones of temperature-responsive, active-ester, diblock copolymers without postpolymerization modification. Specifically, well-defined diblock copolymers were synthesized via RAFT polymerization, where temperature-responsive poly(*N*-

Received: November 24, 2012
Accepted: January 14, 2013
Published: January 17, 2013

isopropylacrylamide), PNIPAM, was used as a macro chain transfer agent (macroCTA) to extend with a hydrophilic block, containing *N*,*N*-dimethylacrylamide (DMA) and 2,3,5,6-tetrafluorophenyl acrylate active ester (TFPA). The resulting block copolymers with active ester groups were used to conjugate with anti-p24 IgG antibody.

RAFT polymerization was chosen because of its functional group tolerance and suitability for synthesizing well-defined active ester[25] and acrylamido polymers.[30−34] Compared to PFPMA, TFPA can potentially achieve higher reaction efficiency with various amines due to relatively less steric hindrance of acrylates.[21] The other benefit of using TFPA over PFPMA includes the ease of polymer characterization via ^{1}H NMR spectroscopy due to the sole aromatic protons (C_5F_4H-) of poly(TFPA) at 7.0 ppm in the diblock copolymer (Figure 1A). Hydrophilic DMA was copolymerized with TFPA as the

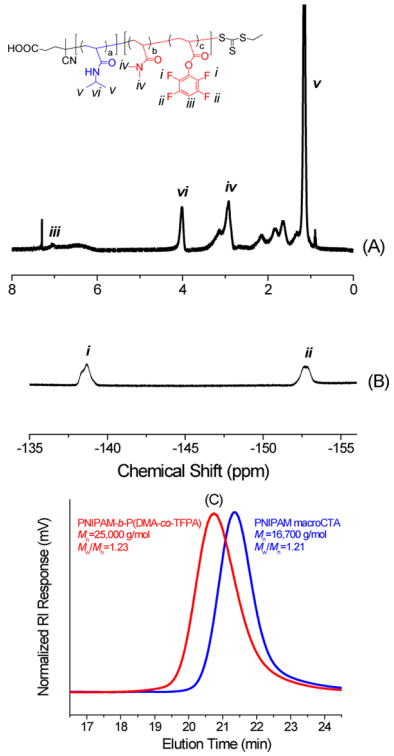
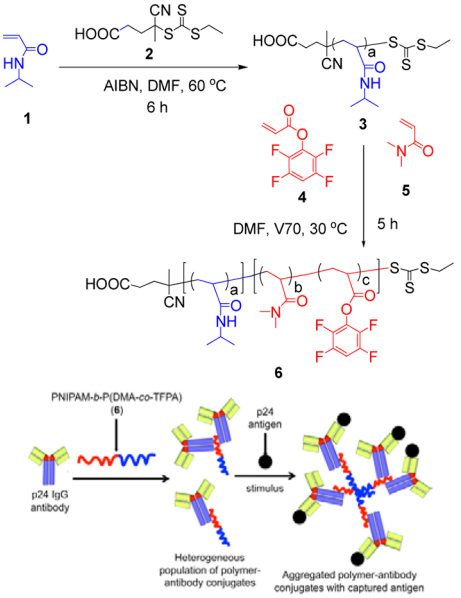


Figure 1. ^{1}H NMR (A), ^{19}F NMR (B) spectra, and SEC trace (C) of PNIPAM-*b*-P(DMA-*co*-TFPA) block copolymer.

second block to ensure aqueous solubility of the block copolymer. The temperature-responsive block, PNIPAM, does not participate in the conjugation, so this block can be engineered independently for the desired stimuli-responsiveness. The diblock copolymer construct can control the polymer orientation by keeping the amine-reactive hydrophilic block (P(DMA-*co*-TFPA)) closer to the protein with the temperature-responsive block away from the protein surface, which preserves the temperature-responsiveness of the resulting conjugates. Therefore, the subsequent polymer−antibody conjugates were temperature-responsive and retained biological activity, so they can potentially be utilized for diagnostics and other bioseparations applications.

