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Preparation of chain-end clickable recombinant protein and its bio-orthogonal modification

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ABSTRACT

Introducing unique functional group into protein is an attractive approach for site-selective protein modification applications. In this report, we systemically investigated four site-selective strategies to introduce azide functionality into recombinant thrombomodulin (TM_{456}), *via* direct recombinant expression with unnatural amino acid, chemical, and enzymatic modification for its bio-orthogonal modification application. First, a straightforward recombinant method to express TM_{456} with azide functionality near C-terminus by replacing methionine with azidohomoanlanine from methionine auxotroph *Escherichia coli* cell was investigated. Next, a sortase-mediated ligation (SML) method to incorporate azide functionality into the C-terminus of recombinant TM_{456} was demonstrated. The third is to add azide functionality to the N-terminal amine of recombinant TM_{456} *via* amidation chemistry, and the fourth is tyrosine selective three-component Mannich reaction to introduce azide functionality to recombinant TM_{456}. Overall, SML of recombinant protein affords the highest overall yield for incorporating azide functionality into the C-terminus recombinant TM_{456} since the key protein expression step uses natural amino acids. Also, single site modification facilitates the highest TM_{456} activity.

1. Introduction

Modification of protein is a very important and necessary approach for studying protein structure and function, and the mechanisms of biological pathways that the protein is involved in. Furthermore, modification can expand protein's functional capacity, especially for therapeutic proteins for enhanced pharmacodynamic and pharmacokinetic properties. The key point for protein modification is to carry out a well-defined site-selective chemistry without reducing the protein's original activity and in high efficiency for generating homogeneous product. Bio-orthogonal chemistry facilitates site-selective modification by targeting to a specific functionality and has shown great potential for protein modifications of different interests. Azide has been proved as a versatile bio-orthogonal chemical reporter, which is small and inert in physiological settings and used for modifying diverse classes of biomolecules [1–5]. In particular, azide-based ligation reactions have been extensively explored for highly selective and biocompatible modification of proteins for biomedical applications [6–8]. Nowadays, three major bio-orthogonal reactions utilizing azide functionality have been fully developed, including Staudinger ligation, click chemistry and copper-free click chemistry (CFCC), among which CFCC is the most convenient and efficient one. Therefore, efficiently introducing azide functionality into proteins has become the key step and versatile approach for site-selective protein modification for both research and applications.

Thrombomodulin (TM) is a membrane glycoprotein mainly expressed by vascular endothelial cells as a major anticoagulation component [9]. TM consists of 557 amino acids divided into five distinct domains: *N*-terminal lectin-like domain (designated as TMD1); six epidermal growth factor (EGF)-like domain (TMD2); Ser/Thr-rich domain (TMD3); transmembrane domain (TMD4); and cytoplasmic tail domain (TMD5). The different domains are responsible for different biological functions of TM [10]. In the past decades, various domains of TM have been cloned and expressed for TM structural and functional study [11]. Further, recombinant TMs of different domains show promising antithrombotic and anti-inflammatory activity in both rodents and primates models. A recombinant soluble TM has been approved for therapeutic

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application in Japan [12]. In addition, TM has been considered as an excellent candidate for biomedical engineering applications. Chaikof et al. reported a truncated TM construct with an azidohomoalanine near *C*-terminus for site-selective modification *via* Staudinger ligation [13] and oriented immobilization through click chemistry [2]. Besides, bioactive modification of pancreatic islets with the azide-containing TM *via* Staudinger ligation was investigated by the same group, showing the potential to reduce procoagulant and pro-inflammatory responses upon transplantation [14,15]. All these results demonstrated the feasibility of recombinant azide-TM for cell surface re-engineering and its promising application for cell transplantation. Recently, we proposed a recombinant TM-liposome conjugate as a bio-inspired antithrombotic agent, which mimics both the protein and lipid membrane structure of the native endothelial membrane TM [16]. Briefly, a TM of EGF-like 4–6 domains with an azidohomoalanine near *C*-terminus (rTM_{456}-N_3) was expressed *via* replacing methionine with azidohomoanlanine from methionine auxotroph *Escherichia coli* cell. Then, site-selective conjugation of the rTM_{456}-N_3 with liposome *via* Staudinger ligation and CFCC afforded recombinant TM_{456}-liposome conjugates, both of which showed enhanced protein C activation activity facilitated by lipid membrane.

