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Site-specific *in situ* growth of a cyclized protein-polymer conjugate with improved stability and tumor retention

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ABSTRACT

A major disadvantage of therapeutic proteins is their instability to external stressors during storage, transport and use. Here, we report site-specific *in situ* growth of a cyclized protein-polymer conjugate with improved *in vitro* and *in vivo* stability. Green fluorescence protein (GFP) was genetically fused at its N- and C-termini with two sortase recognition sequences pentaglycine and LPETG, respectively to yield a linear GFP (*l*-GFP). A cyclized GFP (*c*-GFP) was generated from the *l*-GFP by sortase-catalyzed cyclization. A maleimide-functionalized atom transfer radical polymerization (ATRP) initiator was selectively attached to a free cysteine residue genetically engineered at the C-terminus of GFP to form a macro-initiator (*c*-GFP-Br). Subsequent *in situ* ATRP of oligo(ethylene glycol) methyl ether methacrylate (OEGMA) from the *c*-GFP-Br generated a site-specific (C-terminal) and stoichiometric (1:1) *c*-GFP-POEGMA conjugate with almost quantitative conversion and highly retained activity. Notably, the *c*-GFP-POEGMA conjugate showed 9- and 310-fold increases in thermal stability as compared to the *l*-GFP and its counterpart *l*-GFP-POEGMA, respectively. Additionally, the conjugate displayed significantly improved tumor retention relative to the *l*-GFP and *l*-GFP-POEGMA. The method developed may be applicable to a variety of therapeutic proteins to improve their *in vitro* and *in vivo* stability.

1. Introduction

Covalently conjugating proteins with synthetic polymers, particularly poly(ethylene glycol) (PEG) is widely used as a means to improve the stability and circulatory half lives of proteins and also to lower their immunogenicities [1,2]. Consequently, a number of PEGylated proteins have been approved for the treatment of a variety of diseases [3]. As potential alternatives to PEG, zwitterionic polymers, such as poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC) [4] and poly(carboxybetaine methacrylate) (PCBMA) [5] showed better water solubility, consequently offering better stability to proteins. Polystyrene with pendent trehalose disaccharides stabilized lysozyme against high temperature and repeated lyophilization [6]. Most recently, a heparin-mimicking polymer, a copolymer (P(SS-*co*-OEGMA)) composed of styrene sulfonate (SS) and oligo(ethylene glycol) methyl ether methacrylate (OEGMA) was found to stabilize basic fibroblast growth factor (bFGF) [7]. Cyclizing proteins by intein-mediated protein ligation (IPL) or sortase-mediated protein ligation (SPL) is another way to enhance thermal stabilities [8,9]. More recently, SPL has been used for both site-specific PEGylation and covalent closure of cytokines to form PEGylated circular cytokines that showed increased thermal stability and pharmacokinetics but decreased bioactivity [10]. To our knowledge, these polymers were conjugated to proteins using the conventional “grafting to” method, which typically leads to heterogeneous products with reduced bioactivity and low yield. Furthermore, *in vivo* stability of these non-PEG polymer conjugates have not been studied.

Over the last several years, we have developed two complementary general methodologies for *in situ* growth of stoichiometric PEG-like polymer (POEGMA) conjugates from the N- or C-terminus of proteins with high yield and retained activity [11–13]. In a tumor-bearing mouse model, the POEGMA conjugates showed significantly improved pharmacokinetics and tumor accumulation

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[11,12]. To our knowledge, *in situ* growth of polymer conjugates, the "grafting from" method has not been used to modify cyclized proteins to improve *in vitro* and/or *in vivo* stability of the native protein.

Here, we report a new and general method to improve *in vitro* and *in vivo* protein stability by combination of sortase-catalyzed protein cyclization and site-specific *in situ* polymerization. We select SPL for protein cyclization because SPL is effective to synthesize cyclized proteins with improved thermal stability [8,9]. We choose to directly grow a POEGMA conjugate from a cyclized protein because we have demonstrated that *in situ* growth of stoichiometric POEGMA conjugates not only retained protein activity but also significantly improved pharmacokinetics and tumor accumulation [11,12]. Hence, we hypothesize that *in situ* growth of a POEGMA conjugate from a cyclized protein would improve not only thermal stability but also tumor retention as compared to the native protein and its counterpart a non-cyclized protein-POEGMA conjugate.

2. Materials and methods

2.1. Materials

All molecular biology reagents were purchased from New England Biolabs, unless otherwise specified. All chemical reagents were purchased from Sigma Aldrich and used as received, unless otherwise specified. C26 cells were purchased from cell bank of Chinese Academy of Medical Sciences. Female BALB/c-Nu mice were purchased from Vital River and accommodated in animal research facility of Tsinghua University. The Laboratory Animal Facility at the Tsinghua University is accredited by AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International), and all animal use protocols used in this study are approved by the Institutional Animal Care and Use Committee (IACUC).

