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Site-specific PEGylation of native disulfide bonds in therapeutic proteins

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Native disulfide bonds in therapeutic proteins are crucial for tertiary structure and biological activity and are therefore considered unsuitable for chemical modification[1,2]. We show that native disulfides in human interferon α-2b and in a fragment of an antibody to CD4$^+$ can be modified by site-specific bisalkylation of the two cysteine sulfur atoms to form a three-carbon PEGylated bridge. The yield of PEGylated protein is high, and tertiary structure and biological activity are retained.

It is generally considered that a protein's native disulfide bonds cannot be modified because they are crucial to its structure and function[1,2]. Covalent conjugation of poly(ethylene glycol) (PEG) to therapeutic proteins increases their *in vivo* stability by protecting the protein from degradation, masking its immunogenic sites and reducing clearance[3]. Typically, PEGylation uses nonspecific reactions with nucleophilic residues and produces mixtures of PEGylated positional isomers[4]. To solve this problem, we exploited the reactivity of the two sulfur atoms of a native disulfide for selective conjugation of PEG using a thiol-specific, cross-functionalized PEG monosulfone (**Fig. 1a**). Mechanistically, the conjugated double bond in the PEG monosulfone is necessary to initiate a sequence of addition-elimination reactions[5,6]. After addition of thiol, elimination of sulfinic acid generates another conjugated double bond for the second thiol (**Supplementary Scheme 1** and **Supplementary Methods** online). This leads to the formation of a three-carbon bridge between two sulfur atoms. Disulfide-scrambling reactions are inhibited because of thiol propinquity in the nondenatured protein and by having the bisalkylation functionality at the end of PEG.

We used interferon α-2b (IFN) because it is representative of four-helical-bundle proteins with accessible disulfide bonds. Theoretically, the effect of introducing a three-carbon bridge is determined using stochastic dynamics simulations. The bridged IFN isomers Cys1-CCC-Cys98 and Cys29-CCC-Cys138 are within the conformational flexibility of the crystal and NMR-based structures of interferon α-2a, indicating that IFN's tertiary structure is preserved[7] (**Supplementary Results 1** online).

We found that a three-carbon disulfide-bridged PEG-IFN can be prepared when one protein equivalent (equiv.) of PEG monosulfone is used after reducing both disulfides. Conjugation is conducted at pH 7.8 and 4 °C for 2 h after removal of excess dithiothreitol. If two equivalents of PEG monosulfone are used, both disulfides undergo conjugation. As a control, we conjugated a non-PEG precursor to IFN. SDS-PAGE gels showed IFN's conjugation to precursor and PEG monosulfone, with MALDI-TOF–MS confirming the M_w of the isomers Cys1-CCC-Cys98 and Cys29-CCC-Cys138 (**Fig. 1b–d**) and of their trypsin-digested fragments (**Supplementary Results 2** online). The three-carbon–bridged PEG-IFNs were purified by

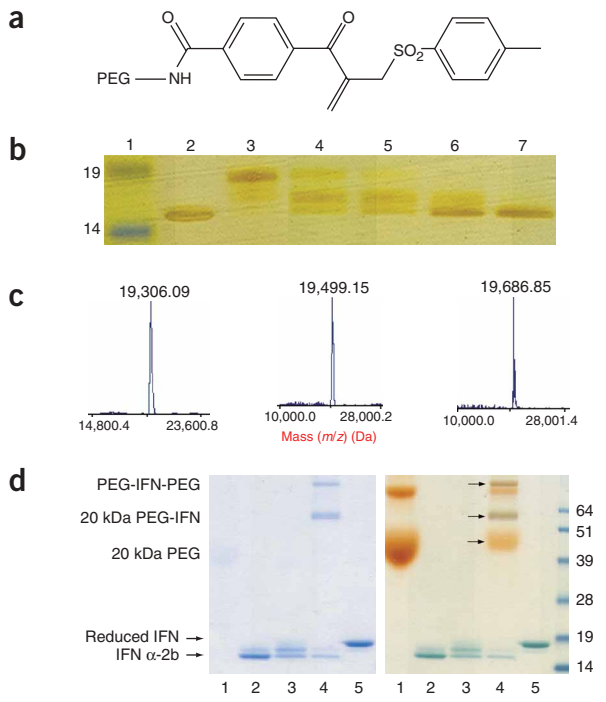

Figure 1 Structural characterization. (**a**) PEG monosulfone. (**b**) Silver-stained gel of the non-PEGylated three-carbon (190 Da) disulfide-bridged IFN. Lanes: (1) M_w markers (kDa); (2) IFN; (3) reduced IFN; (4) 1 equiv. bissulfone showing IFN (upper), single-bridged (middle) and double-bridged (lower) IFN; (5 and 6) 2 and 4 equiv., respectively, showing single-bridged (upper) and double-bridged (lower) IFN; (7) 6 equiv. showing double-bridged IFN. (**c**) MALDI-TOF–MS of IFN (left), Cys-CCC-Cys IFN (middle) and double-bridged IFN (right). (**d**) Gels stained with colloidal blue (protein) and barium iodide (PEG, right). Lanes: (1) 20 kDa PEG; (2) IFN; (3) IFN with reduced disulfide; (4) PEGylation reaction mixture; (5) IFN with both disulfides reduced.

