C-terminal cysteine PEGylation of adalimumab Fab with an engineered interchain SS bond

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Conjugation with PEG is performed to increase serum half-life of the Fab for clinical applications. However, current designs for recombinant Fab only allow PEGylation at the interchain SS bond (disulfide bond) at the C-terminal end of the heavy chain and light chain of the Fab, which the decrease of thermostability occurred by partial reduction of the interchain SS bond. An adalimumab Fab mutant with a novel interchain SS bond (CH1:C177–CL:C160) and one cysteine at the C-terminal end (mutSS FabSH) was designed to maintain Fab thermostability and for site-specific PEGylation. MutSS FabSH was expressed in *Pichia pastoris* and purified mutSS FabSH was conjugated with 20-kDa PEG targeted at the free cysteine. Based on ELISA, PEGylation did not affect the binding capacity of the mutSS FabSH. To confirm the influence of PEGylation on the pharmacokinetic behavior of the Fab, PEGylated mutSS FabSH was administered to mice via tail vein injection. Analysis of the mean serum concentration of the PEGylated mutSS FabSH versus time through ELISA indicated an increase in half-life compared to that of non-PEGylated wild-type Fab. Consequently, we have successfully demonstrated that a Fab mutant with a novel interchain SS bond and one free cysteine at the C-terminal end can be PEGylated without changes in functionality. This design can potentially be used as a platform for modification of other recombinant Fabs.

**Key words**  antibody; Fab; PEG; disulfide bond; protein engineering
INTRODUCTION

Antibody fragments such as the single-chain variable fragment (scFv) and Fab can be efficiently produced using microbial expression systems. Consequently, they are being investigated as alternatives to whole antibodies for application in clinical therapy or diagnosis (1—3). Fab is particularly considered as the suitable antibody fragment for clinical use as it is more stable than the scFv (4). Compared to whole antibodies, Fabs can penetrate tissues more rapidly and are less likely to cause unwanted immunoreaction: the antibody dependent cellular cytotoxicity (ADCC), complement dependent cytotoxicity (CDC).

However, the shorter serum half-life of the Fab compared to that of the whole antibody is a disadvantage for therapeutic application. Conjugation of PEG to proteins, or PEGylation, has been associated with improved circulating half-lives of proteins (5—8). PEGylation of proteins have also been possible to reduce antigenicity, toxicity, or immunogenicity of proteins (5,9,10). Cysteines in protein are generally suitable targets for site-directed labeling with maleimide-activated PEG. Targeting cysteine of the hinge region of Fab’ has proven to be an effective means for site-directed PEGylation (11—14). Certolizumab pegol (Cimzia®), which is used for treating Crohn’s disease, ankylosing spondylitis, and rheumatoid arthritis, is an example of a PEGylated Fab’ (15). PEG can also be added to the free cysteine residue on the hinge region of Fab’ that has been engineered and produced through alternative expression systems. However, free cysteines in the flexible hinge region are also reported to result in lower expression levels or in disulfide scrambling in microbial systems (16). An alternative approach targets the SS bond with the cysteine residue at the C-terminal end of the heavy chain constant region 1 (CH1) and the light chain constant region (CL) in the Fab.
site-specific reduction of the interchain SS bonds can be followed by use of PEG-bis-sulfone reagents, which results in the SS bridge formation between the two thiols attached with PEG (16,17). On the other hand, it has been known that interchain SS bonds contribute to the stability of protein structure.

We have previously prepared a Fab from adalimumab, a therapeutic monoclonal antibody: anti-human tumor necrosis factor α (TNFα), that lacks the C-terminal interchain SS bond between the CH₁ and CL by mutation of cysteine to alanine (FabₐSS) (18). The analysis of Differential scanning calorimetry (DSC) indicated that the melting temperature (Tm) of FabₐSS by measurement of thermal denaturarion was 5 °C lower than that of the wild-type Fab. These results suggested that the interchain SS bond has significantly contributed to the thermal stability of Fab. We then introduced mutations in the adalimumab Fab-H chain (CH₁:V177C) and the adalimumab light chain (CL:Q160C) to form a novel SS bond at the constant region. This resulted in the recovery of the thermal stability of the Fab mutant, thereby mitigating the decrease of thermostability caused by reduction of the original interchain SS bond (18).