Introducing unnatural amino acids into recombinant TM provides versatile tool for site-selective chemistry for protein modification applications. However, the low level of protein expression with unnatural amino acid limits its practical applications. Therefore, to find a way to introduce azide into protein efficiently and site-selectively is highly needed for all promising researches and applications of proteins like TM_{456} described above. In this study, we systemically investigated recombinant, enzymatic and chemical strategies to introduce azide functionality into TM_{456} site-selectively at either *N*-terminus or *C*-terminus. First, we investigated recombinant expression of recombinant TM_{456} with azide functionality near *C*-terminus by replacing methionine with azidohomoanlanine from methionine auxotroph *E. coli* cell. Second, we expressed TM_{456} with sortase A recognizing motif LPETG for introducing azide functionality into the *C*-terminus *via* sortase-mediated ligation (SML). Third, a chemical method was used to add azide molecule to the *N*-terminal amine of TM_{456} *via* amidation chemistry. Finally, multiple azide functional groups were introduced into TM_{456} *via* a tyrosine-selective three-component Mannich reaction (Fig. 1). The azido-TM_{456} derivatives obtained in all four methods were confirmed for site-selective modification and immobilization of recombinant TM through CFCC successfully.

2. Materials and methods

2.1. Materials and reagents

Unless stated otherwise, all reagents and chemicals were obtained from commercial sources and used without further purification. pET 39b vector, pET 28b vector, competent cells, and kanamycin sulfate were purchased from EMD Chemicals (Philadelphia, PA). The mouse monoclonal antibody specific to human TM and Goat anti-Mouse IgG H&L (FITC) were purchased from Abcam (Cambridge, MA). Human protein C, human thrombin and human antithrombin III were obtained from Haematologic Technologies Inc. L-azidohomoalanine was from AnaSpec Inc. (Fremont, CA). Chromogenic thrombin substrate BIOPHEN-CS01 was obtained from Aniara. anti-PEG monoclonal Ab E11 was purchased from Academia Sinica (Taipei, Taiwan). 4N-BOC-Gly-Gly-OH and N-hydroxysuccinimide were purchased from Sigma-Aldrich (St. Louis, USA). DBCO-Cy5, Azido-PEG_3-Amine, Azido-PEG_4-NHS Ester and DBCO-PEG_{5000}-OMe were purchased from Click-Chemistry Tools (Scottsdale, AZ).

2.2. Expression of FLAG-TM3

The gene encoding the EGF domain 4–6 of human thrombomodulin with a FLAG tag (FLAG-TM3) and a C-terminal LPETG motif was designed and synthesized (Genscript Inc.). In order to prevent oxidation and reduce proteolytic susceptibility, three

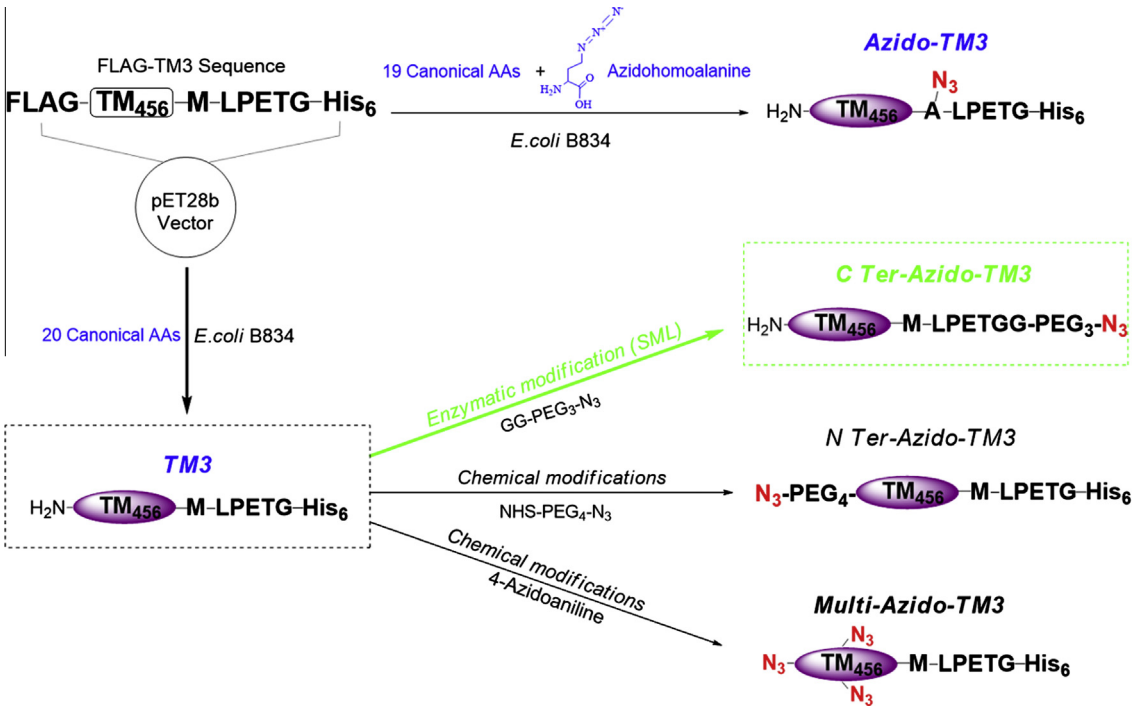

Fig. 1. Multiple strategies for incorporating azide functionality into recombinant TM_{456} for its bio-orthogonal modification applications.