ABSTRACT: The efficacy of protein-based medicines can be compromised by their rapid clearance from the blood circulatory system. Achieving optimal pharmacokinetics is a key requirement for the successful development of safe protein-based medicines. Protein PEGylation is a clinically proven strategy to increase the circulation half-life of protein-based medicines. One limitation of PEGylation is that there are few strategies that achieve site-specific conjugation of PEG to the protein. Here, we describe the covalent conjugation of PEG site-specifically to a polyhistidine tag (His-tag) on a protein. His-tag site-specific PEGylation was achieved with a domain antibody (dAb) that had a 6-histidine His-tag on the C-terminus (dAb-His6) and interferon (IFN) that had an 8-histidine His-tag on the N-terminus (His8-IFN). The site of PEGylation at the His-tag for both dAb-His6-PEG and PEG-His8-IFN was confirmed by digestion, chromatographic, and mass-spectral studies. A methionine was also inserted directly after the N-terminal His-tag in IFN to give His8Met-IFN. Cyanogen bromide digestion studies of PEG-HisMet-IFN were also consistent with PEGylation at the His-tag. By using increased stoichiometries of the PEGylation reagent, it was possible to conjugate two separate PEG molecules to the His-tag of both the dAb and IFN proteins. Stability studies followed by in vitro evaluation confirmed that these PEGylated proteins retained their biological activity. In vivo PK studies showed that all of the His-tag PEGylated samples displayed extended circulation half-lives. Together, our results indicate that site-specific, covalent PEG conjugation at a His-tag can be achieved and biological activity maintained with therapeutically relevant proteins.

INTRODUCTION

During the last 30 years, therapeutic protein and peptide-based pharmaceuticals have become a broad group of medicines that are used to treat many medical indications. With the general exception of monoclonal antibodies, the efficacy of therapeutic proteins and peptides is often compromised because of rapid clearance. The imposition of frequent dosing due to rapid clearance can pose an increased safety risk due to potential immunogenicity and an increased incidence of side effects.

Much effort has been spent to solve the fundamental limitation of suboptimal pharmacokinetics in the development and use of protein-based medicines. Three general approaches are followed to prolong the circulation half-life of proteins: (i) increase the overall size of the protein molecule in circulation while decreasing proteolytic and aggregation pathways (e.g., PEGylation1−8 and glycoengineering9−15), (ii) exploit recycling mechanisms (e.g., protein fusion to Fc or albumin for endocytic recycling, or bispecific motifs to allow binding to a circulating blood protein such as albumin16−22), and (iii) utilize release systems to prolong dosing into the bloodstream (e.g., colloids, pumps).

Of these strategies, protein PEGylation has been clinically proven to be a general approach,1 and in some cases, the PEGylated protein has become a first line treatment.23,24 Much clinical experience has shown that PEGylated proteins are safe and there are at least 10 registered PEGylated medicines in the clinic. PEG is often conjugated to various residues on different amino acids of the same protein yielding positional isomers.25−27 Considering the PEGylated proteins in development,3 site-specific PEGylation strategies are increasingly dominating the development of mono-PEGylated proteins, which tend to be used for cell surface receptor targets.

The site of PEGylation can impact biological and pharmacodynamic (PD)/pharmacokinetic (PK) properties of
the protein.\textsuperscript{25,28} PEGylation can decrease the measured protein binding,\textsuperscript{25} probably due to reduced on-rates caused by steric shielding effects,\textsuperscript{29} and often decreased activity is observed for the PEGylated protein compared to the un-PEGylated protein. Preclinical development could be accelerated if site-specific PEGylation was easier to accomplish, because in some cases, it appears that when PEG is conjugated to the same site on a protein, \textit{in vitro} activity can be broadly maintained as PEG size is changed.\textsuperscript{30} However, there is still much to be learned about how \textit{in vitro} biological properties for PEGylated proteins correlate to what is observed \textit{in vivo}.\textsuperscript{31,32} The possibility exists that, if higher potency can be achieved while the protein displays extended circulation times, then lower doses or less frequent dosing schedules may be possible, either of which may be important as a means to minimize side effects over a given course of treatment.

PEGylation does not cause a protein to display varied biological functions,\textsuperscript{33−35} however, each PEG positional isomer will inevitably display different relative biological activities.\textsuperscript{26,27,36−39} It is becoming increasingly clear that the clinical use of protein-based medicines is complex. Clearly, there are consequences from the processes used in the manufacture of proteins as well as the inherent complexity of protein structure. Unwanted side effects such as an increased susceptibility to infections or malignancies may develop long after treatment has started or even after treatment has stopped.\textsuperscript{40} Post-approval safety-related regulatory actions have been issued for about 23% of these medicines in the U.S. and European Union.\textsuperscript{41} This regulatory vigilance led to the withdrawal of efalizumab in 2009 and gemtuzumab ozogamicin in 2010. The presence of heterogeneous mixtures may contribute to long-term safety related issues of protein-based medicines.\textsuperscript{42} To minimize the formation of positional isomers for future PEGylated products, there is a need for robust strategies that are economically viable to conjugate PEG site-specifically to a protein.

Of the many strategies that have been described to conjugate PEG site-specifically to a protein, few have been scaled and developed clinically.\textsuperscript{43} We now describe how PEG-bis-sulfone \textsuperscript{1}\textsuperscript{30,44} along with two new PEG-bis-sulfones 2 and 3 (Figure 1) can site-specifically PEGylate a polyhistidine-tag (His-tag) on a protein (Scheme 1). These reagents 1−3 are capable of undergoing site-specific conjugation by bis-alkylation with PEG-Mono-Sulfones \textsuperscript{1a−3a} (Figure 1).

![Figure 1. PEG-bis-sulfones 1−3 followed by elimination of sulfinate anion to give the corresponding PEG-mono-sulfones 1a−3a.](image)

![Scheme 1. Possible Mechanism for Site-Specific PEGylation at a His-Tag by Bis-Alkylation with PEG-Mono-Sulfones 1a−3a](image)
function of the protein. However, care must be exercised with the location and size of the His-tag. While His-tagged proteins are being used in the clinic, their widespread therapeutic use may have been hampered by (i) the lack of a defined function for the His-tag post purification and (ii) anxiety that there will be an increased propensity of a secondary antibody response to the tag. PEGylation at a His-tag will remove the first of these concerns and is expected to address the second concern.

Development of a generic approach for the site-specific PEGylation of a protein at a His-tag may provide the opportunity to develop more effective PEGylated proteins by ensuring that both half-life and biological activity can be optimized, since His-tags can often be fused at either the protein C- or N-terminus. Either or both termini in many therapeutic proteins are generally accessible for conjugation and are not generally directly involved with binding. PEGylation at the His-tag may generally result in high retained activity while extending half-life.

