

Published in final edited form as:

Chem Commun (Camb). 2013 March 28; 49(25): 2566–2568. doi:10.1039/c3cc38976f.

Site-Specific Conjugation of RAFT Polymers to Proteins via Expressed Protein Ligation

Yan Xia^{a,b}, Shengchang Tang^a, and Bradley D. Olsen^a

Bradley D. Olsen: bdolsen@mit.edu

^aDepartment of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA, USA. Tel: 617-715-4548

Abstract

Site-specific protein conjugates with RAFT polymers were synthesized using expressed protein ligation. Stable micelles were formed from both linear block copolymer and Y-shaped conjugates.

Protein-polymer conjugates are of increasing interest for pharmaceutical and biotechnological applications, due to their improved pharmacokinetics and decreased immunogenicity as compared to the unmodified proteins.^{1–3} Many of these applications require homogeneous, site-specific conjugates for uniform, consistent performance and biological activity.⁴ The majority of reported protein-polymer conjugation reactions rely on the attachment to lysine or cysteine residues on the protein surface.^{1–3,5} The presence of multiple lysines on the protein surface often leads to heterogeneity in both the position and the number of conjugation sites when lysine is targeted as a reactive site. Cysteine is less abundant in the proteome, but cysteine-based conjugation can be complicated when disulfide bonds are structurally important for the protein secondary or tertiary structures. Numerous alternative methods have been developed to allow site-specific conjugation to proteins.^{6–8} Among them, intein-mediated ligation, or expressed protein ligation (EPL),⁹ takes advantage of self-splicing intein and chemo- and regio-selective native chemical ligation (NCL) between a *N*-terminal cysteine and a *C*-terminal thioester.^{10,11} EPL has been used to conjugate small molecules,^{12–14} nucleic acids,¹⁵ peptides,⁹ and dendrimers.¹⁶ The Chilkoti group recently reported conjugation of an atom transfer radical polymerization (ATRP) initiator onto protein using EPL and subsequent ATRP from the protein initiator to form protein-polymer conjugate.¹⁷ Sumerlin and coworkers first demonstrated protein-polymer conjugates using reversible fragmentation chain transfer (RAFT) polymerization *via* grafting-from.¹⁸ However, to the best of our knowledge, EPL has not been used to directly conjugate proteins to pre-formed, narrowly dispersed polymers synthesized by controlled radical polymerization. Considering the large variety of polymers that can be synthesized using controlled radical polymerization, the extension of EPL to these polymers will enrich the diversity of well-defined protein-polymer conjugates.

Herein, we report the first examples of direct EPL to a range of polymers prepared using RAFT polymerization. Furthermore, we demonstrate the ability to perform orthogonal conjugation reactions at the same site, enabling the synthesis of different bioconjugate architectures that form stable micelles in solution.

RAFT polymerization is a versatile living polymerization technique for preparing functional polymers with pre-defined MW and narrow PDI.¹⁹ However, most RAFT agents are composed of trithiocarbonates or dithioesters, which we observed to react with cysteine rapidly under NCL conditions to form cyclic dithiocarbamate or thioamide, respectively (Figure S1, ESI[†]). Therefore, for conjugation to the C-terminus of a protein, it is important to prepare RAFT agents with protected cysteine and remove the RAFT agent end group after RAFT polymerization to avoid interference and consumption of the cysteine end group before NCL can be attempted.

We synthesized trithiocarbonate and dithioester RAFT agents with protected cysteine groups, and polymerizations using these RAFT agents proceeded smoothly to yield poly(dimethylacrylamide) (PDMA), poly(*N*-isopropylacrylamide) (PNIPAM), and poly(polyethylene-glycol methacrylate) (PPEGMA) with molar mass of 6–21 kDa and PDI < 1.1 (Table 1). The RAFT agent and cysteine end groups were then sequentially removed and deprotected (see experimental detail in ESI[†]).

