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Site-specific *in situ* growth of an interferon-polymer conjugate that outperforms PEGASYS in cancer therapy

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ABSTRACT

Conjugating poly(ethylene glycol) (PEG), PEGylation, to therapeutic proteins is widely used as a means to improve their pharmacokinetics and therapeutic potential. One prime example is PEGylated interferon-alpha (PEGASYS). However, PEGylation usually leads to a heterogeneous mixture of positional isomers with reduced bioactivity and low yield. Herein, we report site-specific *in situ* growth (SIG) of a PEG-like polymer, poly(oligo(ethylene glycol) methyl ether methacrylate) (POEGMA), from the C-terminus of interferon-alpha to form a site-specific (C-terminal) and stoichiometric (1:1) POEGMA conjugate of interferon-alpha in high yield. The POEGMA conjugate showed significantly improved pharmacokinetics, tumor accumulation and anticancer efficacy as compared to interferon-alpha. Notably, the POEGMA conjugate possessed a 7.2-fold higher *in vitro* antiproliferative bioactivity than PEGASYS. More importantly, in a murine cancer model, the POEGMA conjugate completely inhibited tumor growth and eradicated tumors of 75% mice without appreciable systemic toxicity, whereas at the same dose, no mice treated with PEGASYS survived for over 58 days. The outperformance of a site-specific POEGMA conjugate prepared by SIG over PEGASYS that is the current gold standard for interferon-alpha delivery suggests that SIG is of interest for the development of next-generation protein therapeutics.

1. Introduction

Therapeutic proteins are characterized by their high biological activity and specificity, but their clinical use is bottlenecked by their poor stability, short circulating half-life and immunogenicity [1–3]. One typical example is recombinant human interferon alpha (IFN-α), which is Food and Drug Administration (FDA)-approved and widely used in the treatments of viral diseases and cancer. Nevertheless, IFN-α suffers from its short plasma half-life ($t_{1/2}$ = 2–4 h), which necessitates frequent administration with high concentrations to achieve sustained efficacy, causing adverse side effects and poor patient compliance [3].

Conjugating proteins to poly(ethylene glycol) (PEG), known as PEGylation, is widely used as a means to solve these problems [4]. For instance, PEGylated IFN-α (such as PEGASYS developed by Roche and PEGINTRON developed by Schering-Plough) is used clinically to treat chronic hepatitis [5,6]. However, PEGylation has three major drawbacks: (1) it often leads to a heterogeneous product mixture of positional isomers with reduced biological activity [7–9] because a protein typically has numerous ubiquitous reactive residues (Cys, Lys or His) available for PEG attachment, which makes it difficult to control the site of conjugation and the stoichiometry of the conjugate; (2) it is difficult to isolate and purify PEGylated proteins, especially positional isomers [10]; and (3) it involves the reaction between two large macromolecules and the steric hindrance between the two macromolecules typically leads to low yield (<10%). These drawbacks generally complicate the development process of PEGylated proteins, and impede their wide-spread applications. Therefore, it is important to develop a new and general strategy to synthesize well-defined protein-polymer conjugates with high yield, well-retained bioactivity, and significantly improved pharmacological profiles, in which the site of conjugation, the length of the attached polymer, and the stoichiometry of the conjugate (typically 1:1) can be precisely controlled.

Recently, *in situ* growth of polymers from proteins has emerged as an alternative strategy to PEGylation [11–18]. Particularly, we have demonstrated that a PEG-like polymer, poly(oligo(ethylene glycol) methyl ether methacrylate) (POEGMA), can be grown from the N-/C-terminus of model proteins, such as myoglobin (Mb) and

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green fluorescence protein (GFP), by atom transfer radical polymerization (ATRP) to form site-specific (N-/C-terminal) and stoichiometric (1:1) Mb/GFP-POEGMA conjugates with high yield, and retained activity and improved pharmacokinetics as compared with the native proteins [19,20]. Although those results were promising, they only suggested the potential of POEGMA conjugates. As POEGMA was limited to modify the model proteins that are not useful therapeutically, the therapeutic potential of POEGMA conjugates was still in doubt. Furthermore, POEGMA conjugates were not compared with any clinically approved PEGylated proteins.

Herein, we report *in situ* growth of POEGMA solely from the C-terminus of a recombinant IFN-α to yield a site-specific (C-terminal) and stoichiometric (1:1) POEGMA conjugate of IFN-α (IFN-POEGMA) (Fig. 1) and a head-to-head comparison of the POEGMA conjugate with PEGASYS, a PEGylated IFN-α that has been approved by the Food and Drug Administration (FDA) for IFN-α delivery [5,6]. The overall yield of IFN-POEGMA was as high as 66.1%, which was more than 60-fold higher than that (1.1%) of a similar IFN-POEGMA conjugate synthesized by post-polymerization conjugation. IFN-POEGMA not only showed excellent pharmacokinetics that was comparable to PEGASYS but also exhibited a 7.2-fold higher *in vitro* antiproliferative bioactivity than PEGASYS. Particularly, in a murine cancer model, IFN-POEGMA eradicated tumors of 75% mice without apparent systemic toxicity, while at the same dose, no PEGASYS-treated mice survived for over 58 days. These results show that a site-specific POEGMA conjugate of IFN-α can outperforms PEGASYS, the current gold standard for IFN-α delivery, which augurs well for its clinical translation. We herein call this methodology site-specific *in situ* growth (SIG), as an alternative to PEGylation, that is promising for the development of next-generation protein therapeutics.

