

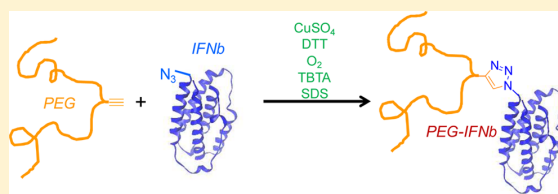
Development of Copper-Catalyzed Azide–Alkyne Cycloaddition for Increased in Vivo Efficacy of Interferon β -1b by Site-Specific PEGylation

Natalie W. Nairn,^{*,†} Kurt D. Shanebeck,[†] Aijun Wang,[†] Thomas J. Graddis,^{†,‡} Michael Pete VanBrunt,[†] Kenneth C. Thornton,[†] and Kenneth Grabstein[†]

[†]Allozyne, 1600 Fairview Avenue E, Suite 300, Seattle, Washington 98102, United States

Supporting Information

ABSTRACT: The development of protein conjugate therapeutics requires control over the site of modification to allow for reproducible generation of a product with the desired potency, pharmacokinetic, and safety profile. Placement of a single nonnatural amino acid at the desired modification site of a recombinant protein, followed by a bioorthogonal reaction, can provide complete control. To this end, we describe the development of copper-catalyzed azide–alkyne cycloaddition (CuAAC, a click chemistry reaction) for site-specific PEGylation of interferon β -1b (IFN β) containing azidohomoalanine (Aha) at the N-terminus. Reaction conditions were optimized using various propargyl-activated PEGs, tris(benzyltriazolylmethyl)amine (TBTA), copper sulfate, and dithiothreitol (DTT) in the presence of SDS. The requirement for air in order to advance the redox potential of the reaction was investigated. The addition of unreactive PEG diol reduced the required molar ratio to 2:1 PEG–alkyne to IFN β . The resultant method produced high conversion of Aha-containing IFN β to the single desired product. PEG–IFN β s with 10, 20, 30, and 40 kDa linear or 40 kDa branched PEGs were produced with these methods and compared. Increasing PEG size yielded decreasing in vitro antiviral activities along with concomitant increases in elimination half-life, AUC, and bioavailability when administered in rats or monkeys. A Daudi tumor xenograft model provided comparative evaluation of these combined effects, wherein a 40 kDa branched PEG–IFN β was much more effective than conjugates with smaller PEGs or unPEGylated IFN β at preventing tumor growth in spite of dosing with fewer units and lesser frequency. The results demonstrate the capability of site-specific nonnatural amino acid incorporation to generate novel biomolecule conjugates with increased in vivo efficacy.



■ INTRODUCTION

The development of biopharmaceutical technology during the past few decades has produced therapeutics with remarkable specificity and activity.¹ Initial products included recombinant forms of naturally occurring proteins such as insulin and interferon. This was followed by development of novel proteins such as monoclonal antibodies generated against specific targets. The next generation of biopharmaceuticals includes highly engineered molecules with modifications that can modulate half-life, combine functions, or perform wholly new functions.

These modifications can sometimes be accomplished by genetic alterations to the protein sequence but in many cases are best generated by chemical conjugation following recombinant expression. Approved protein therapeutics utilizing bioconjugation include PEGylated proteins, glycoconjugate vaccines, and drug- and radio-immunoconjugates. Development of protein conjugation chemistries was initially focused on the reactive amines present in lysine side chains. While this provided for straightforward chemistries, the frequent occurrence of lysine residues throughout protein sequences allows for little control over conjugation site. For instance, PEG-Intron contains 14 positional isomers of polyethylene glycol (PEG) conjugated to

various residues of interferon α -2b.² These isomers have specific in vitro activities varying from 6% to 37% of unPEGylated interferon α due to the steric effects of PEG at various locations within the protein's three-dimensional structure. Bioconjugation to the thiols in cysteine residues provides a more specific approach given the less frequent occurrence of these residues and the ability to control reduction and formation of disulfide bonds. Reduction and alkylation was harnessed for antibody–drug conjugate Adcetris, which contains an average of four monomethyl auristatin E drug molecules per antibody.³ Selective reduction of the four interchain disulfide bonds yields a distribution of species with 0, 2, 4, 6, and 8 conjugated drugs in various isomeric ratios; the species each have different pharmacokinetic profiles and therapeutic indices.^{4,5} N-Terminal conjugation via reductive alkylation allows more, albeit not complete, specific targeting for drugs such as Neulasta (PEG-G-CSF).^{6,7} The recent development of Thiomabs, antibodies possessing engineered free cysteines at accessible sites, has