In this study, an adalimumab Fab mutant, which contains a novel interchain SS bond (CH₁:C177–CL:C160) and one free cysteine at the C-terminal end of CH₁ through the mutation of Cys 214 at that of CL to Ala, was designed to produce a modifiable humanized Fab platform. In addition, we performed site-directed PEGylation of the mutant, followed by characterization of the pharmacokinetic features of the PEGylated mutant Fab.
MATERIALS AND METHODS

Animals

Male SD rats (7 weeks old) weighing 230 to 240 g were purchased from Japan SLC (Hamamatsu, Japan). All experimental animals were humanely carried out according to the guidelines of animal Experiment Ethics Committee of Sojo University.

Construction of co-expression plasmids

The co-expression vector (pPICZαA; Invitrogen, USA) encoding both Fab-H chain gene of adalimumab and L chain gene of adalimumab was constructed according to previous study (18). The genes for the mutants of Fab were obtained by PCR-based site-directed mutagenesis (19). Extra serine residue was attached to each N-terminal end of adalimumab Fab-H chain and adalimumab L chain to perform more efficient processing of the α-factor signal sequence (20).

Expression and purification of Fab

The adalimumab Fab mutant was expressed in the methylotoropic yeast P. pastoris (X-33) for large-scale culture and purified as described previously (18). Yeast transformant cells were grown in shake flask culture of 1 L of BMGY media at 30°C for 3 days. The cultured cells were centrifuged at 3,000 ×g for 15 min at 4°C and the pellets were resuspended in 1 L of BMMY media, and cultivated in shake flask culture at 30°C for 100h. The culture was added daily with 1 ml (to final concentration of 0.1%) of methanol to induce expression. The culture supernatant was precipitated out with 60% ammonium sulfate, and centrifuged.
(10,000 ×g for 15 min at 4°C) to remove the residual medium. The precipitated protein containing Fab was resuspended in 0.05 M sodium acetate buffer (pH 4.0). The protein solution after dialysis against the acetate buffer was then bound to a column packed with SP-Toyopearl resin (22 mm × 120 mm; Tosoh Co., Ltd., Japan) equilibrated with 0.05 M sodium acetate buffer (pH 4.0) and eluted with 1.0 M NaCl in equilibration buffer. The eluted protein containing Fab was dialyzed and was bound to a column packed with Blue Sepharose 6 resin (15 mm × 60 mm; GE Healthcare, USA) and equilibrated with 0.05 M sodium acetate buffer (pH 5.0). The Fab was eluted with 0.4 M NaCl in equilibration buffer. Finally, Fab was purified by a resource S cation exchange chromatography column (Bed volume 1 ml; GE Healthcare, USA) with AKTA purifier system. The column was equilibrated with 0.05 M sodium acetate buffer (pH 5.0) and eluted with a linear gradient of NaCl from 0 to 0.1 M in equilibration buffer at a flow rate of 1 mL/min and total elution volume of 40 mL.

**PEGylation of adalimumab Fab mutant**

Reduction and PEGylation were carried out in 20 mM sodium phosphate buffer (pH 7.5) containing 10 mM EDTA and 50 mM NaCl (PEGylation buffer). A solution of 0.2 mg/ml adalimumab Fab mutant was reacted with 10-fold molar excess of tris(2-carboxyethyl)phosphine with gentle shaking at room temperature for 30 min. The reduced Fab mutant was dialyzed against PEGylation buffer, incubated overnight at room temperature and then reacted with 5-fold molar excess of linear maleimide PEG (MW 20 kDa; SUNBRIGHT ME-200MA0B, NOF corporation) with gentle shaking at room temperature for 16 to 18 h. The reaction mixture of Fab was loaded on a Superdex 75 10/300 GL column (GE Healthcare, USA) equilibrated with 50 mM potassium phosphate buffer,
0.2 M NaCl (pH 7.0) at a flow rate of 0.5 mL/min. Eluted fractions containing PEGylated Fab mutant were collected, and dialyzed against PBS.

**ELISA of adalimumab Fab mutant**

The binding activities of the wild-type adalimumab Fab and PEGylated Fab mutant for human TNFα were determined by ELISA. F96 MaxiSorp Nunc-immunoplate (Thermo Scientific, USA) was coated with human TNFα at 0.2 µM in 50 mM sodium carbonate buffer (pH 9.6), overnight at 4°C. The wells on plate were washed with washing solution (20 mM Tris-HCl, pH 7.6, 0.14M NaCl, 0.05% Tween 20). The wells on plate were blocked with blocking solution (washing solution containing 2% skim milk, 200 µl/well) at room temperature for 1 h, and then washed with washing solution. The solutions containing either wild-type Fab or PEGylated Fab mutant were added (50 µl/well) and incubated at room temperature for 1 h. Then, the wells on plate washed with washing solution and incubated with anti-Fab, human, sheep-poly, conjugated with horseradish peroxidase (Abliance, France) at room temperature for 1 h. After washing step, peroxidase substrate solution (POD Substrate TMB Solution; Nacalai Tesque, Japan) was added (100 µl/well) and incubated at room temperature. The reaction was stopped by the addition of 1 M sulfuric acid (100 µl/well). The absorbance was measured at 450 nm.

