# Site-Specific, Stoichiometric Protein-Polymer Conjugates by *In situ* Atom Transfer Radical Polymerization

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**Statement of Purpose:** The conjugation of protein and cell resistant –stealth– polymers to protein or peptide drugs is a useful strategy to improve the pharmacokinetic profiles and *in vivo* efficacy of biopharmaceuticals; however, there remain significant limitations in the efficient synthesis of monodisperse, biodegradable, stoichiometric polymer conjugates of proteins with high yield, high protein activity, significantly improved pharmacokinetics and improved *in vivo* efficacy. To address these limitations, we have developed two complementary approaches to directly grow a PEG-like polymer at either the N-terminus or C-terminus of a protein to yield stoichiometric (1:1) and site-specific PEG-like polymer conjugates.

**Methods:** A tripartite green fluorescence protein (GFP)-intein-elastin-like polypeptide (ELP) fusion was obtained by recombinant overexpression in E. coli, and was purified by inverse transition cycling (ITC). All other materials and reagents were commercially available except functionalized ATRP initiators that were synthesized in-house. All measurements were carried out at Duke University.

**Results:** *In situ* growth of protein and cell resistant poly(oligo(ethylene glycol) methyl ether methacrylate) (poly(OEGMA)) at the N-terminus of a model protein, myoglobin, is schematically shown in Figure 1 (Gao W. PNAS. 2009; 106: 15231-15236). First, the N-terminus (glycine) is transformed to an aldehyde through a biomimetic transamination reaction (MB-CHO). Second, a hydroxylamine-functionalized ATRP initiator (ABM) is attached to the N-terminus, through a reaction between the aldehyde and the hydroxylamine, to form a macroinitiator (Mb-Br). Third, poly(OEGMA) is directly grown from the protein macroinitiator by atom transfer radical polymerization (ATRP). C-terminal, stoichiometric protein-polymer conjugates were designed and synthesized by C-terminal intein-mediated ligation and *in situ* ATRP as follows (Figure 2). First, a GFP-intein-ELP fusion proteibn was overexpressed in *E. coli* and purified by ITC. Second, cleaving GFP from intein-ELP with a mixture of sodium 2-sulfanylethanesulfonate (MESNA) and 2-amino-N-[2-(2-bromo-2-methyl-propionylamino)-ethyl]-3-mercapto-propionamide (ABMP) yielded the GFP-Br macroinitiator. Third, poly(OEGMA) was grown *in situ* from the C-terminus of GFP by ATRP to form GFP-C-poly(OEGMA). The site-specific (N/C-terminal) modification with the ATRP initiators was confirmed by MALDI-MS, ESI-MS, and LC/MS for proteolytic digest with trypsin, and had > 75% yield. After *in situ* ATRP in aqueous buffer, the stoichiometric protein-N/C-poly(OEGMA) conjugates were purified and characterized with HPLC. Peroxidase activity of myoglobin and fluorescence activity of GFP were completely retained after the modifications.

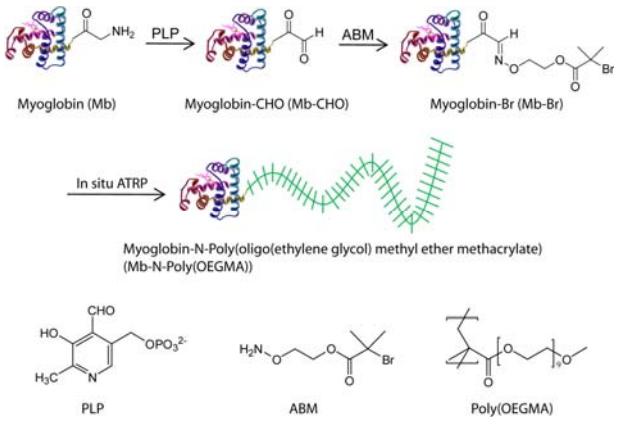


Figure 1. Schematic illustration of *in situ* growth of stoichiometric poly(OEGMA) at the N-terminus of myoglobin.

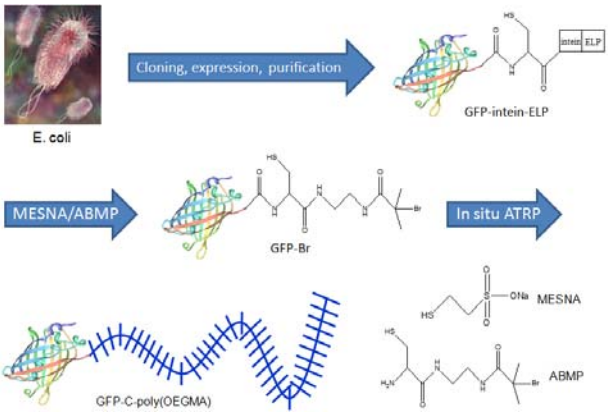


Figure 2. Schematic illustration of synthesis of GFP-C-poly(OEGMA).

Notably, both the Mb-poly(OEGMA) conjugate (N-terminal conjugate) and GFP-poly(OEGMA) conjugate (C-terminal conjugate) showed a 20-40 fold increase in their blood exposure compared to the unmodified protein after intravenous administration to mice (data not shown), thereby demonstrating that comb polymers that present short oligo(ethylene glycol) side-chains are a new class of PEG-like polymers that can significantly improve the pharmacological properties of proteins.

**Conclusions:** We report two general approaches to directly grow stoichiometric (1:1) polymer conjugates from a defined and ubiquitous location on a protein scaffold—the N/C-terminus—via in situ ATRP under aqueous conditions with high yield, with no free polymer byproduct, complete retention of protein activity, and significantly improved pharmacokinetics. Work in progress is focused on quantifying the *in vivo* tissue distribution of these conjugates and on developing conjugates of diverse peptide and protein drugs.