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allowed large scale production of antibody–drug conjugates with 92% as the desired structure.⁸ In spite of these advances, there continues to be a need for generalizable strategies to control bioconjugate structure given the substantial effects conjugation site can have on potency, pharmacokinetics, safety, and manufacturing reproducibility.^{2,4,5,8–14}

A truly site-specific method for protein conjugation has been developed in recent years wherein one or more nonnatural amino acids (NNAAs) are incorporated into the protein sequence during recombinant expression, followed by an orthogonal chemical reaction that does not modify any canonical amino acids.^{15–19} Several strategies have been developed to incorporate NNAAs into recombinant proteins with varying degrees of NNAA incorporation efficiency, sequence fidelity, and scalability. One method to incorporate the NNAA is global substitution of a specific natural amino acid.²⁰ In this approach, the NNAA analogue is incorporated into a recombinant protein via the promiscuity of the aminoacyl-tRNA synthetase when the cognate amino acid is depleted. Here we have introduced the Met analogue azidohomoalanine (Aha) at a single ATG codon to allow for site-specific PEGylation of an engineered human interferon β -1b (IFN β -1b).

Like many cytokines, IFN β has diverse effects on numerous cell types and finds application in the clinic. Some of the described biological activities of IFN β include induction of antiviral activity, inhibition of growth and proliferation, and immunomodulatory effects.^{21–23} First approved in 1993, IFN β is indicated to reduce exacerbations and progressing disability from relapsing forms of multiple sclerosis. The mechanism of action of IFN β in multiple sclerosis is not well understood but has been postulated to involve modulation of T cell activation and proliferation, induction of regulatory T cells, and inhibition of leukocyte migration across the blood–brain barrier.²⁴ Approved recombinant IFN β products include Betaseron, Rebif, Avonex, and Extavia. Dosing frequencies range from every other day to weekly, with common side effects including flu-like symptoms and injection site reactions that can occur after each dose. Thus IFN β is a prime target for the well-known half-life extension afforded by PEGylation,^{10,25} where the concomitant reduced frequency of dosing could decrease side effects and increase efficacy due to increased patient compliance and lesser variation in plasma levels. A PEGylated form of IFN β -1a is in clinical development, wherein a 20 kDa linear PEG is conjugated to the N-terminus by reductive alkylation.^{26,27} The conjugation reaction produces 10–15% multi-PEGylated species and requires facility engineering to safely handle sodium cyanoborohydride, but purification results in a product that is 97% mono-PEGylated at the N-terminus.^{28,29}

In this work, we have developed a PEGylated IFN β -1b using Met substitution by Aha to allow single site PEGylation by copper-catalyzed azide–alkyne cycloaddition (CuAAC) (Scheme 1). The N-terminus was chosen as the PEGylation site because this position is accessible for modification and

suitably distal from receptor binding regions to retain activity after PEGylation.^{30–32} The other three Met residues in native human IFN β were mutated to permissive canonical amino acids that maintain the activity of IFN β , and the penultimate residue was mutated to Glu to retain the N-terminal Aha when expressed in *Escherichia coli*.^{33,34} CuAAC (a reaction of the click chemistry class) was chosen for the conjugation chemistry, as it is unreactive with all natural amino acid residues, can be performed in mild aqueous conditions, results in the formation of a stable aromatic triazole linkage, and is readily accessible by incorporation of the NNAA Aha.^{35–38} Thus PEG containing a single terminal alkyne group was combined with a recombinant IFN β -1b protein containing a single Aha at the N-terminus (Aha-IFN β), along with a Cu(I) source and a ligand, to form the triazole PEG–IFN β conjugate.

We describe the development of CuAAC as applied to a therapeutic protein product candidate. Various PEG structures (Chart 1), choice of catalyst and ligand, optimization of reaction concentrations, and investigation of reducing agent effects are described. Single Aha-specific conjugation was verified analytically. PEGylation was performed while the protein was reduced and denatured, allowing refolding to be performed after PEGylation. Conjugates with various PEG molecular weights and geometries were then compared with respect to their in vitro potencies, in vivo pharmacokinetics, and in vivo potencies as assessed by inhibition of tumor growth in a human tumor xenograft mouse model.