We describe the His-tag-specific PEGylation of two proteins: (i) a domain antibody (dAb) that binds tumor necrosis factor alpha (TNFα) and has a 6-histidine His-tag on the C-terminus (dAb-His<sub>6</sub>) and (ii) interferon α-2a (IFN) that has a 8-histidine His-tag on the N-terminus (His<sub>8</sub>-IFN). The dAb was selected because it exemplifies short half-life antibody fragments and alternative scaffolds, several of which are in clinical development. For many indications, these novel proteins require an increased circulation half-life to become clinically viable. IFN is generally preincubated as described above to generate the corresponding PEG-mono-sulfones in situ before adding to the protein solution. Immediately after PEGylation, each reaction mixture was buffer-exchanged using either a PD-10 or HiPrep 26/10 column to the buffer compatible with ion exchange chromatography. The choice of column was obtained after scouting experiments. At the end of each purification step, the fractions were analyzed by SDS-PAGE to confirm the separation of the PEGylated product from unreacted PEG and PEGylated proteins. PEGylation studies were conducted using PEG molecular weights ranging from 10 to 40 kDa. Depending on the PEG and reaction type, varying protein to PEG molar ratios were used (0.5–6 mol equiv of PEG reagent). During optimization of the PEGylation process, reactions were performed at temperatures ranging from 4 to 25 °C and the reaction time ranged from 2 to 17 h. Reactions that were conducted on a scale necessary to isolate larger amounts of material for PK studies were conducted overnight at 20 °C. The PEG-bis-sulfone reagents 1–3 were generally preincubated as described above to generate the corresponding PEG-mono-sulfones in situ before adding to the protein solution. Immediately after PEGylation, each reaction mixture was buffer-exchanged using either a PD-10 or HiPrep 26/10 column to the buffer compatible with ion exchange chromatography. The choice of column was obtained after scouting experiments. At the end of each purification step, the fractions were analyzed by SDS-PAGE to confirm the separation of the PEGylated product from unreacted PEG and protein.

A representative preparation of PEG-His<sub>8</sub>-IFN is described using 20 kDa PEG-bis-sulfone reagent 1: To a solution of His<sub>8</sub>-IFN (1.37 mg/mL, 0.7 mL) in sodium acetate buffer, pH 5.3, containing 35 μM hydroquinone, was added 2.0 mol equiv of PEG-bis-sulfone reagent 1 (0.096 mL, 20 mg/mL) in 50 mM sodium phosphate, pH 7.4. The bis-sulfone reagent 1 was preincubated in the buffer for 4 h at 20 °C prior to its addition to the His<sub>8</sub>-IFN solution. The reaction solution was incubated for 17 h at 20 °C. The reaction mixture was then treated with sodium triacetoxylborohydride (25 mM final concentration and added as a solid) and further incubated for 1 h at 4 °C. Purification was performed using a HiTrap SP HP 5 mL cation exchange column, followed by polishing purification step using a Superdex 200 prep grade size exclusion column. The PEGylation reaction mixture was first buffer-exchanged into loading buffer (50 mM sodium acetate buffer, pH 4.0) using a PD-10 column. A total of 10 column volumes of loading buffer were then used to wash the column to remove residual PEG. A gradient elution from 0% to 100% elution buffer (50 mM sodium acetate, 1.0 mM sodium chloride, pH 4.0) was carried out, typically over 30 min at 1 mL/min flow rate, to separate the PEGylated proteins. Eluates were fractionated and analyzed by SDS-PAGE. Fractions containing mono-PEGylated product were pooled and concentrated to 2 mL using a Vivaspin concentrator (10 000 MWCO, 3000 g, 4 °C). The solution was then purified with a Superdex 200 prep grade column at a flow rate of 1 mL/min and 50 mM sodium phosphate buffer, 150 mM NaCl, pH 7.5, as a mobile phase.
For preparation of (PEG)$_2$-His$_8$-IFN, 2.5 mol equiv of PEG-bis-sulfone reagent 1 was added to the protein solution, using the same reaction conditions as described for PEG-His$_8$-IFN. The purification conditions were optimized so that the di-PEGylated conjugate could be separated from unconjugated PEG, mono-PEGylated and unreacted His$_8$-IFN using ion exchange chromatography only. Purification was performed using a MacroCap SP 5 mL cation exchange column with 100 mM sodium acetate buffer, pH 4.0, as the loading buffer. A step elution (0%, 27%, 38%, 60%, 100%) was carried out using 100 mM sodium acetate, 1.0 M sodium chloride, pH 4.0, as an elution buffer, typically over 60 min at 2 mL/min flow rate, to separate the PEGylated proteins. Eluates were fractionated and analyzed by SDS-PAGE. Fractions containing di-PEGylated product were pooled and repurified on the MacroCap SP 5 mL cation exchange column using the same method to enhance the purity of (PEG)$_2$-His$_8$-IFN.

**Tryptic Peptide Digestion and MALDI-TOF-MS Analysis.**

Sequence grade modified trypsin was reconstituted (0.2 mg/mL) in 50 mM acetic acid. Tryptsin solution (60 μL) was then added to solutions of dAb-His$_6$ and PEGylated dAb-His$_6$ samples (0.26 mg/mL, 1 mL) in 25 mM ammonium acetate, and the digestion reactions were incubated at 37 °C for up to 30 h. For PEGylated His$_8$-IFN products, trypsin was reconstituted (0.2 mg/mL) in 50 mM ammonium bicarbonate, pH 8.5. Typically, 23.4 μL of trypsin solution was added to 100 μL of a 1 mg/mL protein solution in 50 mM ammonium bicarbonate, pH 8.5, and the digestion reactions were incubated at 37 °C for 8 h. Cyanogen bromide (CNBr) digestion was performed on reduced and denaturated samples (10 mM DTT in 6 M Gu.HCl, 20 mM Tris pH 8.0). Prior to digestion, hydrochloric acid was added to the sample (final concentration 0.5 M), then solid CNBr reagent was added (final concentration of 1 M, protein final concentration of 0.1 mg/mL, 0.1 mL). Digestion was carried out overnight at room temperature in the dark and terminated by buffer exchange using a MacroTrap (C8) column. Digest mixtures were fractionated by RP-HPLC performed on a VariTide RPC column (VARIAN, 5 μm, 100 Å, Column dimensions: 250 × 4.6 mm). Fractions were collected, lyophilized, and then analyzed by MALDI-TOF-MS, SDS-PAGE, Western blot, and dot blot performed with anti-His-tag and anti-PEG antibodies. The masses of the mono-PEGylated and di-PEGylated species and recovered protein were confirmed by MALDI-TOF mass spectrometry using a Voyager DE-PRO MALDI-TOF (Applied Biosystems). The instrument was calibrated...
with the BSA calibration standard kit from AB Sciex. Positive linear mode was used for high molecular mass measurements and reflectron mode was used for low molecular mass measurements. Sinapinic acid (10 mg/mL in acetonitrile/water + 0.1% TFA 50:50 (v/v)) or α-cyano-4-hydroxycinnamic acid (10 mg/mL in acetonitrile/water + 0.1% TFA 50:50 (v/v)) was used as the matrix. Samples were prepared by serial dilution with a matrix solution and allowed to dry before induction into the mass spectrometer. The data were processed using Data Explorer software (AB Sciex) and mMass software.55

**Stability Studies.** A solution of dAb-His6-PEG (0.15 mg/mL) in 50 mM sodium phosphate buffer containing 150 mM sodium chloride, pH 7.4, was aliquoted into 7 different tubes (2 mL) in 50 mM sodium phosphate buffer, 150 mM NaCl pH 7.5 at 4°C for up to 2 months. For samples of dAb-His6-PEG used in the PK study, accelerated stability studies were also performed in 10 mM ammonium bicarbonate at 50°C (1 h) and 90°C (10 min) with or without the addition of 10 mM DTT.