A model protein, green fluorescent protein (GFP), was cloned into the expression vector with the intein-maltose binding domain fused to the C-terminus of GFP to produce a gene encoding a protein suitable for EPL conjugation. The C-terminal residue on the protein of interest is known to have a significant effect on the intein cleavage, and glycine has been reported to maximize the cleavage efficiency.¹³ Therefore, two glycine residues were encoded between the C-terminus of GFP and the intein. A His₆ tag was also coded at the N-terminus of the GFP to allow purification of the protein-polymer conjugate by metal affinity chromatography. The GFP-intein fusion protein was expressed in *Escherichia coli*, and the cell lysate was loaded onto chitin resin mercapto-ethanesulfonate (MESNa) for >12h at 4 °C, GFP was cleaved off the chitin resin with a sulfoethyl thioester group at the C-terminus (Figure S2, ESI[†]).

We first used a PDMA (entry 1 in Table 1) polymer to investigate the conditions and efficiency of polymer conjugation to GFP-thioester. Conjugation conversions reached 50–60% after 6 h at all temperatures investigated, namely 4 °C, room temperature, and 37 °C, and stayed constant afterwards, although the reaction proceeded faster as temperature was increased (Figure S3 (a)–(c), ESI[†]). Increasing the equivalence of polymer from 10 fold to 20 fold or adding additional reducing agent, such as tris(2-carboxyethyl) phosphine (TCEP) did not increase the conversion (Figure S3 (d) and (e), ESI[†]). Therefore, all the subsequent conjugation reactions were performed using 10 fold excess of polymer at room temperature for 12 h without additional reducing agent. Using these conditions, all RAFT polymers were successfully conjugated to GFP at moderate conversions (Fig 1). The lower conversion of

[†]Electronic Supplementary Information (ESI) available: experimental details, NMR, GPC, LC-MS, CD, UV-Vis spectra and additional SDS-PAGE. See DOI: 10.1039/b000000x/

PPEGMA is likely due to the increased steric hindrance at the cysteine chain end as compared to other linear polymers.

Since NCL retains the cysteine residue at the linkage, we next sought to take advantage of this cysteine for subsequent conjugation to the same site on protein to form a Y-shaped protein-polymer conjugates. This strategy provides a complementary strategy to recently reported dibromomaleimide coupling^{20,21} to conjugate proteins to two different functionalities, in the present case placing proteins at the interface between two polymer blocks (Scheme 1). The site of protein to bind the fusion protein. After incubation with 50 mM sodium attachment may have an impact on the stability or activity of proteins.

To enable two subsequent site-specific conjugations at the same site, a GFP mutant was prepared with no accessible surface cysteines, which was confirmed by the absence of conjugation in the presence of excess maleimide-terminated PEG ($M_n = 5$ kDa) and TCEP. To avoid interference with the thiol-maleimide coupling at the newly formed cysteine, the RAFT agent at the polymer chain end was removed via desulfurization by radical-induced reduction.²² Complete removal of RAFT agent was evidenced by the disappearance of the absorption peak of RAFT agent at 310 nm. Cysteine-functionalized PDMA was then conjugated to GFP under our standard NCL conditions with a 60% yield. The reaction mixture was purified to remove excess PDMA, and no attempt was made to isolate the GFP-PDMA from unreacted GFP. The product from the first ligation reaction was then subjected to 10 eq. PNIPAM ($M_n = 7$ kDa) with a maleimide end group. The band corresponding to GFP-PDMA has completely moved to higher MW, as shown by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), indicating successful second conjugation (Fig 2). This resulted in a Y-shaped conjugate, GFP-Y-PDMA-PNIPAM, and the final conjugate was purified by anion exchange chromatography. We also synthesized a PDMA-*b*-PNIPAM with similar MWs in each block (7 kDa each) as compared to the PDMA and PNIPAM used in Y-shaped conjugates. This linear BCP conjugate was simply purified by ammonium sulfate precipitation to remove unconjugated GFP (Fig 2). Circular dichroism (CD) and UV-vis spectra of both types of GFP-polymer conjugates showed minimal change from the spectra recorded with the unconjugated GFP (Figure S9 and S10, ESI[†]), indicating the native protein conformation is reserved by the conjugation procedures.