**Pharmacokinetics of PEGylated adalimumab Fab mutant**

Male SD rats weighing about 240 g were injected intravenously with 100 µg of non-PEGylated the wild-type adalimumab Fab or PEGylated Fab mutant under isoflurane anesthesia (n = 3 per group). Serial blood samples were collected into heparinized syringes
Serum concentration of Fab was determined by ELISA.

RESULTS

Expression and purification of mutSS Fab\textsubscript{SH}

The wild-type Fab has one interchain SS bond at the C-terminal end (Fig. 1). In this study, an adalimumab Fab mutant (mutSS Fab\textsubscript{SH}), which contains a novel interchain SS bond (CH\textsubscript{1}:C177–CL:C160) and one cysteine at the C-terminal end of CH\textsubscript{1} (by a C214A mutation at the C-terminal end of CL), was designed for site-directed PEGylation (Fig. 1). The expression and purification of mutSS Fab\textsubscript{SH} from \textit{P. pastoris} was performed according to our previous methods (18). The elution profile from resource S column (cation exchange chromatography) at the final step of purification indicated a single peak (Fig. 2A). Analysis of non-reducing SDS-PAGE showed that the purified mutSS Fab\textsubscript{SH} was approximately 45-50 kDa in size, which corresponds to the theoretical size (47 kDa) of the wild-type Fab. On the contrary, analysis of reducing SDS-PAGE showed that the purified mutSS Fab\textsubscript{SH} was approximately 25 kDa in size, which corresponds to the theoretical size of Fab-H chain (24kDa) and L chain (23kDa) (Fig. 2B). These results suggest that although the original interchain bond was removed, the two chains formed the Fab through the engineered interchain SS bond. About 10 mg of pure mutSS Fab\textsubscript{SH} was obtained from 1 L culture medium. Therefore, an adalimumab Fab mutant containing a free cysteine at the C-terminal end was successfully expressed in \textit{P. pastoris}.
**PEGylation of mutSS Fab**

The mutSS Fab was reacted with 20-kDa PEG-maleimide for conjugation of PEG through the free cysteine of the C-terminal end. The reaction mixture of Fab was loaded on Superdex 75 column (gel filtration) equilibrated with 50 mM potassium phosphate buffer (pH 7.0). When the mutSS Fab without the free cysteine was PEGylated as control, the elution profile of the gel filtration chromatography revealed a single peak (Fig. 3A). The elution volume of the peak was identical with that of the wild-type Fab, and, based on the analysis of SDS-PAGE, the peak was indicative of non-PEGylated Fab (data not shown). On the other hand, the elution profile of mutSS Fab exhibited two peaks, and the elution volume of the later peak corresponded to that of non-PEGylated Fab (Fig. 3B). From the analysis of SDS-PAGE for first elute (peak 1), the molecular weight of the protein from peak 1 (~119 kDa) was higher than that of the non-PEGylated Fab. The higher molecular weight was attributable to the high hydrodynamic radius of PEG (14). Moreover, SDS and PEG have been reported to form some kind of complex in SDS-PAGE (21, 22). Minor bands (70-80 kDa, >120kDa) appeared after PEGylation although purified mutSS FabSH was used for the reaction (Fig.3B). These bands may have nonspecifically bound PEG to Fab, but this might be characteristic product specific to PEGylated Fab on SDS-PAGE. To confirm to PEG attachment to the free cysteine at the C-terminal of mutSS FabSH, the Fab mutant (mutSS FabASS), which contains a novel interchain SS bond (CH1:C177–CL:C160) and lacks cysteine at the C-terminal end of both CH1 and CL, was used as the control. PEGylation treatment of mutSS FabASS was carried out under the same condition as that of mutSS FabSH. In the SDS-PAGE analysis of PEGylation mixture before gel filtration chromatography, mutSS FabASS did not show the high molecular weight band although mutSS FabSH showed an
apparent high molecular mass (~119 kDa) (supplemental Fig.1). Thus, the mutSS Fab_{SH} was PEGylated at Cys214 at the C-terminal end of CH₁.