The synthesis of TFPA was adopted from a synthetic route (Scheme 1, Supporting Information) by Theato et al.[21] Briefly, tetrafluorophenol was reacted with acryloyl chloride in the presence of an auxiliary base (2,6-luitidine). After removing the

Scheme 1. Synthesis of Tetrafluorophenyl Active Ester Diblock Copolymer via Reversible Addition−Fragmentation Chain Transfer (RAFT) Polymerization and Subsequent Antibody Conjugation and Thermal Precipitation of Captured Protein (p24) Antigens



precipitated 2,6-lutidine hydrochloride, the aqueous workup and distillation resulted in the colorless product (67% yield). The sole aromatic proton (C_5F_4H-) of the monomer appeared at 7 ppm in the ^{1}H NMR spectrum (Figure S1, Supporting Information). The ^{19}F NMR spectrum of the monomer showed the expected signals of the four fluorine atoms of the aromatic ring at −153.8 and −139.9 ppm with 1:1 integral ratio (Figure S2, Supporting Information). Additional experimental details and characterization results are described in the electronic Supporting Information.

The overall diblock copolymer synthesis is illustrated in Scheme 1. NIPAM (**1**) was polymerized with 4-cyano-4-(ethylsulfanylthiocarbonyl) (ECT; **2**) as the CTA and 2,2′-azobisisobutyronitrile (AIBN) as the initiator at 60 °C in DMF. The molar ratio of [monomer]/[CTA]/[initiator] was [200]/[1]/[0.05]. The size exclusion chromatography (SEC) trace of PNIPAM macroCTA is shown in Figure 1. About 65% conversion was obtained in 6 h, yielding polymer with $M_{n,SEC}$ = 16700 g/mol (M_w/M_n = 1.21), which was in good agreement with the theoretical M_n of 15000 g/mol (Table 1). To impart a hydrophilic segment with the bioconjugation sites (active ester

Table 1. Results from RAFT Block Copolymerization of DMA and TFPA Using PNIAPM as a MacroCTA

polymer[a]	[M]/[CTA]/[I][b]	conv.[c] (%)	$M_{n,theory}$[c] (g/mol)	M_n[d] (g/mol)	M_w/M_n[d]
1	[200]/[1]/[0.05]	65	14980	16700	1.21
2	[150]/[1]/[0.05]	30.7/57.5	22960	25000	1.23

[a]**1**: poly(*N*-isopropylacrylamide) macro chain transfer agent, polymerization time = 6 h ; **2**: poly(*N*-isopropylacrylamide)-*b*-poly(*N*,*N*-dimethylacrylamide-*co*-(2,3,5,6-tetrafluorophenyl acrylate), polymerization time = 5 h. [b]Molar ratio of monomer (M)/chain transfer agent (CTA)/initiator or (I). [c]Determined by ^{1}H NMR spectroscopy: NIPAM conversion = 65%, DMA conversion = 30.7%, TFPA conversion = 57.5%. [d]Determined by SEC.

dx.doi.org/10.1021/mz300620v | *ACS Macro Lett.* 2013, 2, 132−136

groups), a block copolymer was synthesized using PNIPAM macroCTA (**3**) with DMA (**5**) and TFPA (**4**). The polymerization condition was optimized to a shorter period of time at low temperature (30 °C) to avoid acrylate monomer side reactions.[35] $^{1}H/^{19}F$ NMR spectroscopy and SEC analyses of the resulting block copolymer (**6**) confirmed successful incorporation of the active esters and hydrophilic acrylamido groups. Compared to the PNIPAM macroCTA ($M_{n,SEC}$ = 16700 g/mol, M_w/M_n = 1.21), the molecular weight of the resulting block copolymer increased to $M_{n,SEC}$ = 25000 g/mol ($M_{n,theory}$ = 22960 g/mol, PDI = 1.23). The peak at 7.0 ppm in the ^{1}H NMR spectrum confirmed the presence of the sole aromatic protons (C_5F_4H-) of poly(tetrafluorophenyl acrylate) and the peak at 2.9 ppm is attributed to the methyl (CH_3-) protons of poly(*N*,*N*-dimethylacrylamide) (Figure 1A). For the second block the feed ratio of DMA/TFPA was 90:10. However, due to the relatively higher reactivity of acrylates compared to acrylamides, the final mol % of DMA and TFPA in the block copolymer were 80 and 20%, respectively, based on ^{1}H NMR of purified PNIPAM-*b*-P(DMA-*co*-TFPA). Further, the broad signals at the ^{19}F NMR spectrum of the block copolymer at −152.7 and −138.7 ppm with a 1:1 integral ratio also confirmed the successful incorporation of active ester groups in the diblock copolymer (Figure 1B). The polymeric fluoro peaks were slightly shifted from the monomer peaks (Figure S2, Supporting Information) and no monomeric peaks were observed. Relatively good blocking efficiency was observed by SEC (Figure 1C) along with unimodal and narrow molecular weight distributions (Table 1).