■ EXPERIMENTAL PROCEDURES

General Materials. Azidohomoalanine (Aha) hydrochloride and tris(benzyltriazolylmethyl)amine (TBTA) were custom synthesized by Medchem Source (Federal Way, WA) and SAFC. Branched 40 kDa mPEG₂-PA₃, mPEG₂-PA₆, and mPEG₂-PA₉ (approximately 20 kDa per branch), linear 30 and 40 kDa mPEG-PA, and 30 kDa PEG diol (polyethylene glycol, alcohol groups on both ends) were obtained from NOF, Japan. CuSO₄ pentahydrate (99.999% pure), CuBr (99.999% pure), TCEP (tris(2-carboxyethyl)phosphine hydrochloride), and oxidized DTT (*trans*-4,5-dihydroxy-1,2-dithiane) were from Sigma-Aldrich. Bathophenanthroline disulfonic acid disodium salt (BPA) and DTT (DL-dithiothreitol) were from Fluka.

Molecular Biology and Expression of IFN β . Molecular biology and expression was performed as described previously.³³ Briefly, the synthetic gene encoding the engineered IFN β sequence was codon-optimized for the *E. coli* expression host then cloned into the pQE30 expression vector (Qiagen) under control of the T5/lac/lac promoter using standard molecular biology methods. After confirming the DNA sequence, the expression vector was transformed to a Met auxotrophic *E. coli* expression host (B834 from Novagen or MAM15 from D. Tirrell). Recombinant IFN β was produced by IPTG induction in the presence of either Met (for Met–IFN β), or Aha (for Aha–IFN β). After approximately 3 h of induction, *E. coli* cells were harvested. Fermentation was performed at 1 L scale in a shaker flask or at 5 L scale in a fermentor with typical titers of 0.6–1.0 g IFN β per L (fermentor). The theoretical MW of the engineered IFN β protein is 20.0 kDa.

Isolation of IFN β . Cells were lysed by microfluidizing in 100 mM sodium phosphate, 150 mM NaCl, pH 7.4 (PBS), followed by centrifuging to isolate the inclusion bodies. Inclusion bodies were washed by microfluidizing and centrifuging in PBS two more times, followed by two washes in 50 mM Tris, 1 mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 M

Scheme 1. PEGylation of Aha-IFN β by CuAAC

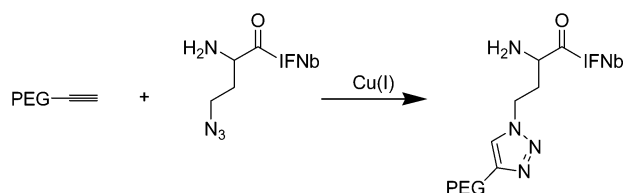
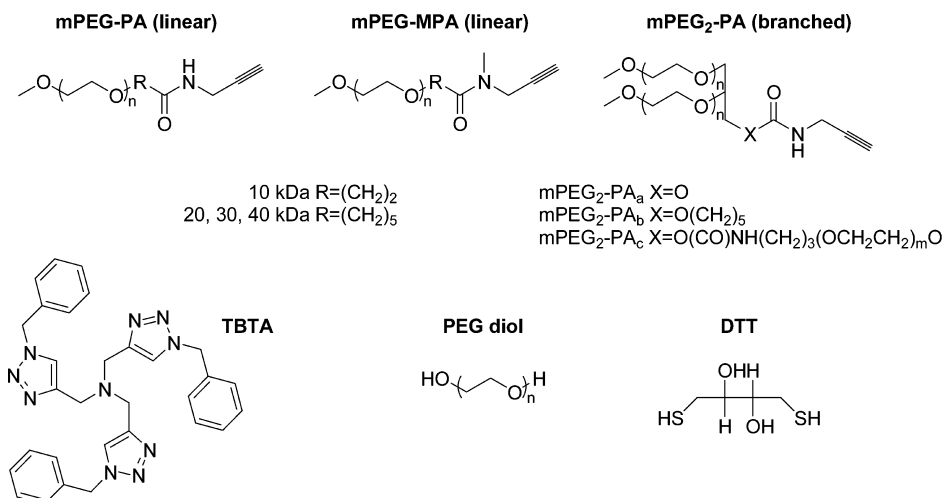