PEG-His6-IFN was incubated in 50 mM sodium phosphate buffer, 150 mM NaCl pH 7.5 at 4°C, room temperature or 37°C. To each tested sample was added 1 mM sodium azide to prevent bacterial growth and protease inhibitor cocktail (dilution 1:500) to inhibit proteolytic digestion. For samples used in the pharmacokinetic studies, accelerated stability studies were also performed in 10 mM ammonium bicarbonate at 50°C (1 h) and 90°C (10 min) with or without the addition of 10 mM DTT.

**RESULTS**

**Site-Specific PEGylation of a C-Terminal His6 Domain Antibody (dAb-His6).** PEG-bis-sulfone 1 (10 and 20 kDa PEG molecular weight) was preincubated to allow the formation of the corresponding PEG mono-sulfone 1a before addition (1.5 mol equiv) to a solution of the dAb-His6 to give a mono-PEGylated domain antibody (dAb-His6-PEG) product (Figure 2A, lanes 2 and 3). Addition of the PEG-bis-sulfone 1 (20 kDa) to a dAb variant without the His6 tag under the same conditions did not result in any PEGylation of the dAb (Figure 2A, lane 1).

Using dAb-His6, the PEGylation conditions were optimized with the 20 kDa PEG-bis-sulfone 1. The mono-PEGylated product (dAb-His6-PEG) was the major product (40–50%), with a di-PEGylated dAb product (dAb-His6-(PEG)2) also being formed (10–15%). A trace amount of a tri-PEGylated product could also be observed by SDS-PAGE. The optimal concentration for PEGylation of the dAb-His6 was in the range of 1–2.5 mg/mL. The PEGylation reaction was reproducible and was conducted at scales of up to 30 mg dAb-His6.

Increasing the stoichiometry of the PEG-bis-sulfone 1 (20 kDa) to 2.4 equiv allowed the formation of a greater amount of the di-PEGylated dAb (dAb-His6-(PEG)2). Using optimized conditions with PEG-bis-sulfone 1 (20 kDa), purification of both dAb-His6-PEG and dAb-His6-(PEG)2 was achieved by ion exchange chromatography (Figure 2A, lanes 4 and 5, respectively). When an IMAC column was used, the PEGylated species displayed decreased binding affinities compared to the starting dAb-His6. IMAC binding decreased with increased PEGylation. Hence, dAb-His6-(PEG)2, eluted first, followed by dAb-His6-PEG, and then unreacted dAb-His6 was sequentially eluted from the IMAC column with increasing imidazole concentration.

A dAb with a C-terminal 2-histidine tag (dAb-His2) was also prepared (Supporting Information (SI) Figure S1-D). The dAb-His6 was found to undergo reaction with 1 equiv of PEG-bis-sulfone 1 (20 kDa) in conditions where no conjugation was observed for the dAb without a His-tag. The PEGylated product, dAb-His2-PEG, was purified (Figure 2A, lane 6). Additional His-tag motifs were considered by examining the PEGylation of C-terminal His-tags of chemerin-9, which corresponds to the C terminus of processed chemerin (149-YFPGQFAFS-157). Chemerin-9 peptides with C-terminal His-tags of 2, 4, and 6 histidines were allowed to react with PEG-bis-sulfone 1 (SI Figure S2-A). In each case, PEGylation was observed by RP-HPLC. No PEGylation was observed for the non-His-tag version of chemerin-9. A variant of chemerin-9 with a HGHGHG tag was also observed to undergo PEGylation (SI Figure S1-E).

The mass, as recorded by MALDI-TOF-MS of the purified dAb-His6-PEG (20 kDa PEG) was 32.7 kDa (SI Figure S1-E). The PEGylation product from the reaction of 20 kDa PEG-bis-sulfone 1 and dAb-His6 was subjected to trypsin digestion (Figure 2B, lanes 4–7). There is a cleavage site (lysine-arginine) adjacent to the His-tag (SI Figure S1-A, Figure 2C). It was found in multiple (>12) experiments that this cleavage site was digested more quickly than the other cleavage sites within the dAb (Figure 2B, lane 3). Digestion of dAb-His6-PEG (20
kDa) followed by SDS-PAGE analysis produced a band that is consistent for the dAb fragment and a band consistent for His6-PEG that was slightly below that of the dAb-His6-PEG (Figure 2B, lanes 5 and 7). MALDI-TOF-MS analysis of the digestion mixture (SI Figure S1-F) was consistent with the SDS-PAGE analysis. Masses of fragments obtained by digestion were observed that were consistent with the non-His-tag dAb (11.9 Da) and the expected mass of 20.8 kDa for His6-PEG (0.8 for His6 tag + ~20 kDa for PEG) (SI Figure S1-F).

Further analysis of the trypsin digestion mixture by RP-HPLC (Figure 2D) showed a new fraction in the digestion mixture at 22.4 min for the 20 kDa dAb-His6-PEG compared to the digestion mixture of dAb-His6. This fraction contained the PEG conjugated fragment (Figure 2E, lane 2). MALDI-TOF-MS analysis of this fraction (Figure 2F) indicated that its mass was approximately 20.8 kDa, which is the expected mass for His6-PEG. The difference in mass between dAb-His6-PEG and His6-PEG is 11.9 kDa, which is the expected mass of the dAb fragment without the His6 fragment. A mass for the free dAb (11.9 kDa) was also observed in the digestion mixture (SI Figure S1-F). The observed (theoretical) masses of the starting dAb-His6 and dAb are 12755 (12756.2) and 11996 (11933.3) Da, respectively (SI Figures S1-C and S1-B, respectively).

The purified di-PEGylated product, dAb-His6-(PEG)2 (2 × 20 kDa PEG) displayed a mass as recorded by MALDI-TOF-MS of ~55.4 kDa (SI Figure S1-G) and appeared to undergo digestion more slowly than the mono-PEGylated dAb-His6-PEG. A new peak in the RP-HPLC (SI Figure S1-H) was observed at 23 min from the digestion mixture of dAb-His6-(PEG)2 and the mass of this peak as determined by MALDI-TOF-MS was ~41.3 kDa (Figure 3B). Tryptic digestion and SDS-PAGE analysis of dAb-His6-PEG (20 kDa) was also consistent with PEGylation having occurred at the His-tag (SI Figure S1-I).