The protein conjugation capability of the active ester diblock copolymer was investigated using anti-p24 IgG antibody as a model protein because biologically active polymer−antibody conjugates are relevant for various biomedical applications. For example, p24 is protein biomarker for diagnosing HIV infection.[36,37] More sensitive p24 immunoassays are needed for earlier diagnosis of HIV infection. So, stimuli-responsive polymer-anti-p24 IgG conjugates may be clinically relevant reagents in future HIV and other diagnostic assays.

The polymer−antibody conjugation is illustrated in Scheme 1. We used a "grafting-to" approach in which an active ester diblock copolymer stock solution (in anhydrous DMSO) was injected into the anti-p24 IgG solution (sodium bicarbonate buffer, pH 10.8) and mixed at 25 °C for 2 h. The diblock copolymer was completely soluble in the aqueous buffer at all concentrations investigated (up to ∼45 mg/mL). At the conjugation pH of 10.8, TFPA ester hydrolysis was a concern. However, rapid (2 h) conjugations were achieved at pH 10.8 and 25 °C. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) was utilized to confirm conjugation before removing unreacted antibodies from the reaction solutions by thermal precipitation (Figure 2). The polymer did not stain in these gels or interfere with analyses (lane B). Polymer/antibody molar ratios from 11:1 to 180:1 (lanes H−D) were investigated. As the molar ratio increased, IgG bands shifted to higher molecular weights and exhibited a smeared migration pattern, which indicated successful polymer conjugation to anti-p24 IgG. Based on the disappearance of free IgG bands, the conjugation efficiency reached 100% between polymer:antibody molar ratios of 90:1 and 180:1 (lanes D and E). The conjugation efficiency reached 100% at lower polymer/antibody ratios (∼50−75:1) by optimizing the reaction time, pH, and temperature (data not shown). Conjugation efficiencies will likely improve further as the second amine-

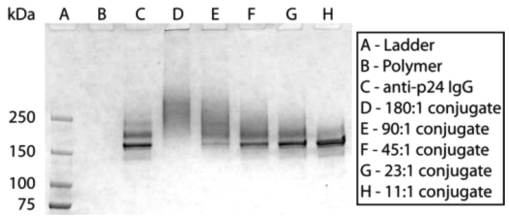


Figure 2. SDS-PAGE analysis of diblock copolymer (lane B), native anti-p24 IgG (lane C), and polymer−antibody conjugates at the following molar ratios: 180:1 (lane D), 90:1 (lane E), 45:1 (lane F), 23:1 (lane G), and 11:1 (lane H).

reactive block and conjugation conditions are optimized in future studies. In other work with fluorophenyl ester-containing polymers, conjugations with small molecule amines achieved quantitative yields at much lower molar ratios.[21,24] However, these conjugations were performed at 50 °C, which is unacceptable for most biomolecules. Additionally, lower conjugation efficiencies with antibody were expected due to steric hindrance. During the conjugation reaction, accessible amines on the IgG molecules (i.e., lysine residues, terminal $-NH_2$) were deprotonated at pH 10.8, and they randomly reacted with one or more pendant TFPA ester groups of the polymer backbone to form covalent amide bonds. With this polymer design, conjugation reactions (Scheme 1) could yield one or more polymer chains attached to a single antibody as well as antibody−polymer−antibody conjugates (a single polymer chain conjugated to more than one antibody).[2] Thus, the population of conjugates would be heterogeneous, creating the smeared migration pattern observed in SDS-PAGE, which is similar to other results with both grafting-to and grafting-from approaches.[8,9,12,14,38] The diblock copolymer developed with pendant amine-reactive TFPA esters is a new reagent for efficient protein conjugations.