**Characterization of the PEGylated mutSS Fab_{SH}**

Binding of PEGylated mutSS Fab_{SH} with human TNFα was evaluated by ELISA. The increase in absorbance at 450 nm was proportional with the increase in the concentration of the wild-type Fab as shown in Fig. 4. The curve of PEGylated mutSS Fab_{SH} was almost identical with that of the wild-type. The circular dichroism spectrum of PEGylated mutSS Fab_{SH} was also similar to that of the wild-type (data not shown). These results indicate that PEGylation of Cys214 at the C-terminal end of CH₁ has no involvement in the binding activity against human TNFα and the conformation of the Fab.

**Pharmacokinetics of PEGylated mutSS Fab_{SH}**

To confirm the effect of PEGylation, PEGylated mutSS Fab_{SH} was administered to mice through tail vein injection. Serial blood was drawn from tail vein at several hours after administration. The pharmacokinetic features of the PEGylated mutSS Fab_{SH} were compared with those of the non-PEGylated wild-type under the same conditions and dose. The concentration of mean serum versus time profiles of the wild-type and PEGylated mutSS Fab_{SH} in mice are shown in Fig. 5. The pharmacokinetic parameters of the wild-type and PEGylated mutSS Fab_{SH} are summarized in Table 1. The half-life of PEGylated mutSS Fab_{SH} was remarkably higher than that of the non-PEGylated wild-type Fab. Concomitantly, compared to non-PEGylated wild-type Fab, the PEGylated mutSS Fab_{SH} had a 40-fold reduction in clearance and a 40-fold increase in the area under the curve (AUC).
DISCUSSION

The Fab consists of a part of the heavy chain, which has a variable domain (VH₁) and a constant domain (CH₁), and of the light chain, which also has a variable domain (VL) and a constant domain (CL). The Fab structure is held together by two interdomain packing interactions at VH and VL interfaces, CH₁ and CL interfaces, and by one interchain SS bond that connects the C-terminal ends of the CH₁ and CL. Expression of Fab’ in microbial systems can result in protein aggregation and in disulfide scrambling due to solvent exposure of the free cysteine in the Fab’ (16,17). Due to the lack of the flexible hinge site, which carries the free cysteine, Fab can be more efficiently expressed in microbial systems than the Fab’. However, cysteine residues are the most reactive nucleophiles in proteins. In general, free cysteines are the suitable targets for site-directed labeling of whole IgG or antibody fragments for PEGylation or for production of antibody-drug conjugates (ADC). Although the interchain SS bond between CH₁ and CL at the C-terminal end of the Fab is a good target due to its accessibility and due to lack of steric hindrance, site-specific reduction of interchain SS bonds may lead to destabilization of protein. In a previous study, we prepared an adalimumab Fab mutant that lacks a internal SS bond at the C-terminal end (FabΔSS). This mutant had lower thermostability than the wild-type, suggesting that the interchain SS bond is important for maintaining the integrity of the Fab structure (18). Protein destabilization leads to the formation of aggregates or to early degradation (23,24). To recover the stability of FabΔSS, a engineered interchain SS bond outside the C-terminal end was introduced through the mutations CH₁:V177C and CL:Q160C within the constant region of FabΔSS (mutSS FabΔSS), followed by confirmation of the formation of the internal SS bond between
CH_{1} and CL. The melting temperature of mutSS Fab_{ASS} was 5 °C higher than that of Fab_{ASS} (18). In this paper, we created Fab mutant (mutSS Fab_{SH}), which contains a novel interchain SS bond (CH_{1}:C177–CL:C160) and one cysteine at the C-terminal end of CH_{1}. Although the free cysteine residue in protein might cause lower expression levels or disulfide scrambling in microbial systems (16), mutSS Fab_{SH} was expressed from \textit{P. pastoris} in sufficient quantity (Fig.2A). As shown in Fig.2B, the formation of the engineered interchain SS bond was confirmed by SDS-PAGE. It was demonstrated that the free cysteine residue at the C-terminal end of CH_{1} did not affect the formation of the three-dimensional structure of Fab. The purified mutSS Fab_{SH} was conjugated with PEG targeted at the free cysteine. Another reported approach for PEGylation is to target the interchain SS bond between C-terminal region (25) or the N-terminal \alpha-amino groups (14) of Fab. Our approach using mutSS Fab_{SH} does not require the reduction of intermolecular SS bonds or modification of the active site; therefore, it is expected to have productive advantage for important clinical applications.