Chart 1. Structures of Various Reagents Used



NaCl, pH 8.0, followed by one more wash in PBS. IFN β was then isolated by butanol extraction.³⁹ The inclusion bodies were solubilized in 100 mM sodium phosphate, 100 mM DTT, 2% SDS, pH 7.4, by sonication or microfluidization. An equal volume of 2-butanol was added, and the IFN β was extracted into the organic phase by agitation. The mixture was centrifuged at 6000g for 15 min to speed phase separation, and the organic phase was collected. This phase was then dripped into 4–5 parts of 100 mM sodium phosphate, 0.1% SDS, pH 7.4, with stirring. The pH was lowered to 5.0 with glacial acetic acid to precipitate IFN β , which was then harvested by centrifugation at 14000g for 2 h. Acid precipitated IFN β was stored at -80°C until use.

Synthesis of 10 and 20 kDa mPEG-PA and 10 kDa mPEG-MPA. Monomethoxy-PEG-SPA, MW 10 kDa (Nektar, San Francisco, CA), was dissolved at 2.5% in 1,4-dioxane under argon and dissolved by heating in a warm water bath. Propargylamine or *N*-methylpropargylamine (Sigma-Aldrich) was added at 50 molar excess and allowed to react for 1.5 h under argon at room temperature. The product was precipitated by 5–10 $\times$ dilution in cold hexane and captured on a 0.2 μm nylon filter. The precipitate was dissolved in water, treated with 1 M NaOH, neutralized with 1 M HCl, dialyzed against 0.1 M NaCl and then H_2O , and isolated by lyophilization. 20 kDa mPEG-PA was made similarly using Sunbright ME-200-HS (methoxy-PEG- $(\text{CH}_2)_5\text{-COO-NHS}$) from NOF or purchased from NOF.

PEGylation of Aha-IFN β . The reaction occurred in a buffer of 100 mM sodium phosphate, SDS, and DTT at pH 7.55. Aha-IFN β acid precipitate and, separately, mPEG-PA and PEG diol were dissolved in the buffer. TBTA was dissolved in DMSO or added as a solid. CuBr was suspended in degassed H_2O immediately before use, and CuSO_4 was dissolved in H_2O with no special precautions. Reactants were combined in the following order: Aha-IFN β and buffer, PEG, TBTA, Cu. The reactions were mixed immediately after addition of TBTA (to disperse the TBTA that precipitates immediately upon dilution in buffer) and after addition of Cu. Specific concentrations of reagents for each experiment are given in the legends. Reactions were incubated at room temperature with mixing overnight, unless otherwise noted. Reactions were performed at 100 μL to 250 mL scale, typically with an air headspace at least twice the volume of the reaction. For the investigation of starting redox ratio, the total concentration of DTT (reduced, DL-dithiothreitol) and oxidized DTT (*trans*-4,5-dihydroxy-1,2-dithiane) was set to 10 mM at the

beginning of the reaction, but the initial ratio between reduced and oxidized DTT was varied. Redox potential was measured with a micro redox electrode (Lazar Research Laboratories).