Prior to biological evaluation, the PEGylated dAb was examined for its stability (SI Figure S3-A). There was no evidence of dePEGylation by SDS-PAGE analysis when dAb-His6-PEG (20 kDa PEG) was stored at a concentration of 0.15 mg/mL in PBS solution at pH 7.4 for 60 d at 4 °C. Storage at 37 °C in the same solution for 5 d also did not appear to cause the loss of PEG from the dAb. Since the PEG-hex-sulfone 1 undergoes bis-alkylation by a sequence of conjugate addition and elimination reactions, stability was also evaluated at pH 2 and at pH 13. When dAb-His6-PEG was stored as a solution at a concentration of 0.15 mg/mL at 37 °C for up to 55 min at both of these pH values, the conjugate was stable.

The PEGylated dAb appeared to maintain the same relative conformational structure as determined by circular dichroism (CD) (SI Figure S3-B). The thermal denaturation profiles of dAb-His6, dAb-His6-PEG, and dAb-His6-(PEG)2 were also conducted using CD (SI Figure S3-C). Attaching 1 or 2 PEG molecules did not alter the secondary structure of dAb-His6 or cause thermal instability. Further corroboration that the conformational structure of the dAb-His6 was maintained after PEGylation was obtained by molecular dynamics simulation studies (SI Figure S3-D). These computational modeling experiments suggested that when two PEGs are conjugated to the His-tag they may both extend independently from the protein, but from the same relative site on the protein without effecting protein conformation.

Figure 4. (A) In vitro activity of dAb-His6 in L929 cytotoxicity assay. dAb-His6 (closed circle), dAb-His6-PEG (20 kDa PEG; open square) and dAb-His6-(PEG)2 (2 × 20 kDa PEG; open triangle), and dAb-His6 (open circle) and dAb-His2-PEG (20 kDa PEG; closed square). (B) Pharmacokinetic profile of iodinated dAb-His6 and PEGylated dAb-His6 in mice. dAb-His6 (closed circle), dAb-His6-PEG (20 kDa PEG; open square), and dAb-His6-(PEG)2 (2 × 20 kDa PEG; open triangle).

The dAb and its PEGylated products were evaluated for their ability to inhibit TNFα-mediated cytotoxicity in a L929 cells-based assay (Figure 4A, SI Figure S4). The activity of the dAb-His6 was not significantly different (1 way ANOVA with Bonferroni's multiple comparison test) with the nontagged dAb (2.4 ± 1.0 μg/mL (n = 10) and 3.1 ± 0.6 μg/mL (n = 4), respectively). Following PEGylation, the mono-PEGylated product, dAb-His6-PEG (20 kDa PEG), retained more than 90% of its activity with an ED50 value of 2.6 ± 0.3 μg/mL (n = 16). The di-PEGylated product, dAb-His6-(PEG)2 (2 × 20 kDa PEG), was also active with an ED50 value of 4.8 ± 0.6 μg/mL (n = 5). The mono-PEGylated product, dAb-His6-PEG (10 kDa PEG), also appeared to retained most of its activity (ED50 = 1.0 μg/mL, n = 1). Similarly for dAb-His6, the in vitro activity for both dAb-His6 and dAb-His6-PEG (20 kDa PEG) displayed similar ED50 values (2.7 ± 1.6 μg/mL (n = 3) and 2.7 μg/mL (n = 1), respectively). These results indicate a high retention of activity in vitro remained after PEGylation at the C-terminal His-tag on the dAb.

Blood circulation half-lives of 1125 radiolabeled dAb-His6, dAb-His6-PEG (20 kDa PEG) and dAb-His6-(PEG)2 (2 × 20 kDa PEG) were estimated following their administration to mice and determination of radioactivity levels in plasma (Figure 4B). The non-PEGylated dAb-His6 had a half-life of approximately 5 min. With one 20 kDa PEG conjugated to His6 the half-life was extended to 4.8 h, and with two 20 kDa PEG molecules attached, it was extended further to 18 h. The AUC for non-PEGylated dAb-His6 was 14.4 %ID·h/mL, which
Figure 5. (A) Representative SDS-PAGE (colloidal blue) for the PEGylation of His8-IFN with PEG-bis-sulfones 2 and 3. Lane M, protein standards; Lane 1, control reaction of 20 kDa PEG-bis-sulfone 2 (1.5 equiv) and IFN; Lane 2, reaction mixture of 20 kDa PEG-bis-sulfone 2 (1.5 equiv) and His8-IFN; Lane 3, purified PEG-His8-IFN (20 kDa PEG); Lane 4, purified PEG-His8-IFN (30 kDa PEG); Lane 5, purified PEG-His8-IFN (40 kDa PEG); Lane 6, purified (PEG)$_2$-His8-IFN (2 × 20 kDa PEG); and Lane 7, purified (PEG)$_2$-His8-IFN (2 × 30 kDa PEG). Lanes 8 and 9 show the b-PEG-His8-IFN derived from PEG-bis-sulfone 3. Lane 8, purified b-PEG-His8-IFN (40 kDa PEG) and Lane 9, b-PEG-His8-IFN (60 kDa PEG). (B) Computationally calculated (AMMP molecular mechanics) lowest energy conformations of PEG-bis-sulfones 1 and 2 to illustrate the change in conformation of PEG (22 repeat units) with the two different leaving groups used in these reagents. (C) Table of expected relevant peptide fragments from both His8-IFN (fragments A-C) and PEG-His8-IFN (fragments D and E) after tryptic digestion. (D) Comparative tryptic digestions of native His8-IFN (lanes 1−3) and PEG-His8-IFN (20 kDa PEG) (lanes 4−6); Lane M, protein standards; Lane 1, His8-IFN; Lane 2, tryptic digestion of His8-IFN; Lane 3, DTT treatment of His8-IFN followed by tryptic digestion; Lane 4, PEG-His8-IFN; Lane 5, tryptic digestion of PEG-His8-IFN; and Lane 6, DTT treatment of PEG-His8-IFN followed by tryptic digestion. Lanes 1′ to 6′ correspond to anti-His-tag Western blots of lanes 1 to 6. (E) SDS-PAGE (colloidal blue, barium iodide, Western blot) of the RP-HPLC fractions for the tryptic digestion fragments obtained for His8-IFN (A, C, lanes 1−2) and PEG-His8-IFN (20 kDa PEG) (D, E, lanes 3−4). Arrow indicates the presence of a faint band corresponding to fragment C.
was increased by 13-fold (188 %ID.h/mL) and 64-fold (919 %ID.h/mL) following the addition of one or two 20 kDa PEG molecules, respectively.

**Site-Specific PEGylation of an N-Terminal His$_8$ Interferon $\alpha$-2a (His$_8$-IFN).** PEG-bis-sulfone 1 underwent reaction with His$_8$-IFN as it did with dAb-His$_8$ (SI Figure S5-A). Control reactions under the conjugation conditions with non-histidine-tagged IFN indicated that IFN did not undergo PEGylation with PEG-bis-sulfone 1 unless a His$_8$-tag was present in the protein (SI Figure S5-A, lane 1). To examine different reagent variants, PEG-bis-sulfones 2 and 3 were prepared (Figure 1). PEG-bis-sulfone reagent 2 was prepared directly from reagent 1 (Scheme 2) to examine the effects of the sulfinic acid leaving group (e.g., 4 vs 5). Reagent 3 was prepared (Scheme 3) to conjugate two PEG chains through the conjugation of one molecule of the reagent to the protein.