PEGylation is widely adapted to improve drug bioavailability and pharmacokinetics (15). Certolizumab pegol, PEGylated Fab’, is different from other TNF\alpha inhibitors such as adalimumab and does not have an Fc region, and therefore, it can suppress CDC and ADCC caused by the Fc region. In vitro experiments have demonstrated that certolizumab pegol does not exert CDC or ADCC (26). Moreover, it has been reported that certolizumab pegol preferentially penetrates inflamed tissue compared to uninflamed tissue and the persistence of certolizumab pegol in the inflamed tissue is more prolonged than adalimumab, IgG molecule (27). Thus, the Fab fragments exhibit high therapeutic effects as a TNF\alpha inhibitor.

In this study, mutSS Fab_{ASH} containing a free cysteine at the C-terminal end was successfully expressed in \textit{P. pastoris}. The mutant was designed to recover the decrease of
thermostability caused by the lack of the interchain SS bond at the C-terminal end and to produce a Fab for site-directed modification with 20 kDa PEG. PEG molecules with molecular mass of 20 — 40 kDa are widely used to investigate the pharmaceutical properties of proteins (9,28,29). As shown in Fig. 5 and Table 1, there was 27-fold increase in the serum half-life of the PEGylated mutSS FabSH compared to that of the non-PEGylated wild-type Fab. This improvement is consistent with reports for other Fabs that have been conjugated with 20 kDa PEG (13).

Thus, we have successfully established a modifiable Fab platform that allows functionalization of the cysteine in the C-terminal end with PEG or with a pharmaceutical compound. This platform can therefore be used to engineer Fabs for clinical applications.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Materials

The online version of this article contains supplementary materials (Supplementary Fig. 1).
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Table 1
Pharmacokinetic parameters of the wild-type Fab, the PEGylated mutSS Fab_{SH}

|                  | Wild-type      | PEGylated mutSS Fab_{SH} |
|------------------|----------------|--------------------------|
| t1/2 (hr)        | 0.97 ±0.54     | 26.95 ± 2.46             |
| CL (L/hr)        | 0.12 ±0.02     | 0.0030 ± 0.0001          |
| AUC (mg·hr/L)    | 3.58 ±0.33     | 146.4 ±3.57              |

t1/2: elimination half-life; CL: clearance; AUC: area under the curve
Fig. 1. Schematic representation of the wild-type Fab and mutSS Fab\textsubscript{SH}.

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\includegraphics[width=0.8\textwidth]{figure1.png}
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Fig. 2. Cation-exchange chromatography (A) of mutSS Fab$_{SH}$ and SDS-PAGE analysis (B) of the eluted fraction (peak a) from resource S column. A mutSS Fab$_{SH}$ was purified by a resource S column in AKTA purifier system. The column was equilibrated with 0.05 M sodium acetate buffer (pH 5.0) and eluted with a linear gradient of NaCl from 0 to 0.1 M in equilibration buffer at a flow rate of 1 mL/min and total elution volume of 40 mL. The samples of the eluted fraction were analyzed by 12% SDS-PAGE under reducing and non-reducing condition.
Fig. 3. Gel filtration chromatography of control Fab (A) and mutSS Fab_{SH} (B) with PEGylation. The reaction mixture was loaded on a Superdex 75 equilibrated with 50 mM potassium phosphate buffer (pH 7.0) containing 0.2 M NaCl at a flow rate of 0.5 ml/min. The eluted fraction (peak 1) were analyzed in non-reducing 10% SDS-PAGE.
Fig. 4. ELISA assay of the wild-type adalimumab Fab (open circles) and PEGylated mutSS FabSH (closed triangles) for human TNFα. ELISA plate was coated with human TNFα, overnight at 4°C, followed by the reaction of wild-type Fab or PEGylated mutSS FabSH at the concentration of 12.5, 6.25, 3.125, 1.56, and 0.78 nM.
Fig. 5. The mean serum concentration versus time profiles of the wild-type Fab (open circles) and PEGylated mutSS Fab$_{SH}$ (closed triangles) in mice. Male SD rats weighing about 240 g were injected intravenously with 100 µg of non-PEGylated the wild-type Fab of or PEGylated mutSS Fab$_{SH}$ under isoflurane anesthesia. Serial blood samples were collected into heparinized syringes from tail vein at 0.017 (1min), 0.5, 1, 2, 4, 6, 8, 10, 24, 48, 72 and 168 h after administration. Serum concentration of Fab was determined by ELISA.