PEG-IFN β Purification and Refolding. After PEGylation of IFN β , 100 mM DTT and 10 mM EDTA were added to the reactions and stirred at room temperature for 80 min, followed by filtration with a 0.22 μm filter. PEGylated and unPEGylated IFN β were then separated by running over a Superdex 200 column (GE-Healthcare) with 20 mM sodium phosphate, 150 mM NaCl, 5 mM EDTA, 10 mM DTT, 0.02% SDS, pH 7.5 as the running buffer. Alternatively, separation occurred by diluting the mixtures 1:20 with 10 mM DTT, 0.1% SDS, pH 7.2, loading onto a ceramic hydroxyapatite type II column (80 μm , Biorad) and eluting in a gradient from 5 to 500 mM sodium phosphate in 10 mM DTT, 0.1% SDS, pH 7.2. PEG-IFN β -containing fractions were then pooled and refolded by dilution into 10–20 parts rapidly stirring refolding buffer comprising 10 mM Tris, 10 mM NaCl, 1 mM EDTA, 6 μM CuSO_4 , 0.2–0.6% zwittergent 3–14, pH 10.⁴⁰ Refolding proceeded for 2.5 h at room temperature or overnight at 5°C . Refolding was stopped by lowering the pH to 5.0 with acetic acid. PEG-IFN β was concentrated by loading onto a MacroCap SP CEX column (GE-Healthcare). Elution was achieved with steps up to 2 M NaCl in 50 mM sodium acetate, 5 mM EDTA, 0.1% polysorbate 80 (NOF), pH 5.0. The resultant pool was concentrated with a YM-30 Centriprep (Millipore) prior to SEC polish to remove aggregate. The concentrate was loaded onto a Sephacryl S300HR column (GE-Healthcare) and eluted in 20 mM sodium acetate, 100 mM NaCl, 5 mM EDTA, 0.1% polysorbate 80, pH 5. The monomer pool was concentrated with a YM-30 Centriprep (Millipore) and buffer exchanged over a PD-10 column (GE-Healthcare) into 50 mM sodium acetate, 50 mM NaCl, 5 mM EDTA, 3% mannitol, 1% polysorbate 80, pH 5. The final material was stored at 5°C .

SDS-PAGE. PEGylation yields were determined by applying the reaction mixtures to SDS-PAGE under reducing conditions. After mixing, reaction solids were pelleted by centrifugation or were removed by a 0.2 μm hydrophilic PVDF filter. The supernatant was combined with reducing 2 $\times$ sample loading buffer (Tris-Glycine 2 $\times$ SDS sample buffer, Invitrogen plus 10% β -mercaptoethanol), heated at 90–95 $^\circ\text{C}$ for 3–10 min, and applied to 4–20% Tris-Glycine gels (Invitrogen). Gels were washed with water and then stained for 1 h with Simply Blue Safestain (Invitrogen) and destained with water. Gels were

imaged by densitometry, and % PEGylation was determined by dividing the PEG–IFN β intensity by the sum of the PEG–IFN β and free IFN β intensities.

Peptide Mapping and MALDI. The primary structure and conjugation site of samples were confirmed by LC–MS peptide mapping. Following trypsin digestion of purified Aha–IFN β or PEG–IFN β conjugates overnight at 37 °C, the reaction was quenched by adding TFA. The tryptic peptides were separated by C18 RP–HPLC with in-line UV detection. Fractions were collected, dried down under vacuum, and then resuspended in 0.1% trifluoroacetic acid. The peptide(s) in each fraction were identified by MALDI (MS and MS–MS) analysis at the Institute of Systems Biology (Seattle, WA). Tryptic digests were also analyzed directly by MALDI as described previously.³⁴

Antiviral Assay for Potency. Potency was measured by taking advantage of the antiviral properties of IFN β . IFN β inhibits the ability of the Encephalomyocarditis virus (ATCC no. VR-1298) to cause lysis of the A549 lung carcinoma cell line (ATCC no. CCL-185). Titrations of test samples and standards were added to 96-well flat-bottom tissue culture plates in a volume of 50 μ L. A549 cells were added as 28000 cells/well in 100 μ L. After overnight incubation, 50 μ L of EMC virus (of sufficient titer to ensure 100% lysis) was added. After an additional 3 days of culture, the supernatants were removed and 50 μ L of 0.2% crystal violet in Histochoice MB fixative (Electron Microscopy Sciences, Hatfield, PA) was added and incubated for 20 min. Excess stain was removed by washing the plates four times with deionized water. Absorbance was measured using a microplate reader at 550 nm. EC₅₀ values were determined using a four-parameter logistic equation with GraphPad Prism (GraphPad Software Inc., San Diego, CA).

Pharmacokinetic Analysis. Pharmacokinetic properties of the PEG–IFN β s were determined following subcutaneous or intravenous administration in cynomolgus monkeys (*Macaca fascicularis*) or subcutaneous administration in rats (Sprague Dawley, Charles River Laboratories, Wilmington, MA). Three male animals of each species were dosed for each test article. In rats, blood samples were taken via jugular vein catheter at 0 (predose), 0.25, 0.5, 1, 2, 4, 6, 24, 48, and 72 h post dose. In monkeys, blood samples were taken at 0 (predose), 0.17 (iv dose only), 0.5, 2, 4, 6, 8, 10, 12, 18, 24, 72, 96, 120, 144, 168, 216, and 240 h post dose. Plasma IFN β concentrations were determined using the A549 antiviral assay. Pharmacokinetic parameters were determined using standard noncompartmental analysis with WinNonlin v.5.2 (Pharsight Corp., Mountain View, CA). Area under curve (AUC) values were calculated using the linear/log trapezoidal method.