PEG-bis-sulfone 2 (20 kDa PEG) was preincubated to allow the partial formation of the corresponding PEG-mono-sulfone 2a before 1.5 mol equiv was added to a solution of His$_8$-IFN to give the mono-PEGylated product (PEG-His$_8$-IFN) (Figure SA, lane 2). Several PEG-His$_8$-IFN variants including di-PEGylated products (i.e., (PEG)$_2$-His$_8$-IFN) derived from the conjugation of two molecules of PEG-mono-sulfone 2a were produced and purified (Figure SA, lanes 3–7). As with PEG-bis-sulfone 1, control reactions with non-His-tagged IFN indicated that IFN did not undergo PEGylation with PEG-bis-sulfone 2 in the conditions used for conjugation unless a His$_8$-tag was present in the protein (Figure SA, lane 1). During purification and as with dAb-His$_8$-PEG, differential binding was observed between PEGylated and unPEGylated His$_8$-IFN species when metal affinity columns were used.

Generation of the PEG-mono-sulfones (Figure 1) can be tailored by reaction conditions (e.g., pH$_4$ concentration) so that PEGylation can be optimized for a given protein. The structure of the leaving sulfinic acid moiety ($4$ or $5$) was found to influence the rate of PEGylation. The PEG-bis-sulfone 2 appeared to be less reactive during conjugation than the PEG-bis-sulfone 1. For example, during optimization for the PEGylation of His$_8$-IFN, a larger equivalence of PEG-bis-sulfone 2 (6 equiv) was required than was used for the PEG-bis-sulfone 1 (2–3 equiv) to prepare the di-PEGylated product (PEG)$_2$-His$_8$-IFN. Once the PEGylation reaction is complete, both the PEG-bis-sulfones 1 and 2 generate the same three-carbon linkage and conjugate (e.g., structure 9, Scheme 1).

Reduction of the reactivity of PEG-bis-sulfone 2 was initially thought to be due to the sulfinic acid 5 being a weaker leaving group compared to sulfinic acid 4. Semiempirical calculations, carried out using MOPAC 2009 and PM6 methods, indicated that there was little difference in the charges and bond orders of sulfinic acids 4 and 5. However, conformational searches did suggest that there is a greater association of the PEG polymer with the succinic acid moieties of the PEG-bis-sulfone 2 compared to the PEG polymer with the tolyl leaving groups in the PEG-bis-sulfone 1 (Figure SB). These increased interactions of PEG could contribute to reducing the reactivity of the PEG-bis-sulfone 2 or its mono-sulfone analogue by a relative reduction of the steric accessibility of the reacting moiety with the protein.

The PEG-bis-sulfone 3 was also incubated to generate the corresponding mono-sulfone 3a, which then underwent reaction with His$_8$-IFN to give the “branched” product, b-PEG-His$_8$-IFN (Figure SA, lanes 8 and 9). PEG-bis-sulfone 3 reagents were prepared with either a total of 40 kDa or 60 kDa PEG bound to the conjugating linker (i.e., two 20 kDa or two 30 kDa PEG chains bound to each linker moiety, respectively). The PEG-bis-sulfone 3 also displayed a lower apparent relative reactivity compared to the PEG-bis-sulfone 1. Modulation of the PEGylation reactivity by simple changes in the structure of the PEG-bis-sulfone reagents 1–3 and the conditions used to generate the corresponding PEG-mono-sulfone provide a unique means to optimize site-specific PEGylation with a wide range of His-tag variant proteins.

Conducting tryptic digestion of His$_8$-IFN without prior treatment with dithiothreitol (DTT) results in the formation of two disulfide-containing peptide fragments, A and B (Figure SC; SI Figure S5-B). In native IFN, there are two disulfides: one at the N-terminus between Cys1 and Cys98 and the other between Cys29 and Cys138. Fragments A and B are higher in molecular weight than the other tryptic fragments in IFN due to the presence of the disulfides. Fragment A has the N-terminal disulfide and in His$_8$-IFN also includes the His-tag. Fragment D is analogous to fragment A but with PEG conjugated to the His$_8$-tag. Fragment D would be expected for PEG-His$_8$-IFN when digested without prior DTT treatment. If DTT treatment occurs either before or after tryptic digestion, then fragment E is expected for PEG-His$_8$-IFN.

Both His$_8$-IFN and PEG-His$_8$-IFN (derived from PEG-bis-sulfone 2, 20 kDa PEG) were subjected to trypsin digestion with and without prior reduction with DTT (Figure SD). Tryptic digestion of His$_8$-IFN is shown in lanes 1–3 and for PEG-His$_8$-IFN is shown in lanes 4–6 (Figure SD). Lanes 1–6 are dyed with both colloidal blue to detect protein and barium iodide to detect PEG. The corresponding Western blots for lanes 1–6 are shown in lanes 1′–6′ (Figure SD). Two bands were observed in lane 2 for the digestion of His$_8$-IFN without prior DTT treatment. The migration of the higher molecular weight band is consistent with this band being the disulfide-containing fragment A. The lower band is consistent with the other disulfide-containing fragment B along with other fragments. Only the higher molecular weight fragment in lane 2 was detected in the corresponding anti-His-tag Western blot (Figure SD, lane 2′) indicating that this band was indeed fragment A.

The band for fragment A disappeared when the digestion of His$_8$-IFN was preceded by DTT treatment (Figure SD, lane 3). Depending on the extent of tryptic digestion and disulfide reduction, fragment A is expected to yield at least two separate fragments, with at least one fragment containing the His-tag. This fragment is labeled as fragment C (Figure SC). There are multiple lysine-arginine cleavage sites for trypsin in His$_8$-IFN (SI Figure S5–B), so a His$_8$-tag fragment C (Figure SD, lane 3') could also be composed of incomplete or mis-cleaved fragments along with a trace amount of fragment A as was observed in the anti-His-tag Western blot (Figure SD, lanes 3 and 3').

Tryptic digestion of PEG-His$_8$-IFN (20 kDa PEG) without any DTT treatment (Figure SD, lane 5) gave one band for a PEG peptide fragment. Western blot analysis using an anti-His-tag antibody indicated that this PEG protein band also possessed the His-tag. This band is labeled fragment D (Figure SC), which is analogous to fragment A that was observed from the tryptic digestion of the unPEGylated protein, His$_8$-IFN. Fragment A is not present in lane 5 as it was in lane 2, which suggests that the His-tag containing fragment was PEGylated. The other band in lane 5 (Figure SD), which is analogous to
the second band (fragment B) in lane 2, is not PEGylated and therefore appears to be fragment B.

