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Genetically Encoded Initiator for Polymer Growth from Proteins

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Abstract: Despite the importance of protein−polymer bioconjugates, there is no general method for producing homogeneous recombinant protein that contains polymer initiators at defined sites. To address this deficiency, we designed the amino acid 4-(2′-bromoisobutyramido)phenylalanine (**1**) as an initiator in atom-transfer radical polymerization (ATRP) that would provide a stable linkage between the protein and growing polymer. We synthesized **1** and evolved a *Methanococcus jannaschii* tyrosyl-tRNA synthetase/$tRNA_{CUA}$ pair to genetically encode this initiator in response to an amber codon. To demonstrate the utility of this initiator, we produced green fluorescent protein (GFP) with **1** site-specifically incorporated on its surface (GFP−**1**). Purified GFP−**1** was then used as an initiator under standard ATRP conditions with a monomer, oligo(ethylene oxide) monomethyl ether methacrylate, efficiently producing a polymer−GFP bioconjugate where the polymer is connected at our selected site on GFP.

The wide functional diversity of proteins — catalysis, regulation, transport, and structure — has made them desirable for integration into materials and medicine. Protein−polymer conjugates have already shown an impressive range of altered or improved properties.[1] To date, protein polymers have been prepared in two general ways: either by graft-to methods where the functionalized polymer is attached to an amino acid, cofactor, or end group, or by the graft-from method where a location(s) on the purified protein is functionalized with an initiator and then the polymer is grown from that site.[1d,2] Thus far, the graft-from methods employed for residue-specific incorporation of polymerization initiators into proteins are limited to the N-terminal position or specific natural amino-acid-directed linkages.[1b,c,3] Both methods suffer from challenging purification of intermediates and/or the inability to efficiently control the number or location of polymer connections, which compromises protein structural integrity. While the many graft-to and graft-from experiments using natural amino acids on proteins have illustrated the immense potential impact of well-defined protein−polymer conjugates, their application is limited by technical shortcomings.

Despite the importance of protein−polymers, there is no general method for producing homogeneous recombinant proteins that contain polymer initiators at defined sites.[4] To address this deficiency, we designed the amino acid 4-(2′-bromoisobutyramido)-phenylalanine (**1**, Figure 1A), since it should function as an initiator in atom-transfer radical polymerization (ATRP)[5] and would provide a stable linkage between the protein and growing polymer. We synthesized **1** and evolved a *Methanococcus jannaschii* (*Mj*) tyrosyl-tRNA synthetase (RS)/$tRNA_{CUA}$ pair to genetically encode this initiator in response to an amber codon.[6] To demonstrate the utility of this initiator, we produced green fluorescent protein (GFP) with **1** site-specifically incorporated on its surface. Purified GFP−**1** was then used as an initiator under standard ATRP conditions with a monomer, oligo(ethylene oxide) monomethyl ether methacrylate ($OEO_{300}MA$), efficiently producing a polyMPEG−GFP bioconjugate where the polymer is connected at the selected site on GFP.

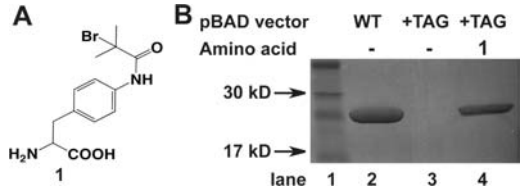

Figure 1. Genetic incorporation of ATRP initiator into proteins. (A) Initiator 4-(2′-bromoisobutyramido)phenylalanine (**1**). The synthetic route need not be asymmetric, since the *Mj*RS utilizes only the L form. (B) The evolved *Mj*RS/$tRNA_{CUA}$ pair in *pDule-BIBAF* allows for site-specific incorporation of **1** in response to an amber codon. Lane 2 shows expression levels of GFP-wt from pBad-GFP-His_6. Production of GFP−**1** from pBad-GFP-134TAG-His_6 is dependent on **1** in the growth media: lane 3 without **1** present, lane 4 with 1 mM **1** present. Protein was purified by Co^{2+} affinity chromatography, separated by SDS−PAGE, and stained with Coomassie.

It is important to synthesize **1** in large quantities since relatively large quantities of initiator-containing protein are needed for polymerization experiments. The initiator **1** was synthesized in two steps in 63% yield from commercially available material (see Supporting Information for details).

To evolve the orthogonal *Mj*TyrRS/$tRNA_{CUA}$ pair capable of incorporating **1** in response to an amber codon, we used a library of the synthetase (RS) gene that was randomized for the codons corresponding to six active-site residues (Y32, L65, F108, Q109, D158, I159) within 7 Å of the bound tyrosine.[6] We performed two rounds of alternating positive and negative selection on this library (see Supporting Information for details). The clones that survived the selection were transformed into cells with a plasmid containing a GFP gene interrupted with an amber codon.[7] A total of 92 colonies were assessed for UAA-dependent expression of GFP. The eight top performing clones showed greater than 400 mg/L of GFP−**1** expression in the presence of **1** and no detectable GFP fluorescence over background in the absence of **1** (Supporting Information Figure 2). Sequencing these eight clones revealed six different RS sequences (Supporting Information Table 2).