Tumor Xenograft Model. Efficacy was assessed using a human tumor xenograft model in SCID mice. CB.17 SCID mice (Charles River Laboratories) were implanted with 5–10 $\times$ 10⁶ Daudi cells (ATCC no. CCL-213) subcutaneously, and tumor growth was measured three times per week using digital calipers. Tumor volume was determined by measuring the longest (a) and shortest (b) diameter and calculated using the formula volume = $ab^2\pi/6$.

All in vivo experiments were carried out in accordance with approved Institutional Animal Care and Use Committee (IACUC) protocols.

RESULTS

In developing CuAAC for IFN β bioconjugation, reaction conditions were screened by reacting PEG–alkynes with Aha and with Aha–IFN β . mPEG–PA reacted well with Aha when

using the TBTA ligand, whereas mPEG–MPA was surprisingly unreactive with Aha (Supporting Information). mPEG–PA was then used to PEGylate Aha–IFN β (Figure 1). No reaction

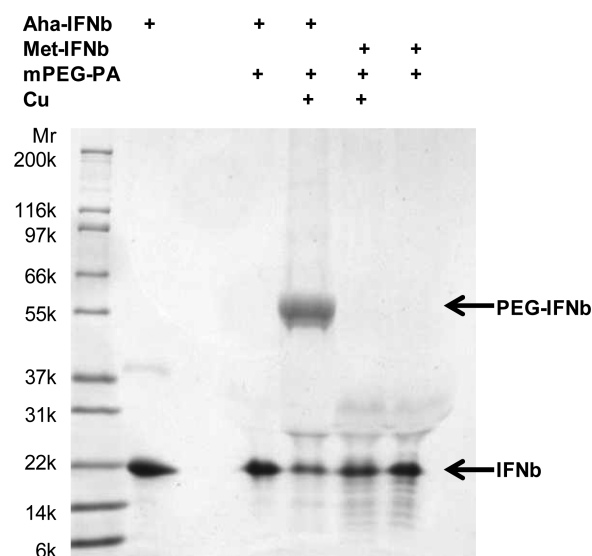


Figure 1. SDS-PAGE analysis of mPEG–PA 20 kDa reactions with IFN β . No reaction occurs with Met–IFN β or in the absence of Cu. PEG–IFN β migrates as a diffuse band due to polydispersity of the polymer. The high loading of excess PEG onto the gel causes smearing in the 25–60k M_r range (unpurified reaction mixtures were applied directly to the gel).

occurred in the absence of copper or when Met–IFN β was used. As expected, the migration position of the PEG–IFN β conjugate ran higher than the predicted 40 kDa when compared to the protein calibration standards, as PEG has a different relative mobility than proteins in SDS-PAGE due to differences in conformation and SDS association ratios.^{41–43} Peptide mapping confirmed that PEGylation occurred at the N-terminus of the protein (Figure 2). The N-terminal peptide was absent from PEG–IFN β conjugates, while all other peptides were retained. As expected, Aha was only detected at the N-terminal site of

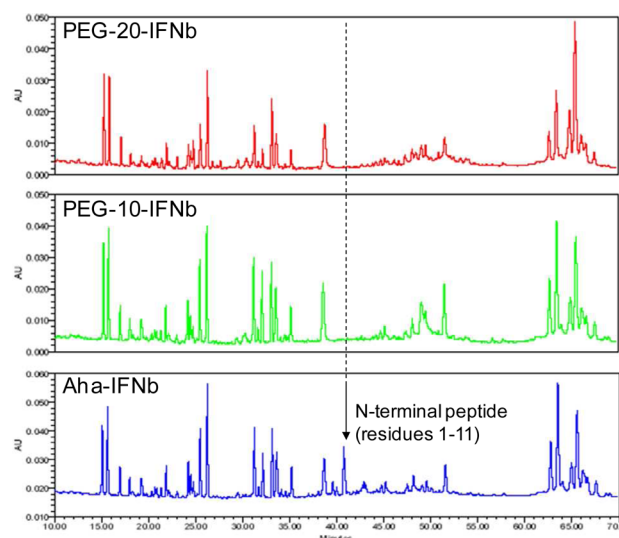


Figure 2. Peptide mapping of Aha–IFN β and of mPEG–PA 10 or 20 kDa conjugated to Aha–IFN β .