Thermo-responsive micellization of these protein-polymer conjugates was observed regardless of the molecular architecture, as studied using dynamic light scattering (DLS). At 25 °C, both conjugates existed in the monomer form with R_h of 5.5 and 3.5 nm for the linear and Y-shaped BCP conjugates, respectively; the lower R_h for the Y-shaped conjugate is consistent with the more compact molecular configuration of the branched polymer. Upon heating to 50 °C, above the lower critical solution temperature (LCST) of PNIPAM, micelles were rapidly formed in both cases: the linear conjugate had a R_h of 15.3 nm and the Y-shaped conjugate had a R_h of 12.7 nm. Micellization was completely reversible. Upon cooling back to 25 °C, the conjugate monomer form was restored within minutes. Transmission electron microscopy (TEM) of dehydrated micelles adsorbed to Formvar-coated grids and negatively stained revealed the formation of spherical micelles for both bioconjugate architectures.

Conclusions

We demonstrated the successful, site-specific conjugation of RAFT homopolymers and block copolymers to a model protein, GFP, via EPL. Sequential EPL and thiol-maleimide ligation allowed the synthesis of Y-shaped conjugate with two different polymers attached at the same site on protein. Both linear and Y-shaped GFP-PDMA-PNIPAM BCP conjugates formed stable, thermo-responsive micelles. This demonstrates that the general EPL/RAFT bioconjugation strategy can be applied to prepare various protein-polymer conjugate materials for encapsulated enzymes and protein therapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank DTRA for funding under project BA12PHM159, the Biophysical Instrumentation Facility for the Study of Complex Macromolecular Systems and the Center for Materials Science and Engineering (NSF-0070319 and NIH GM68762) for the use of instruments. We thank Prof. Hadley Sikes for the use of FPLC, Carla Thomas for assistance with TEM, and Christopher Lam for providing the GFP gene.

Notes and references

1. Hoffman AS, Stayton PS. *Progress in Polymer Science*. 2007; 32:922.
2. Gauthier MA, Klok H-A. *Chemical Communications*. 2008:2591. [PubMed: 18535687]
3. Broyer RM, Grover GN, Maynard HD. *Chemical Communications*. 2011; 47:2212. [PubMed: 21229146]
4. Kochendoerfer GG. *Current Opinion in Chemical Biology*. 2005; 9:555. [PubMed: 16233987]
5. Jones MW, Mantovani G, Ryan SM, Wang X, Brayden DJ, Haddleton DM. *Chemical Communications*. 2009:5272. [PubMed: 19707643]
6. Antos JM, Francis MB. *Current Opinion in Chemical Biology*. 2006; 10:253. [PubMed: 16698310]
7. de Graaf AJ, Kooijman M, Hennink WE, Mastrobattista E. *Bioconjugate Chemistry*. 2009; 20:1281. [PubMed: 19191567]
8. Sletten EM, Bertozzi CR. *Angewandte Chemie International Edition*. 2009; 48:6974.
9. Muir TW, Sondhi D, Cole PA. *Proceedings of the National Academy of Sciences*. 1998; 95:6705.
10. Dawson P, Muir T, Clark-Lewis I, Kent S. *Science*. 1994; 266:776. [PubMed: 7973629]
11. Hackeng TM, Griffin JH, Dawson PE. *Proceedings of the National Academy of Sciences*. 1999; 96:10068.
12. Tolbert TJ, Wong CH. *Journal of the American Chemical Society*. 2000; 122:5421.
13. Lue RYP, Chen GYJ, Hu Y, Zhu Q, Yao SQ. *Journal of the American Chemical Society*. 2004; 126:1055. [PubMed: 14746473]
14. Wood RJ, Pascoe DD, Brown ZK, Medlicott EM, Kriek M, Neylon C, Roach PL. *Bioconjugate Chemistry*. 2004; 15:366. [PubMed: 15025533]
15. Lovrinovic M, Fruk L, Schroder H, Niemeyer CM. *Chemical Communications*. 2007:353. [PubMed: 17220968]
16. van Baal I, Malda H, Synowsky SA, van Dongen JLJ, Hackeng TM, Merckx M, Meijer EW. *Angewandte Chemie International Edition*. 2005; 44:5052.
17. Gao W, Liu W, Christensen T, Zalutsky MR, Chilkoti A. *Proceedings of the National Academy of Sciences*. 2010; 107:16432.
18. De P, Li M, Gondi SR, Sumerlin BS. *Journal of the American Chemical Society*. 2008; 130:11288. [PubMed: 18665597]