Cloud point analysis was used to characterize the temperature-responsiveness (lower critical solution temperature, LCST) of the polymers and polymer−antibody conjugates (Figure 3). The PNIPAM macroCTA exhibited a sharp LCST

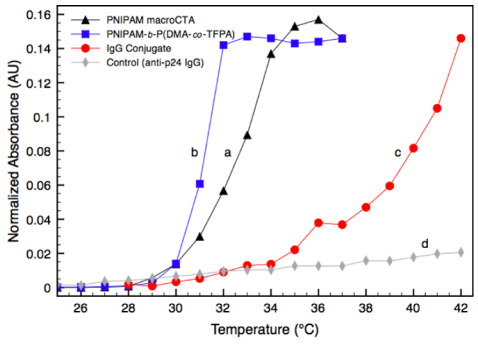


Figure 3. LSCT measurements of (a) PNIPAM macroCTA, (b) PNIPAM-*b*-P(DMA-*co*-TFPA), (c) IgG conjugate, and (d) control antibody (anti-p24 IgG).

at 30−32 °C (curve a), as expected. The LCST of the PNIPAM-*b*-P(DMA-*co*-TFPA) diblock copolymer (curve b) was nearly identical to the PNIPAM macroCTA, although the transition was more sharp. The second block contained both hydrophobic (TFPA) and hydrophilic (DMA) monomers, but the TFPA incorporation was insufficient (20 mol % by ^{1}H NMR) to significantly increase the polymer hydrophobicity (or

dx.doi.org/10.1021/mz300620v | *ACS Macro Lett.* 2013, 2, 132−136

decrease the LCST). The native anti-p24 IgG antibody was included as a control, and it showed no thermoresponsive behavior (Figure 3, curve d). The polymer−antibody conjugates (IgG conjugates, Figure 3, curve c) exhibited a broad transition from about 34−40 °C. Therefore, we conclude that the thermoresponsive properties of the diblock copolymer were conferred to the polymer-antibody conjugates. However, the bulky, hydrophilic antibody attenuated the sharp temperature-responsiveness of the polymer and shifted the LCST of the polymer−antibody conjugates to a higher temperature. This observation is consistent with some reports of thermoresponsive protein conjugates[2,6,12,39] but not others.[11,38,40] Presumably, steric hindrance plays a greater role in inhibiting coaggregation between polymer−antibody conjugates than conjugates of smaller proteins, which could explain our observations. The effect of protein conjugation on the thermoresponsive properties of PNIPAM is likely a complex interplay between the number of grafted polymer chains, the molecular weight of the polymer and protein, and potentially the type of conjugation chemistry,[2,39] and future polymer designs will be explored to sharpen the temperature transition. Importantly, the stimuli-responsive behavior of PNIPAM was conferred to the polymer-antibody conjugates. Above the LCST, the aggregated polymer-antibody conjugates were captured via centrifugation (Figure S3, Supporting Information), which further demonstrated the thermoresponsive behavior of these conjugates. This proof-of-concept experiment will inform future immunoassay work in isolating protein antigens with stimuli-responsive polymer−antibody conjugates.