Aha-IFN β . PEGylation was also confirmed by the comigration of PEG and protein as visualized by differential staining of SDS-PAGE (Supporting Information).

Reaction conditions were optimized for PEGylation of Aha-IFN β . Increasing concentrations of mPEG-PA increased the reaction product (Figure 3a). A TBTA/Cu ratio of 2 appeared

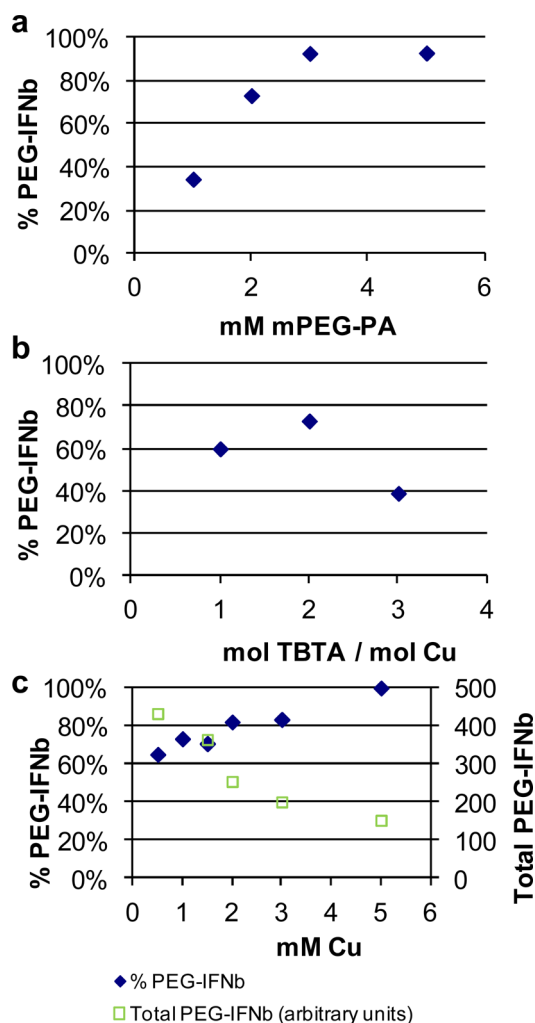


Figure 3. Reaction optimization of mPEG-PA 10 kDa with Aha-IFN β . Default conditions were 0.025 mM (0.5 mg/mL) protein, 2 mM (2 wt %) mPEG-PA, 2 mM TBTA, 1 mM CuBr, 2 wt % SDS, and 10 mM DTT. (a) Optimization of PEG concentration. (b) Optimization of TBTA ligand to Cu molar ratio, holding Cu at 1 mM. (c) Optimization of Cu concentration, holding TBTA to Cu molar ratio at 2.

optimal (Figure 3b), as successfully used in previous reports,^{37,38,44} although our general experience has suggested only a mild dependence on the ratio within the range of 1–3. The effect of copper concentration on reaction yield and kinetics is more complex. Figure 3c shows that protein recovery was reduced at high copper concentrations. This could be degradation caused by reactive oxygen species, such as hydrogen peroxide and hydroxyl radical, which can be formed when combining copper, air, and a reducing agent.⁴⁵ However, due to the low molar concentration of protein that is typically present in bioconjugations, high concentrations of copper catalyst relative to the reactant alkyne and azide concentrations are required in order to achieve reasonable reaction kinetics. Figure 4 shows that reaction rates fell off dramatically at copper concentrations below