Treatment of PEG-His$_8$-IFN (20 kDa PEG) with DTT followed by trypptic digestion gave the bands shown in lanes 6 and 6' (Figure 5D). The PEGylated peptide fragment in lane 6 was the only band detected in the corresponding anti-His-tag Western blot in lane 6' and this fragment was labeled peptide fragment E (Figure 5D), which is thought to be derived from the disulfide-containing His$_8$-tag fragment D.

RP-HPLC was used to separate the digestion mixtures of His$_8$-IFN and PEG-His$_8$-IFN (20 kDa PEG, derived from PEG- bis-sulfone 2) that were obtained with and without prior treatment with DTT (SI Figures S5 C–F). SDS-PAGE and Western blot analyses of the separated fractions indicated that PEG was conjugated only to the His$_8$-tagged fragments D and E (Figure 5E). MALDI-TOF spectra were consistent for the expected masses for fragments D (5.8 kDa for the peptide fragment + ∼20 kDa for PEG) and E (2.5 kDa for the peptide fragment + ∼20 kDa for PEG) (SI Figure S5 G,H).

Several variants of PEGylated His$_8$-IFN derived from the PEG-bis-sulfones (1–3) of different molecular weights were subjected to analogous trypptic digestion studies (SI Figures S6 A–E). All these digestion studies consistently showed that PEGylation occurred on the His-tag fragment as determined by SDS-PAGE and anti-His-tag Western blots. No nonspecific PEGylation occurred when there was a His-tag present in the protein. Further confirmation of this was obtained by analyzing the RP-HPLC fractions after trypptic digestion by dot blot analysis using both anti-PEG and anti-His-tag antibodies. Trypptic digestion of b-PEG-His$_8$-IFN (60 kDa PEG) without prior treatment with DTT was followed by RP-HPLC (Figure 6A). This allowed isolation of fragment D (listed in Figure 5C), which after treatment with DTT gave two fragments (Figure 6B). One fragment was detected by both anti-PEG and anti-His-tag antibodies in a dot blot analysis (Figure 6B) and corresponded to fragment E (listed in Figure 5C).

To further evaluate methodologies to avoid potential ambiguity due to incomplete trypptic digestion or the possibility that conjugation had occurred at another site in the His-tag containing fragment, His$_8$Met-IFN was cloned and produced in E. coli. A methionine was inserted directly after the N-terminal His-tag as a specific site for cyanogen bromide (CNBr) digestion. The His$_8$Met-IFN was PEGylated using PEG-bis-sulfone 1 (10 kDa PEG). Both the His$_8$Met-IFN and PEG-His$_8$Met-IFN were subjected to CNBr digestion and the digest mixtures were separated by RP-HPLC (Figure 7A). The fraction at 46.2 min was detected by both anti-His-tag and anti-PEG antibodies in a dot blot analysis (Figure 7B). MALDI-TOF-MS analysis of this fraction (Figure 7C) displayed a mass of approximately 11.3 kDa, which is consistent with the conjugation of 10 kDa PEG to a His$_8$ fragment (1.2 kDa).

It was thought that, compared to PEG-bis-sulfones 1 and 2, the two urethanes in PEG bis-sulfone 3 would result in lower electron withdrawing character in the PEG reagent ketone needed for conjugate addition to covalently link the PEG to the protein. The PEG–IFN conjugates were thus treated with sodium triacetoxyborohydride to reduce the reagent ketone after conjugation to prevent any possibility of dePEGylation by an elimination reaction. A control reaction with His$_8$-IFN indicated that the observed ED$_{50}$ of His$_8$-IFN in the in vitro EMCV-A549 antiviral assay was 10.5 ± 1.1 pg/mL (n = 2) after treatment with the hydride reagent. This was comparable to the observed ED$_{50}$ of His$_8$-IFN when it was stored in the same conditions without the hydride reagent. Prior to biological evaluation, both mono- and di-PEGylated His$_8$-IFN were evaluated for their stability in a wide range of conditions including higher temperature (up to 90 °C) and in the presence of DTT to ensure that there was no dePEGylation (SI Figure S7 A–E). No evidence of free protein was observed by SDS-PAGE.

The His$_8$-IFN and its PEGylated variants were then evaluated for their in vitro EMCV-A549 antiviral activity (Figure 8A). His$_8$-IFN which was used for all the PEGylation reactions displayed an ED$_{50}$ of 7 pg/mL, which was comparable to that reported for interferon α-2a in terms of specific activity. All the mono-PEGylated His$_8$-IFN products derived from both PEG-bis-sulfones 1 and 3 displayed an ED$_{50}$ in the range of 50–300 pg/mL. Conjugating two separate 20 kDa PEGs onto the His$_8$ resulted in ED$_{50}$ values within the range of 370–720 pg/mL. Similarly, b-PEG-His$_8$-IFN products displayed ED$_{50}$ values in the range of 230–680 pg/mL. Blood circulation half-lives following subcutaneous injection were estimated in both mice and rats from the IFN activity in serum determined using the EMCV-A549 antiviral assay. The half-lives increased with
increasing PEG molecular weight and the relative amount of PEG (Table 1, Figure 8B), with those products conjugated with two PEGs or with a β-PEG showing the longest $t_{1/2}$ (30−49.6 h) compared to the native His$_8$-IFN (1 h).

**DISCUSSION**

Targeting a His-tag for stable, covalent conjugation using the PEG-bis-sulfones 1−3 does not rely on metal chelation. Metal chelation has previously been used to label and to conjugate moieties to a His-tag (for example, ref 58), often for purification purposes.59 Some proteins do have clusters of histidines at their surface, 60 but most do not. Histidines generally have a lower $pK_a$ than other nucleophilic residues in a protein, i.e., lysine and arginine, and so are generally more reactive at lower pH. Only the histidines within a polyhistidine sequence appear to display enhanced reactivity with the PEG-bis-sulfone reagents 1−3. Isolated histidine residues in the protein mainchain are less reactive toward the PEG-bis-sulfone reagents, and so we consider a His-tag a good reactive moiety for conjugation with our reagents.

The PEG-bis-sulfones are thought to undergo bis-alkylation$^{30,44}$ by a sequence of addition−elimination reactions as drawn in Scheme 1. The PEGylations were conducted in reaction conditions that are thought to lead to the formation of the thermodynamically favored conjugate. DePEGylation was generally not observed to have occurred, and the dAb-His$_6$-PEG products appeared to be stable in a range of conditions (SI Figure S3-A). The same was also generally true for the PEGylated His$_8$-IFN products. Analogous to reductive amination, it is possible to avoid any risk of dePEGylation by reduction of the ketone derived from the PEG reagent with a hydride reagent (e.g., sodium triacetoxyborohydride) after PEGylation. To explore hydride reduction of our reagents post-PEGylation, we elected to treat the PEGylated His$_8$-IFN products that were made. These PEGylated conjugates were stable to the loss of PEG (SI Figure S7 A−E), even in what are considered to be harsh conditions. By using our PEG-bis-sulfone reagents 1−3, the resulting PEGylated protein can, in principle, also be easily labeled with a tritiated reducing agent for use in preclinical PK studies.