19. Moad G, Rizzardo E, Thang SH. *Accounts of Chemical Research*. 2008; 41:1133. [PubMed: 18700787]
20. Smith MEB, Schumacher FF, Ryan CP, Tedaldi LM, Papaioannou D, Waksman G, Caddick S, Baker JR. *Journal of the American Chemical Society*. 2010; 132:1960. [PubMed: 20092331]
21. Jones MW, Strickland RA, Schumacher FF, Caddick S, Baker JR, Gibson MI, Haddleton DM. *Journal of the American Chemical Society*. 2011; 134:1847. [PubMed: 22188166]
22. Chong YK, Moad G, Rizzardo E, Thang SH. *Macromolecules*. 2007; 40:4446.

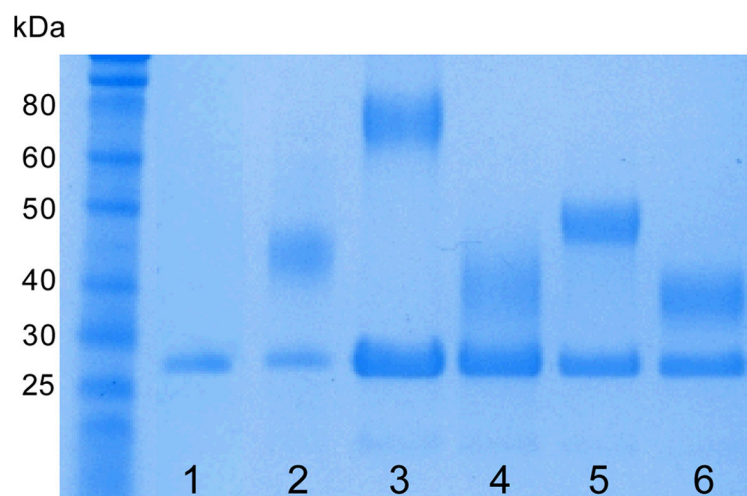


Fig 1. SDS-PAGE of (1) GFP-thioester after chitin column purification; crude conjugates of GFP with (2) PDMA (entry 1 in Table 1), (3) PDMA (entry 2 in Table 1), (4) PPEGMA (entry 3 in Table 1), (5) PDMA-*b*-PNIPAM (entry 4 in Table 1), (6) PDMA (entry 5 in Table 1).

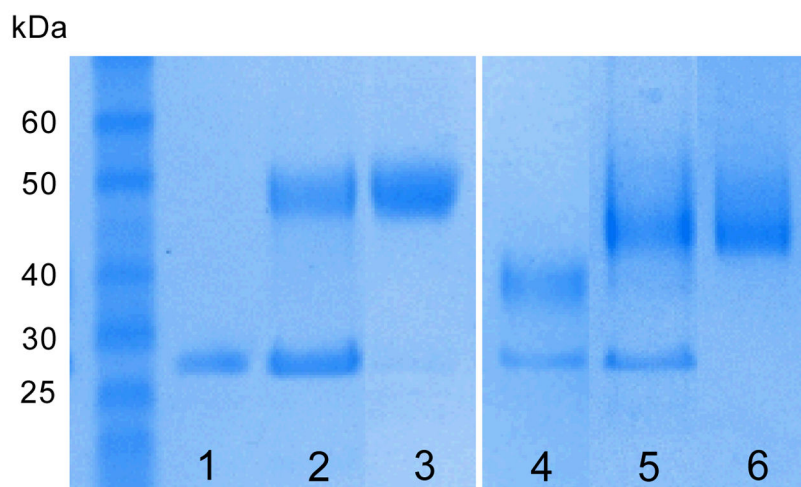


Fig 2. SDS-PAGE of the conjugation reactions and purification of the conjugates. (1) GFP-thioester after intein cleavage; (2) crude conjugate of GFP with PDMA-*b*-PNIPAM (entry 4 in Table 1); (3) purified conjugate of GFP with PDMA-*b*-PNIPAM by ammonium sulfate precipitation; (4) crude conjugate of GFP with PDMA (entry 5 in Table 1); (5) crude Y-shaped conjugate of GFP-PDMA and PNIPAM; (6) purified Y-shaped conjugate of GFP-Y-PDMA-PNIPAM by anion exchange chromatography.