2.2. Synthesis of the ATRP initiator DBMP

2.2.1. 1-(2-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)ethyl)-1H-pyrrole-2,5-dione

Diisopropyl azodiformate (DIAD, 985 μL, 5 mmol) was dropwise added to a 20 mL anhydrous tetrahydrofuran solution of triphenylphosphine (1.31 g, 5 mmol), and the reaction solution was kept under stirring for 15 min. To the reaction system, 5 mL of anhydrous tetrahydrofuran containing 971 mg (5 mmol) of tetraethylene glycol was added dropwise. After stirring for another 15 min, 10 mL of anhydrous tetrahydrofuran containing 485 mg (5 mmol) of maleimide was added dropwise. The reaction mixture was allowed to stir at room temperature overnight. After removal of the solvent via rotary evaporation, the residue was purified via silica gel column chromatography (DCM: MeOH = 100:3 as eluent, Rf = 0.24), to yield colorless oil ($C_{12}H_{19}NO_6$, 505 mg, 37.0%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.688 (s, 2H), 3.552−3.732 (m, 16H). ESI-mass *m*/*z*: 296.1 ($[M + Na]^+$).

2.2.2. 2-(2-(2-(2-(2,5-dioxo-2H-pyrrol-1(5H)-yl)ethoxy)ethoxy)ethoxy)ethyl 2-bromo-2-methylpropanoate (DBMP)

1-(2-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)ethyl)-1H-pyrrole-2,5-dione (500 mg, 1.8 mmol) was dissolved in 20 mL of anhydrous dichloromethane, and the solution was cooled with an ice-bath. To the cooled solution, 250 μL (1.8 mmol) of triethylamine was added at 0 °C, then 5 mL of anhydrous dichloromethane containing 225 μL (1.8 mmol) of 2-bromo-2- methylpropionyl bromide was added dropwise at 0 °C. After stirring for 2 h at room temperature, the solvent was removed via rotary evaporation, the residue oil was further purified via silica gel column chromatography (PE:EA = 1:1 as eluent, Rf = 0.6), the product was yielded as colorless oil ($C_{16}H_{24}BrNO_6$, 309.4 mg, 40.0%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.702 (s, 2H), 4.324 (m, 2H), 3.604−3.749 (m, 14H), 1.938 (s, 6H). ESI-mass *m*/*z*: 444.1 ($[M + Na]^+$), 446.1 ($[M + Na]^+$).

2.3. Construction, expression and purification of *l*-GFP and sortase A

The gene encoding *l*-GFP was polymerase chain reaction (PCR)-amplified from a previously constructed GFP-containing pET-32b(+) vector and ligated into pET-25b(+) (Novagen) through *Nde* I and *Bam* HI restriction sites. The oligonucleotides used are as follows:

F-primer: 5′ TACGCGCATATGGGTGGCGGTGGCGGTGCTAGCAAAGGAGAAGAA 3′

R-primer: 5′ CGGGATCCTTAATGATGATGATGATGGTGGCCACCGGTTTCCG GCAGGC 3′

The recombinant plasmid was confirmed by DNA sequencing, then transformed into *Escherichia coli* BL21(DE3) competents (Invitrogen), and further incubated in Luria Bertani medium supplemented with ampicillin (100 μg/mL) at 37 °C. This culture was used to inoculate a large scale of sterile Terrific Broth medium with shaking at 37 °C until the optical density at 600 nm (OD_{600}) is 0.5. Overexpression of *l*-GFP was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 500 μM at 25 °C for 12 h. Cells were harvested and resuspended in 50 mM Tris·HCl, 150 mM NaCl, pH 7.4. After sonication and centrifugation, the extract was treated with polyethyleneimine (1% w/v) in order to precipitate nucleic acids, followed by centrifugation to spin down the precipitate. The supernatant was then applied to a 5 mL HisTrap column (GE Healthcare). The column was washed with equilibration buffer (50 mM Tris, 500 mM NaCl, 10% glycerol, 5 mM imidazole, pH 7.4) and then washing buffer (50 mM Tris, 500 mM NaCl, 10% glycerol, 50 mM imidazole, pH 7.4). His_6-tagged protein was finally eluted using buffer containing 500 mM imidazole. The eluted *l*-GFP was further purified on a HiPrep 26/10 desalting column (GE Healthcare) for buffer exchanging to 50 mM Tris·HCl, 150 mM NaCl, pH 7.4 and stored at −80 °C for further use. The identity and purity of *l*-GFP was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The concentration of protein was estimated by UV-visible spectroscopy (Thermo Scientific) with the absorbance of 280 nm.