2. Materials and methods

2.1. Materials

All chemical reagents were purchased from Sigma Aldrich or J&K Scientific and used as received, unless otherwise specified. All molecular biology reagents were purchased from New England Biolabs and used as received, unless otherwise specified. All cell culture reagents and media were purchased from Gibco or Hyclone, unless otherwise specified. Daudi B cells and human ovarian carcinoma cells (OVCAR-3) were purchased from cell bank of Chinese Academy of Medical Sciences. Female BALB/c nude mice were purchased from Vital River Laboratories (Beijing, China) 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 protocols used in this study are approved by the Institutional Animal Care and Use Committee (IACUC).

2.2. Synthesis of the ATRP initiator AEBM (See Scheme S1)

2.2.1. 2-(2-(tert-butoxycarbonyl)amino)ethyl 2-bromo-2-methylpropanoate (compound 1)

Tert-butyl 2-hydroxyethylcarbamate (1.6 g, 10 mmol), diisopropylethylamine (1.8 mL, 11 mmol, 1.1 eq.) were dissolved in dichloromethane (20 mL) in an ice-water bath. 2-bromo-2-methylpropanoyl bromide (1.25 mL, 10 mmol) was added dropwise to the cooled solution over 15 min. After 30 min, the ice-water bath was removed, and the reaction solution was allowed to warm to room temperature and stirred for 16 h. The solvent was removed via rotary evaporation. The product was purified via silica gel column chromatography (DCM:EA = 1:1). The product was yellow oil ($C_{11}H_{20}BrNO_4$, 2.13 g, 68.6%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.83 (s, 1H), 4.24 (m, 2H), 3.42 (m, 2H), 1.95 (s, 6H), 1.45 (s, 9H). ESI-mass *m/z*: 332.1([M + Na] $^+$), 334.1([M + Na] $^+$).

2.2.2. 2-(2-(2-(2-(tert-butoxycarbonyl)acetamido)acetamido)acetamido)ethyl 2-bromo-2-methyl- propanoate (compound 2)

2-(2-(tert-butoxycarbonyl)amino) ethyl 2-bromo-2- methylpropanoate (2.13 g) was deprotected with 50 mL of 6 M HCl ethyl acetate solution, and shaken for 2 h. After the deprotection reaction, the reaction mixture was filtered. The filter cake was collected and dried to yield 2-aminoethyl 2-bromo-2-methylpropanoate hydrochloride quantitatively. Subsequently, 2-(2-(2-(tert-butoxycarbonyl)acetamido)acetamido)acetic acid (289 mg, 1 mmol), 2-aminoethyl 2-bromo-2-methylpropanoate hydrochloride (246 mg, 1 mmol), EDC (288 mg, 1.5 mmol), and diisopropylethylamine (175 μL, 1 mmol) were dissolved in 10 mL of dichloromethane. The reaction mixture was allowed to stir at room temperature for 16 h. After reaction, the solvent was removed, the white solid residue was washed sequentially with 4 mL × 2 of water, 1 mL × 3 of methanol, and 4 mL of ethyl ether, yielding a white powder ($C_{15}H_{26}BrN_3O_6$, 269 mg, 56%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.26 (t, 2H), 3.91 (s, 2H), 3.87 (s, 2H), 3.78 (s, 2H), 3.54 (t, 2H), 1.94 (s, 6H),

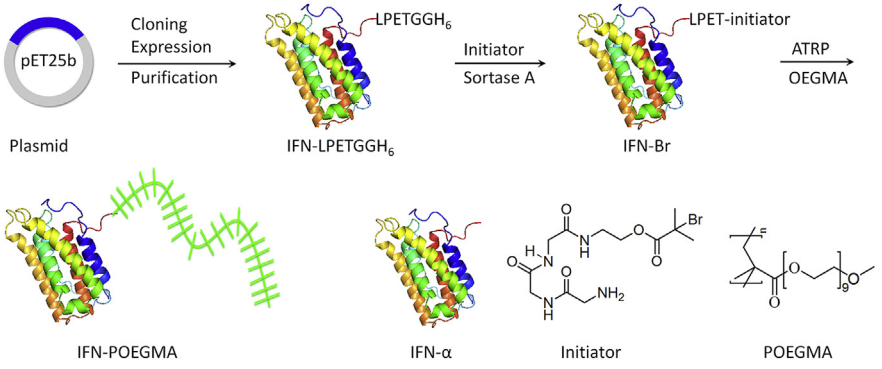

Fig. 1. Schematic illustration of *in situ* growth of POEGMA from the C-terminus of IFN-α to form a site-specific (C-terminal) and stoichiometric (1:1) IFN-POEGMA conjugate. First, the triglycine-functionalized atom transfer radical polymerization (ATRP) initiator (AEBM) is selectively attached to the C-terminus of IFN-α by sortase A catalyzed ligation to form a macroinitiator (IFN-Br). Second, POEGMA is site-specifically grown from the IFN-Br via ATRP to form an IFN-POEGMA conjugate. The crystal structure of IFN-α was generated from PDB code 1ITF [21].