![Figure 7](image.png)

Figure 7. (A) CNBr digestion of His$_8$Met-IFN and PEG-His$_8$Met-IFN (10 kDa PEG), RP-HPLC chromatograms of digested His$_8$Met-IFN (dotted line) and PEG-His$_8$Met-IFN (10 kDa PEG) (continuous line); (B) dot blot analysis of each fraction with anti-His-tag and anti-PEG antibodies; and (C) MALDI-TOF mass spectrum analysis of the fraction (46.2 min) showing the mass of the PEG-His$_8$Met fragment (10 kDa PEG) (∼11.3 kDa).
Figure 8. (A) In vitro activity of PEG-His$_8$-IFN (A549 cytotoxicity): (Left) His$_8$-IFN $\alpha$-2a (closed circle), PEG-His$_8$-IFN $\alpha$-2a (20 kDa PEG; open circle), PEG-His$_8$-IFN $\alpha$-2a (30 kDa PEG; crossed circle), PEG-His$_8$-IFN $\alpha$-2a (40 kDa PEG; open reverse triangle), and (PEG)$_2$-His$_8$-IFN $\alpha$-2a (2 x 20 kDa PEG; open rhombus). (Right) His$_8$-IFN $\alpha$-2a (closed circle), b-PEG-His$_8$-IFN (40 kDa PEG; open square) and b-PEG-His$_8$-IFN (60 kDa PEG; open triangle). (B) Pharmacokinetic profile of PEG-His$_8$-IFN in rats; His$_8$-IFN $\alpha$-2a (closed circle), b-PEG-His$_8$-IFN (40 kDa PEG; open square), (PEG)$_2$-His$_8$-IFN (2 x 20 kDa PEG; open rhombus), b-PEG-His$_8$-IFN (60 kDa PEG; open triangle).

Table 1. Pharmacokinetic Data of PEGylated Analogues of His$_8$-IFN in Rats and Mice

<table>
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<tr>
<th>Parameter</th>
<th>Unit</th>
<th>His$_8$-IFN (20 kDa PEG)</th>
<th>(PEG)$_2$-His$_8$-IFN (2 x 20 kDa PEG)</th>
<th>b-PEG-His$_8$-IFN (40 kDa PEG)</th>
<th>b-PEG-His$_8$-IFN (60 kDa PEG)</th>
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<td>29.3</td>
<td>24200</td>
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</table>

Mouse

| Parameter | Unit | His$_8$-IFN (20 kDa PEG) | (PEG)$_2$-His$_8$-IFN (2 x 20 kDa PEG) | b-PEG-His$_8$-IFN (40 kDa PEG) | b-PEG-His$_8$-IFN (60 kDa PEG) |
|-----------|------|--------------------------|----------------------------------------|---------------------------------|                                 |
| Dose      | μg/kg| 100                      | 100                                    | 100                             | 100                             |
| t$_{1/2}$ | h    | 1.2$^a$                   | 13.3$^a$                               | 25.4                            | 34.1                            | 49.3                            |

$^a$Conducted in a preliminary PK study separate from the other three samples. $^b$Derived from PEG-bis-sulfone 3.

The PEG-bis-sulfone reagents 1–3 undergo elimination of a sulfinic acid (either 4 or 5) to give the corresponding PEG-mono-sulfones 1a–3a. It is the PEG-mono-sulfone, which then is thought to undergo PEGylation with the protein. The rate of generation of the PEG-mono-sulfone can be tailored by reaction conditions (e.g., pH, concentration) so that PEGylation can be optimized for any given protein. Our computational modeling experiments comparing PEG-bis-sulfones 1 and 2 and their observed reactivity suggest that the structure of the leaving sulfuric acid groups may also interact with PEG to influence conjugation rates. This provides another means to tailor the PEGylation reagent to the structure of a given protein. To our knowledge, these simple means to modulate reactivity while maintaining specificity are not possible for any other type of PEGylation reagent. Additionally, the structure of the leaving group can be used in unique ways, potentially to aid conjugation. For example, the acidic moieties of PEG-bis-sulfone 2 can be used to bind this reagent to an anion exchange column. In this case, the PEG reagent could be bound to a column and then made to undergo reaction without the product being required to bind to the column.

Once reaction is complete, the PEG-bis-sulfones all generate the same three-carbon linkage that is conjugated to the protein. PEG-bis-sulfones 1 and 2 generate the same conjugate, while PEG-bis-sulfone 3 generates a conjugate that is derived from two PEG chains linked to the reacting moiety. This is often referred to in the literature as a “branched” PEGylation reagent. Extension of protein half-life is often a function of PEG size and the biochemical attributes of the protein, e.g., recycling mechanisms and susceptibility to proteolytic degradation. In the case of nonendogenous proteins, simple conjugation of PEG will cause extended half-life primarily due to the increased size of the PEG. The “branched” PEG reagents are often described as giving conjugates that have more extended circulation half-lives than conjugates derived from the corresponding linear PEG reagent.

Since PEGylation using the PEG-bis-sulfone reagents is selective for a His-tag and since more than one PEG molecule can be conjugated to the His-tag, there may be the possibility to further modify the circulation half-life for a given molar mass of PEG.

During optimization of the PEGylation reaction of His$_8$-IFN it was observed by SDS-PAGE that PEG-bis-sulfone 1 could occasionally undergo a small observable amount of PEGylation with non-His-tag interferon $\alpha$-2a (IFN) depending on the pH or the reactant concentrations that were used. There was, however, no indication from any of the digestion or MALDI studies that nonspecific reactions occurred when there was a His-tag present in the protein. Histidine is less reactive as a nucleophile than a free thiol, so a longer incubation time was required to maximize the PEGylation yields than is necessary when the PEG-bis-sulfone 1 is used in reaction with thiols. Conducting control reactions with the non-His-tagged version of a protein in conditions optimal for His-tag PEGylation may therefore be problematic. In many reactions, a slight excess of the PEG reagent is used, and at appropriate conditions and in the absence of free thiol, reaction appears to be most favored at the His-tag rather than at other nucleophilic residues along the protein backbone.

Digestion studies of the PEGylated dAb-His$_8$ and His$_8$-IFN products (Figures 2,3, 5–7; SI Figures S1, S5, and S6) were all consistent with PEGylation occurring at the His-tag. The most available and labile digestion cleavage point in the dAb-His$_8$ was adjacent to the His-tag, which after partial digestion left essentially the intact dAb without the His$_8$-tag (Figures 2–3). Although the His-tag could be detected in the starting dAb-His$_8$ in Western blot experiments, it could not be detected after...
digestion of either the starting dAb-His₆ or its PEGylated products. For His₈-IFN, the cleavage point is at least 12 amino acids away from the His-tag. The relative size of the His-tag fragment in His₈-IFN compared to dAb-His₆ may have facilitated Western blot detection of the His-tag in IFN after digestion. Interestingly, in the CNBr digestion experiments the cleavage point is adjacent to the His-tag, and in this case, it was still possible to observe the His-tag fragment by anti-His-tag Western blot. The length of the 8-histidine tag in both His₈-IFN and His₈-Met-IFN may therefore have aided these Western blot detection experiments.