The gene for recombinant sortase A (SrtA) with a N-terminal His_6 tag was inserted into pET-25b(+) via *Nde* I and *Bam* HI restriction sites. The expression and purification process of SrtA was the same as that of *l*-GFP.

The plasmid for GFP containing a His_6 tag at its C-terminus as a control was constructed and verified via DNA sequencing.

Full amino acid sequences for all proteins used are listed in Table S1.

2.4. Circularization

l-GFP (50 μM) was circularized by incubation with SrtA (25 μM) in a sortase reaction buffer (50 mM Tris·HCl, 150 mM NaCl, 10 mM $CaCl_2$, pH 7.4) overnight at 25 °C without agitation. The reaction mixture was diluted into 5 volumes with 20 mM Tris·HCl, 100 mM NaCl, pH 7.4. The resulting solution was then applied to a HiTrap Capto Q column (GE Healthcare) for anion exchange chromatography to remove SrtA. Continuous gradient chromatography was performed with 20 mM Tris·HCl, pH 7.4 as equilibration buffer and 20 mM Tris·HCl, 1 M NaCl, pH 7.4 as eluent. Peaks containing the desired product mixture were collected, exchanged with 50 mM Tris·HCl, 150 mM NaCl, pH 7.4 via a desalting column, and concentrated with ultrafiltration (Amicon Ultra-0.5 centrifugal filter, 3K MWCO, Millipore). *c*-GFP was further separated from its oligomers by gel filtration chromatography, performed on AKTA purifier.

2.5. In situ ATRP

2.5.1. Attachment of the ATRP initiator to c-GFP and l-GFP

2 mL of 100 μM GFP (*c*-GFP or *l*-GFP) in 50 mM Tris·HCl, 150 mM NaCl, pH 7.4 was mixed with 20 μL of 50 mM Tris(2-carboxyethyl) phosphine (TCEP) (Strem Chemical Inc.) in Tris·HCl buffer at 25 °C. After 30 min, 20 μL of 50 mM DBMP in H_2O was added into the reaction mixture, and the final reaction solution was allowed to sit without agitation for 1 h at room temperature. The unreacted DBMP and TCEP, and other small species were cleared by ultrafiltration.

2.5.2. In situ ATRP of OEGMA from c-GFP and l-GFP

A deoxygenated solution of 0.02 mmol CuCl and 0.087 mmol 1,1,4,7,10,10-hexamethyltriethylene- tetramine (HMTETA) in 1 mL MilliQ water was transferred into 1 mL of 50 μM deoxygenated GFP-Br (*c*-GFP-Br or *l*-GFP-Br) and 1.05 mmol poly(ethylene glycol) methyl ether methacrylate (OEGMA) (MW = 475) in 50 mM Tris·HCl, 150 mM NaCl, pH 7.4. The polymerization was allowed to proceed for 1 h at room temperature under nitrogen protection and was quenched by exposing to air. The protein conjugates were purified from the reaction mixtures via a desalting column as described above.

2.6. Physicochemical characterization

2.6.1. Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

l-GFP, *c*-GFP, *l*-GFP-Br or *c*-GFP-Br was digested with trypsin or endoproteinase Asp-N (Promega) in 40 mM ammonium bicarbonate, 10% (v/v) acetonitrile aqueous solution at 37 °C overnight and the peptide fragments were analyzed by a Q-Exactive LC-MS/MS (Thermo Scientific). The digested samples were extracted twice with 1% (v/v) trifluoroacetic acid in 50% (v/v) acetonitrile aqueous solution for 30 min. Peptides were separated by a 65 min gradient elution at a flow rate of 0.3 μL/min with DIONEX Ultimate 3000 RSLC nano system equipped with the Q-Exactive. The analytical column was a homemade fused silica capillary column (75 μm i.d., 150 mm length; Upchurch) packed with C-18 resin (300 Å, 5 μm; Varian). Mobile phase A consisted of 0.1% (v/v) formic acid, and mobile phase B consisted of 80% acetonitrile and 0.08% formic acid. The MS was operated in the data-dependent acquisition mode using Xcalibur 2.2 software with a single full-scan mass spectrum from 400 to 1500 *m*/*z* with 140,000 resolution, followed by ten data-dependent MS/MS scans in the ion trap at 27% normalized collision energy. The LC-MS/MS spectra were searched against the selected databases using an in-house Proteome Discovery searching algorithm, and validated manually. Static mass modifications corresponding to carbamidomethylation on Cys residues and oxidation on Met residues were included.