For further characterization of the incorporation of **1** into proteins in response to the amber codon, the most active RS was cloned into a *pDule* vector that contains one copy of *Mj* $tRNA_{CUA}$ to create *pDule-BIBAF*.[7,8] Expression of GFP gene interrupted by an amber codon at site 134 in the presence of *pDule-BIBAF* was efficient and dependent on the presence of **1** (Figure 1b). Using 1 mM **1**, 0.42 g of GFP−**1** was purified per liter of medium, while wild-

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type GFP (GFP-wt) yielded 1.27 g/L under similar conditions (no GFP is produced in the absence of **1**). To further demonstrate that **1** can be incorporated into recombinant proteins using *pDule-BIBAF*, we compared the masses of GFP−**1** to GFP-wt using electrospray ionization−quadrupole time-of-flight mass analysis to verify that only a single **1** is incorporated in GFP (Supporting Information Figure 4). The site of **1** incorporation was confirmed by analysis of the tandem mass spectrometry (MS/MS) fragmentation series of the relevant tryptic peptide (Supporting Information Figures 5 and 6). Overall, the results of protein expression with affinity purification, sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), and MS analysis demonstrate the high fidelity and efficient incorporation of **1** at a genetically programmed site in GFP using *pDule-BIBAF*.

ATRP's tolerance of mild temperatures and aqueous conditions has been established for a variety of monomers on many biomolecules. The use of **1** as an initiator has never been reported. Since polyMPEG−protein bioconjugates have shown efficient pharmacokinetics and therapeutic potency,[1c−f,3b] the monomer $OEO_{300}MA$ was selected to demonstrate that the genetically incorporated initiator **1** can be used in ATRP. The GFP−**1** initiator and $OEO_{300}MA$ monomer were degassed with nitrogen, and the reaction was initiated by adding a degassed stock solution of 2,2′-bipyridine and Cu^{+}/Cu^{2+} (see Supporting Information for details). ATRP performed in degassed phosphate-buffered saline (PBS) at 24 °C was quenched at different time points to assess polymer growth by SDS−PAGE and size exclusion chromatography (SEC) (Figure 2A,B). As expected, polymer growth from GFP−**1** was evidenced by shifts to higher molecular weight (MW) with increasing ATRP reaction time, whereas no change was evident on GFP-wt under ATRP conditions. While the radical process of ATRP can have early stage termination leading to residual unreacted GFP−**1**, characterization by SEC showed that 93% of the GFP−**1** initiator was incorporated into high-MW polymers at 180 min (Figure 2A, lane 7; Figure 2C, green trace). Since it is very stable, the amide linkage connecting the polymer to GFP prevents separation via hydrolysis for analysis. To verify that the polymers grown from the surface of GFP−**1** did not significantly affect the structure of the protein, we compared fluorescence/protein concentration ratios, since GFP fluorescence intensity correlates with the structural integrity of the protein (Supporting Information Table 3).[9] To further characterize the polyMPEG−GFP bioconjugate, the 180 min reaction was fractionated by SEC, yielding purified protein−polymers of different sizes. The fractionated soluble fluorescent polyMPEG−GFP samples exhibited the expected MW increase when characterized by SDS−PAGE (Supporting Information Figures 7 and 8).

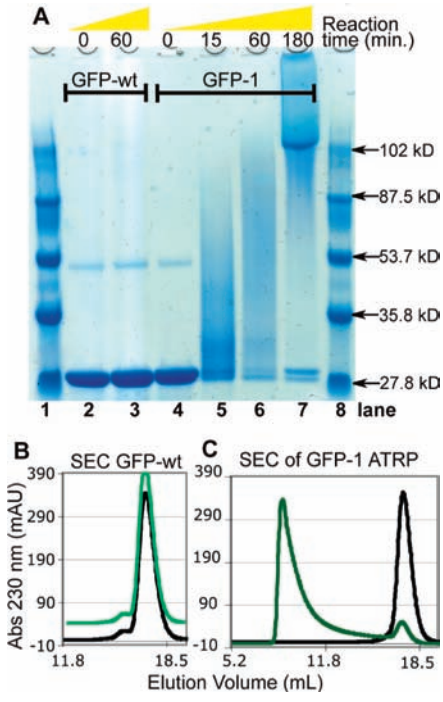

Figure 2. Characterization of ATRP grafting from GFP-wt and GFP−**1** with $OEO_{300}MA$ monomer in PBS at 24 °C. (A) SDS−PAGE of crude time points (5 μg of protein was loaded on each lane of a 4−12% gel). The reaction produced no size change for GFP-wt (lanes 2 and 3), while the majority of GFP−**1** showed significant size increases with increasing ATRP reaction time (lanes 4−7). (B,C) SEC of 0.1 mg of desalted reaction time points on Superdex 200 at a flow rate of 0.8 mL/min of PBS buffer monitored at 230 nm: (B) GFP-wt eluted at the expected volume of 17.3 mL (black) and was unaltered by the ATRP reaction (offset green); (C) GFP−**1** ATRP reaction showed the protein significantly increasing in size (black, time = 0; green, time = 180 min).

In conclusion, we report a general method for the quantitative, site-specific incorporation of a polymer initiator, **1**, into recombinant proteins. While other reports have shown the ability to control and manipulate polymer growth from proteins,[1c,3b,5c] this method overcomes the technical challenges of attaching an initiator to the protein of interest prior to polymerization and provides facile access to a diversity of sites on proteins. GFP-wt was unable to grow polymers, but the single addition of **1** at site 134 allowed for efficient polymer growth. We have demonstrated the utility of this new initiator in a protein by growing polymers of $OEO_{300}MA$ from GFP−**1** and showing that attached polymer does not affect the general structure or solubility of GFP. The resulting amide linkage between the protein and polymer should be stable to drug delivery and material science applications. While we have shown that this initiator on GFP functions well for generating protein−polymers in aqueous conditions by standard ATRP chemistry, it should also function with other controlled radical polymerization agents as well.[1e,f,2a] Our future efforts will focus on application of this method for preparation and study of unique protein−polymer hybrid materials and pharmaceuticals.

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Supporting Information Available: Experimental details, selection methods, MS characterization of proteins, and protein−polymer characterizations. This material is available free of charge via the Internet at http://pubs.acs.org.

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