The clinical use of proteins that have a His-tag appears to be safe. Most therapeutic proteins do elicit some type of an immunogenic response in a proportion of patients in which they are used. As far as we can discern from the literature, no immunogenic response due specifically to a His-tag on a therapeutic protein has been reported in humans. It is known that the steric shielding provided by PEG acts to reduce the immunogenicity of a therapeutic protein. PEGylation at a His-tag would be expected to reduce any potential immunogenic response that could be directed at a His-tag in a protein. A strategy to further reduce the likelihood of any potential His-tag derived immunogenicity may be to simply reduce the size of the His-tag that is PEGylated. Our data do indicate that it is possible to shorten or modify a His-tag for site-specific PEGylation.

Many different chemical and biological strategies are being examined for the site-specific conjugation of polymers and other molecules to proteins (for example, refs 69–72). Efforts to conjugate PEG site-specifically to a protein have been the focus of various strategies including the incorporation of non-native amino acids and free unpaired cysteines. Incorporation of non-native amino acids probably requires that cellular biochemical pathways be robustly modified to ensure that the efficiency of protein production is not compromised by reversion to the wild-type phenotype.

Incorporation of a single cysteine is best exploited for site-specific PEGylation of therapeutic proteins that do not have any disulfides. For example, a protein scaffold derived from a fibronectin type III domain which does not have a disulfide in its structure has been engineered to have a free cysteine for PEGylation and is currently in clinical trials. Thiol conjugation is efficient and site-specific as long as the reagent that is used is stable during and after conjugation. Many of the maleimide-based reagents are known to be hydrolytically labile, which can result in contamination by acidic byproducts. These reagents also undergo exchange reactions in vivo.

In addition to monocysteine-specific conjugation, other amino acids (e.g., arginine, glutamic acid) and glycosyl moieties have also been described as targets for conjugation. PEGylation of either the C-terminal or N-terminal of proteins has also been described. Of these, reductive amination at the N-terminus appears to have been investigated more. Reductive amination is often considered to be a site-specific method for PEGylation. Imine formation is the first step in reductive amination with water as the byproduct. While reaction conditions have been described that indicate that reductive amination can lead to broadly site-specific PEGylation and can be used to produce a clinical product, exploiting a slight difference in the pKₐ of a single N-terminal amino acid to achieve high yielding and homogeneous imine formation in an aqueous medium is not generally efficient in terms of reagent stoichiometry or site specificity. One advantage of the His-tag as a functional unit is that it appears to offer a more efficient moiety for conjugation than is generally observed for a single amino group at the amino terminus of a protein.

As a chemical process, protein PEGylation is often thought of as simple to implement. Considering some of the reagents that are used to produce currently marketed therapeutic products, the perception that PEGylation is straightforward can be misplaced. While reductive amination is a sensitive reaction that often requires a very narrow set of conditions to be useful, it is generally inefficient due to the need for imine formation. Another example involves PEGylation with active ester reagents (e.g., N-hydroxysuccinimide esters), which tend to be hydrolytically labile and not selective. Optimization and scalability of PEGylation with these reagents often requires considerable effort and tedious purification processes to obtain a reproducible and robust process that is scalable. This is true even with maleimide reagents due to their propensity for hydrolysis to acidic byproducts and to undergo exchange with other thiol reactive molecules in vivo.

His-tagged proteins can often be purified effectively using immobilized metal affinity chromatography (IMAC). The scalability of IMAC for commercial production has been described elsewhere and its limitations have been solved and clinical grade proteins can be produced. While PEGylation at a histidine tag allows the protein to be purified by IMAC, the use of IMAC is not a requirement. Ion exchange chromatography was often found to be adequate for our purposes, since the amount of PEG-bis-sulfone reagent that was generally required was only a slight excess. There may be advantages for considering IMAC. One example would be if PEGylation is conducted during early development of a new protein where a lysate mixture may contain some His-tag variant of the protein(s) of interest.

The needs we are addressing are to develop a robust and easy to implement site-specific PEGylation methodology. Many new proteins are not endogenous, so engineering a tag for PEGylation may be appropriate for their development. We believe this will allow for more cost-effective medicines that in some cases will be more efficacious. As our data show, His-tag conjugation at either terminus of a protein may be a general strategy to achieve relatively high activity without the need to try to place a conjugation site within the protein main chain. Different classes of proteins (e.g., α-helical barrel proteins, antibodies) and peptides have emerged as structural motifs for therapeutic applications. In many therapeutic proteins, one or both termini are not involved with the binding associated with the biological activity of the protein. Often the termini of a protein can be much more flexible than other regions of the protein. Terminal His-tag PEGylation of both dAb-His₆ and His₈-IFN resulted in good retention of activity in vitro.

In conclusion, we describe a general strategy for the covalent, site-specific PEGylation at a His-tag in therapeutically relevant proteins. Site-specific PEGylation using PEG-bis-sulfones 1–3 at a His-tag has general utility. Excluding conjugation to cysteine thios, PEGylation to a His-tag may be the next most efficient method available for site-specific conjugation of PEG to native amino acids in proteins.
and proteins, (2) PEGylation of chemerin, (3) determination of protein concentration, in vitro activity and circulation half-lives, and (3) computational modeling of dAb-His₈-PEG and PEG-bis-sulfones 1–2. Additional data include the following: For dAb-His₈: (i) MALDI-TOF spectra of starting dAb variants and peptide fragments obtained after proteolytic digestion; (ii) SDS-PAGE analysis of the stability of dAb-His₈-PEG; (iii) CD and denaturation profiles of dAb-His₈-PEG; (iv) comparative ribbon representations of the protein domain for dAb and dAb-His₈-PEG; (v) images depicting the visual assessment used to determine TNF-α mediated cytotoxic activity; and (vi) comparative HPLCs of different His-tag motifs of chemerin. For His₈-IFN: (i) comparative SDS-PAGE gels showing the mobilities of His₈-IFN conjugated to PEG-bis-sulfone 1 of different molecular weights; (ii) RP-HPLC chromatograms of trypsin digested His₈-IFN and PEG-His₈-IFN (20 kDa PEG) derived from PEG-bis-sulfone 2 and digestion data for several other PEG-His₈-INF conjugates; (iii) SDS-PAGE gels from stability studies of several PEG-His₈-INF conjugates. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions
Equal contribution for first authorship.

Notes
The authors declare the following competing financial interest(s): S.B. is a full-time academic employee of the UCL School of Pharmacy. As a co-founder of PolyTheris Ltd he has equity in the company, but receives no financial compensation. He is a non-paid director and acts as a non-paid CSO. M.M. is also a full time employee of the UCL School of Pharmacy. His position is funded as a postdoctoral researcher by the UK government (Knowledge Transfer Partnership, 67%) and PolyTherics (33%).

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■ REFERENCES

(24) Yang, B. (2006) Integration of pharmacokinetics and pharmacodynamics into the drug development of Pegfilgrastim, a PEGylated Protein, in Pharmacokinetics and Pharmacodynamics of...


