In situ Polymerization of a PEG Monomer from the C-Terminus of an Intein Fusion Protein Significantly Improves Pharmacokinetics and Tumor Accumulation

Weiping Gao, Ashutosh Chilkoti
Department of Biomedical Engineering, Center for Biologically Inspired Materials and Material Systems, Duke University, Durham, NC 27708, USA

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 a combined protein- and polymer-engineering approach to directly grow a PEG-like polymer from the C-terminus of an intein fusion 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 C-terminus of a model protein, green fluorescence protein (GFP), is schematically shown in Figure 1 (Gao W. Proc. Natl. Acad. Sci. USA 2010; 107: 16432-16437). First, a GFP-intein-ELP fusion protein 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-poly(OEGMA). The site-specific (C-terminal) modification with the ATRP initiator was confirmed by ESI-MS and LC/MS of the GFP-Br after proteolytic digestion with Lys-C, and was > 80% yield. After *in situ* ATRP in aqueous buffer, the stoichiometric GFP-poly(OEGMA) conjugate was purified and characterized by HPLC. Fluorescence activity of GFP was completely retained after the modifications.

In vivo pharmacokinetics was studied by tracking the blood concentration of radio-iodine labeled conjugate and unmodified GFP. Unmodified GFP was rapidly cleared from blood with a high clearance rate of 7 mL/h, and exhibited a short distribution phase ($t_{1/2\alpha}$ = 0.1 h) and a rapid terminal elimination phase ($t_{1/2\beta}$ = 4 h) . In contrast, the clearance rate of the conjugate decreased to 0.5 mL/h, the distribution phase of the conjugate increased by 20 times ($t_{1/2\alpha}$ = 2 h) relative to GFP, and the terminal elimination phase was prolonged to 26 h. These pharmacokinetic differences resulted in a 15-fold increase

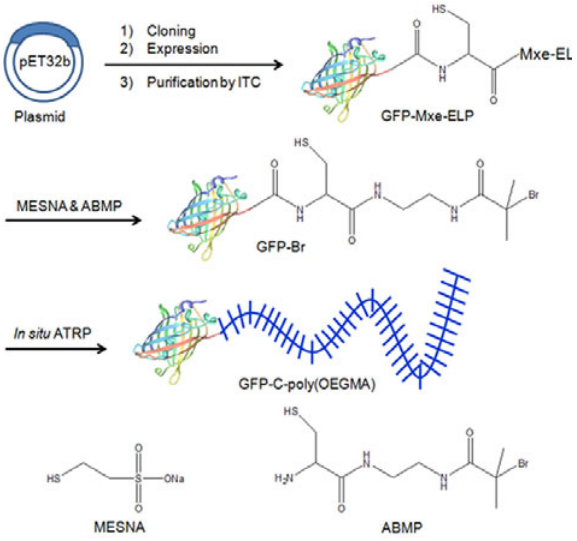

Figure 1. Schematic illustration of *in situ* growth of stoichiometric poly(OEGMA) at the C-terminus of GFP.

in the area under the curve (AUC) of the conjugate (214 %*h/mL) compared to unmodified GFP (14 %*h/mL), which indicates that C-terminal site-specific POEGMA modification significantly improved the blood exposure of GFP.

Tumor accumulation was also investigated in mice implanted with *s.c.* C26 murine tumors. After bolus injection, the concentration of unmodified GFP in the tumor rapidly decreased from 2% ID/g at 30 min to 0.4% ID/g at 6 h. In contrast, the concentration of the conjugate in the tumor increased from 2.5% ID/g tumor tissue at 30 min to 3.4% ID/g tumor tissue at 6 h, and *was 50 times greater than that of unmodified GFP at 24 h*. This finding demonstrates that an important consequence of a long plasma half-life is enhanced accumulation of the protein-polymer conjugate in a solid tumor, and hence strongly suggest that this protein conjugation technology may be useful to improve the efficacy of protein and peptide drugs and targeting agents under development for cancer imaging and therapy.

Conclusions: We report a new and general approach to directly grow stoichiometric (1:1) polymer conjugates from a defined and ubiquitous location on a protein scaffold—the 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 and tumor accumulation. Work in progress is focused on developing conjugates of diverse peptide and protein drugs.