In addition to the temperature-responsiveness, we also characterized the binding capability of the polymer−antibody conjugate against p24 protein (antigen). This property is crucial for the intended bioseparations applications because polymer conjugation to proteins can affect protein activity via a number of mechanisms.[2] The antigen binding efficiency of the polymer-anti-p24 IgG antibody conjugates was compared to native anti-p24 IgG by competitive enzyme-linked immunosorbent assays (ELISAs, Figure 4). In these experiments, p24 antigen was

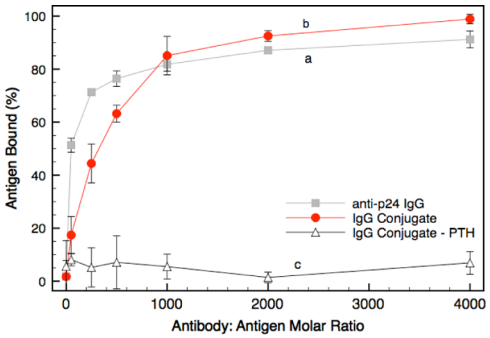


Figure 4. Competitive ELISA binding curves demonstrated polymer−antibody conjugate binding activity and specificity (a) anti-p24 IgG, (b) IgG conjugate, and (c) IgG conjugate binding to an alternative antigen (PTH).

mixed with polymer−antibody conjugates or native antibody at defined antibody/antigen molar ratios, and the free (unbound) p24 was quantified by ELISA. As the antibody/antigen molar ratio was increased, both the anti-p24 IgG antibody (Figure 4, curve a) and polymer−antibody conjugate (Figure 4, curve b) bound more p24. The native antibody exhibited a plateau with maximum p24 binding at 91 ± 3.2%. The polymer−antibody conjugates showed a slightly higher maximum p24 binding efficiency at 99 ± 1.8% and a slightly lower binding affinity or K_D. This small difference in binding behavior could be attributed to enhanced bioactivity due to modification of the protein microenvironment after polymer conjugation.[2] Also, an error is likely in the estimated antibody content of polymer−antibody conjugate solutions (calculated by the ratio of protein and polymer absorbance values with UV−vis spectroscopy) because the conjugate population is heterogeneous. For example, if multiple polymer chains are conjugated to a single antibody, the high polymer absorbance might result in underestimating the actual antibody content of the conjugate solution. The estimation error influences the molar ratio in the antigen binding experiments, and it could explain the slight variation between the conjugate and native antibody. Because the trend of the polymer−antibody conjugate binding data closely matched that of the native antibody, further examination of the K_D and maximum binding differences was not performed for this study. To confirm the specificity of polymer−antibody conjugates for p24 antigen, the binding efficiency of these conjugates for a different antigen, parathyroid hormone (PTH), was investigated. Across all molar ratios, nonspecific binding of PTH by polymer−antibody conjugates was negligible (5.7 ± 2.2%, Figure 4, curve c). Therefore, we can conclude the polymer−antibody conjugates are biologically active and retain binding specificity for the target antigen.

To our knowledge, this is the first report of RAFT polymerization of TFPA monomer. The stimuli-responsive PNIPAM diblock copolymer with multiple tetrafluorophenyl-containing active ester groups is a new polymer for efficient protein conjugation with control of polymer orientation on the conjugate. This new reactive polymer was used to generate thermoresponsive polymer−antibody conjugates that retained antigen binding efficiency and specificity. These stimuli-responsive polymer−antibody conjugates are valuable reagents for future immunoassays because they rapidly bind antigens with solution phase kinetics, unlike antibodies that are constrained to solid supports (i.e., microtiter plate surfaces, microparticles). After antigen binding, temperature stimuli, about 40 °C, could enable separation of aggregated polymer−antibody conjugates via centrifugation, filtration or other methods for downstream analysis. This system is modular; other molecular recognition moieties (i.e., biotin, nucleic acids, receptors) may be conjugated to the PNIPAM-*b*-P(DMA-*co*-TFPA) for the development of additional bioseparations and sample enrichment applications.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and additional figures including ^{19}F and ^{1}H NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): The polymers described in this manuscript are being utilized by Nexgenia Inc. that has licensed technology from U.W. P.S.S. and J.J.L. are co-founders of this company and have a significant financial interest in the company.

■ ACKNOWLEDGMENTS

The authors acknowledge financial support from the Life Sciences Discovery Fund, the Coulter Foundation, and the University of Washington Center for Commercialization (C4C).

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■ NOTE ADDED AFTER ASAP PUBLICATION

This letter posted asap on January 17, 2013. Scheme 1 and Figure 1 have been revised. The correct version posted on January 24, 2013.