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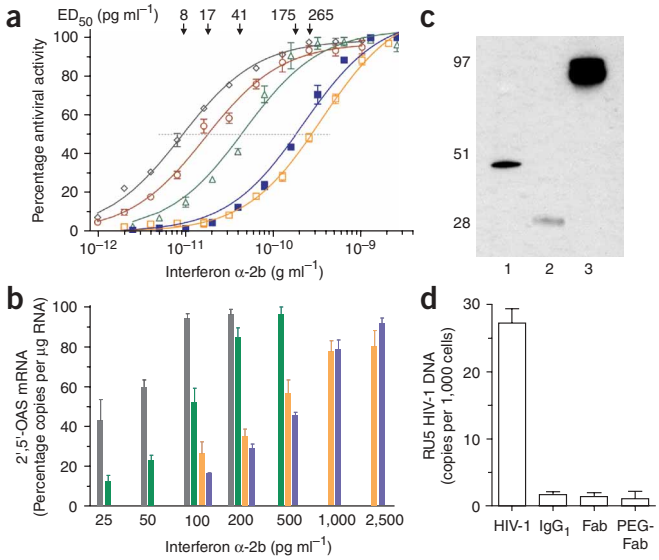

Figure 2 Biological activities. (**a**) Antiviral activity in A549 cells infected with EMC virus ($n = 6$). (**b**) 2′,5′-OAS mRNA synthesis in Molt-4 cells ($n = 3$). IFN (gray); unreacted IFN recovered after SEC-HPLC (red); non-PEGylated three-carbon disulfide-bridged IFN (green); three-carbon disulfide single-bridged 10 kDa PEG-IFN (orange); three-carbon disulfide single-bridged 20 kDa PEG-IFN (blue). (**c**) Immunoblot with an antibody to Fab. M_w markers (left) are in kDa. Lanes: (1) Fab; (2) reduced Fab; (3) three-carbon disulfide single-bridged 20 kDa PEG-Fab. (**d**) Inhibition of HIV-1 entry into human C8166 (T-lymphoblastoid) cells as determined by real-time PCR for RU5, the first DNA transcript of HIV-1 to be synthesized after viral entry ($n = 3$). Data presented as mean ± s.e.m.

cation-exchange chromatography followed by size-exclusion chromatography (SEC)-HPLC with confirmation by western immunoblotting. The SEC-HPLC chromatogram showed a three-carbon disulfide single-bridged PEG-IFN (that is, Cys1-CC[PEG]C-Cys98 or Cys29-CC[PEG]C-Cys138, yield 65%), a three-carbon disulfide double-bridged PEG-IFN (Cys1-CC[PEG]C-Cys98 and Cys29-CC[PEG]C-Cys138, yield 23.5%), IFN (yield 4.9%) and aggregated IFN (yield 6.6%) (**Supplementary Results 3** online).

The reaction can be simplified by *in situ* conversion of the PEG bissulfone to the PEG monosulfone at pH 7.8 during protein conjugation. Competitive reactions of the PEG monosulfone with other nucleophilic residues are not seen (**Supplementary Results 4** online). MALDI-TOF–MS confirmed the M_w of the two-bridged PEG-IFN isomers, and CD confirmed the preservation of IFN's α-helical structure (**Supplementary Results 2**).

Interferon α-2b has distinct effects *in vitro*: it blocks infection of human A549 (lung epithelial) cells by encephalomyocarditis (EMC) virus, it induces 2′,5′-oligoadenylate synthetase (2′,5′-OAS) mRNA synthesis, and it upregulates major histocompatibility (MHC) class I expression on immunoregulatory cells (**Supplementary Methods**). Using SEC-HPLC, we found that the unreacted IFN and the non-PEGylated three-carbon disulfide single-bridged IFN both showed a small reduction in antiviral activity compared to IFN (**Fig. 2a**,**b**). Our results also showed that insertion of a three-carbon disulfide bridge contributed ~11%, and addition of PEG contributed ~89% to the reduction in the PEG-IFN's biological activity. Because PEG reduces protein immunogenicity, the PEG-IFNs have a lower affinity for MHC class I molecules than IFN (**Supplementary Results 5** online). Uniquely, the PEG's length does not affect its biological activities. The PEG-IFN's biological activities (~8% of IFN) are similar to those of the PEG-IFN in clinical use (~7%)[8–10]; the enhanced *in vivo* therapeutic efficacy compensating for the reduced *in vitro* activity[10]. Our PEG-IFNs are stable in aqueous solution for 3 months at 4 °C; and in human serum for 30 h at 37 °C. After subcutaneous administration in mice, the 20 kDa PEG-IFN's half-life is 12 h compared to 1 h for IFN.

We applied this approach to a human CD4 receptor–blocking antibody fragment (Fab). Entry of HIV-1 into cells requires viral gp120 to bind the D1 domain of human CD4. The IgG_1 monoclonal antibody Q4120/ADP318 (which binds the D1 domain of CD4; ref. 11) was digested to make Fab and PEGylated after reduction of its interchain disulfide (**Fig. 2c**). At a saturating dose, the PEG-Fab was as effective as Fab at blocking HIV-1 entry into $CD4^+$ T-lymphocyte cells (**Fig. 2d**).

Our studies also include the PEGylation of L-asparaginase without loss of enzyme activity or immunogenicity[12]. The accessible native disulfide bonds of proteins can therefore be modified by the site-specific insertion of a three-carbon PEGylated bridge. Our approach differs fundamentally from conjugation of PEG to amine residues[8–10], where the biological activity of the PEGylated positional isomers depends upon conjugation conditions and the size of PEG[4]. It also makes engineering free cysteines into proteins for thiol-selective PEGylation unnecessary. As the biological activities of our PEGylated proteins are independent of PEG size, only their *in vivo* pharmacokinetics need optimizing before clinical trials.

Note: Supplementary information is available on the Nature Chemical Biology website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Chemical Biology* website for details).

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