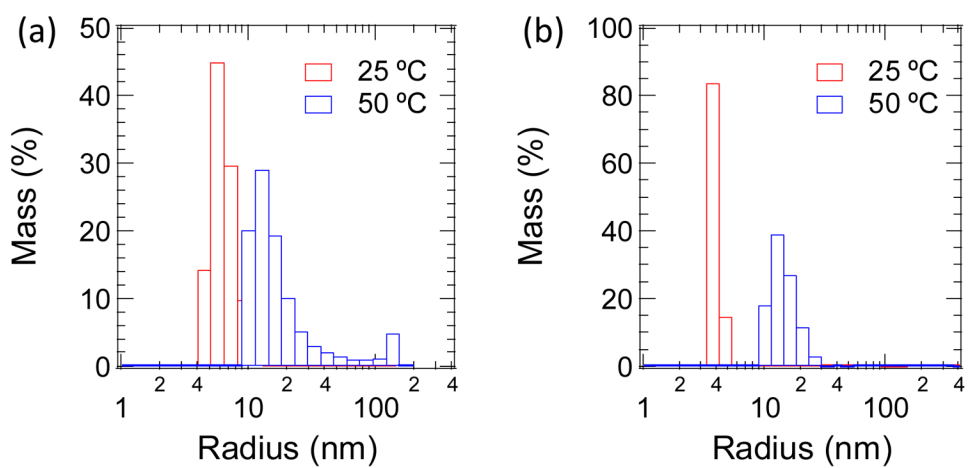


Fig. 3. DLS of (a) linear and (b) Y-shaped BCP conjugates showing reversible temperature dependent micellization.

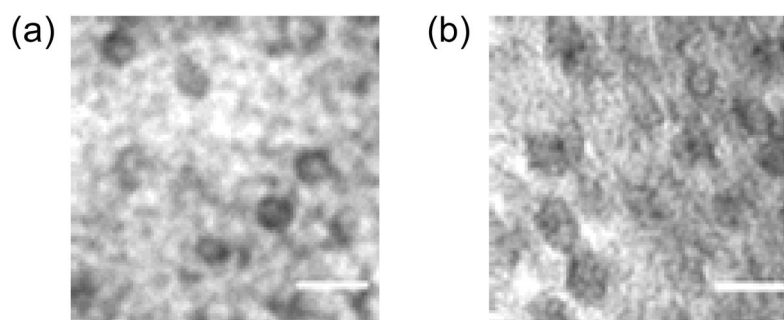
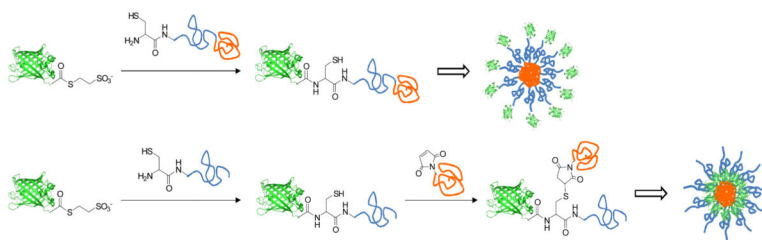


Fig. 4. TEM of the micelles resulted from (a) linear and (b) Y-shaped BCP conjugates at 50 °C. The scale bar corresponds to 50 nm.

**Scheme 1.**

Synthesis of linear (top) and Y-shaped (bottom) GFP-block copolymer conjugates and their proposed micellar assembly with different protein location in respective micelles.

Table 1

Characteristics and conjugation of RAFT polymers to GFP

polymer	M_n^a (kDa)	PDI ^a	Conjugation yield ^b
PDMA	6.7	1.02	62%
PDMA	21.0	1.06	52%
PPEGMA	9.2	1.04	29%
PDMA- <i>b</i> -PNIPAM	14.8	1.08	57%
PDMA ^c	6.4	1.04	60%

^aDetermined by GPC in DMF (0.02M LiBr) using refractive index (RI) and MALLS detectors.

^bCalculated using densitometry from SDS-PAGE analysis.

^cPDMA with a hydride end group.