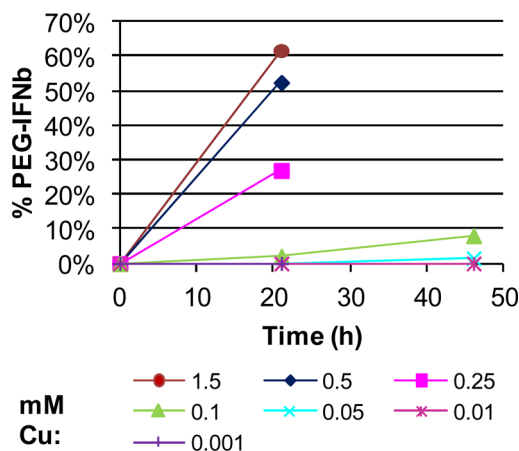


Figure 4. Reaction kinetics versus copper concentration. Aha-IFN β (0.05 mM, 1 mg/mL) was reacted with 40 kDa mPEG₂-PA₃ (0.15 mM, 0.6 wt %) in the presence of TBTA at 2 molar excess of CuSO₄, 2 wt % SDS, 2 mM DTT, and 1.4 wt % 30 kDa PEG diol.

0.5 mM. While the reaction can occur with either CuBr alone or with CuSO₄ and reducing agent, we have found it more robust and reproducible in the latter conditions.

As thiols are well known to interact with metals such as copper, the interplay between reaction kinetics, redox potential, reducing agent concentration, and oxygen were further investigated. Bioconjugation using DTT was high yielding in the presence of excess oxygen but yielded no product when conducted under an inert atmosphere (Table 1, reactions 1 and 2). When enough moles of oxygen were present to oxidize the DTT, the reaction again proceeded well, but adding more reducing agent as TCEP prevented reaction in the 18 h time frame tested (reactions 3 and 4). A single addition of fresh air to replace depleted oxygen was sufficient to move the reaction forward (reaction 5). This pointed to the importance of DTT oxidation for reaction progression. The redox potential was measured during the course of reactions using DTT in the presence of excess air (Figure 5a). The environment was very reducing at first, then increased over more than 15 h to a plateau in an oxidizing environment. Concurrently, no product was formed until the redox potential had inflected and plateaued, then the reaction advanced to completion over a number of hours. These rates are expected to depend on various factors including mixing efficiency, the ratio of air/liquid interface area to reaction volume, and dissolved oxygen content in the reaction buffer. We hypothesize that much of the copper is initially strongly complexed by the free thiols of DTT. As oxygen begins to oxidize the DTT, copper is released from DTT and may complex with the weaker ligand TBTA,⁴⁶ allowing productive access to the metal center. The initial redox state of the reaction was optimized by starting with 10 mM total of reduced and oxidized DTT at various ratios and monitoring kinetics (Figure 5b). The reaction was fastest with 1–2 mM (reduced) DTT present at time zero, presumably by reducing the delay time to reaction initiation while still providing sufficient reducing agent to reduce Cu(II) and provide Cu(I) to the system. Further experiments have shown that using 2 mM of (reduced) DTT with no added oxidized DTT provides the same results.

The molar excess of reactive PEG to Aha-IFN β is critical given the substantial price of high quality activated PEG reagent. When preparing conjugates with varying PEG MWs, we noted that the reaction efficiency was more dependent on the mass

Table 1. Reaction Dependence on Oxygen and Reducing Agent^a

no.	DTT (mM)	TCEP (mM)	atmosphere	% PEGylation	mmol component at t0			
					DTT	TCEP	O ₂	Cu
1	10	0	excess air	58	1.0×10^{-3}	0	4.8×10^{-3}	1.0×10^{-4}
2	10	0	argon	0	1.0×10^{-3}	0	0	1.0×10^{-4}
3	10	0	minimal air	60	1.5×10^{-3}	0	8.2×10^{-4}	1.5×10^{-4}
4	10	2	minimal air	0	1.5×10^{-3}	3.0×10^{-4}	8.2×10^{-4}	1.5×10^{-4}
5	10	2	minimal air, refreshed	46	1.5×10^{-3}	3.0×10^{-4}	8.2×10^{-4}	1.5×10^{-4}

^aAha-IFNb (0.1 mM, 2 mg/mL) was reacted with 20 kDa mPEG-PA (1 mM, 2 wt %) in the presence of 2 mM TBTA, 1 mM CuSO₄, 0.5 wt % SDS, and reducing agent(s) under various atmospheres. The reaction with excess air had a large air headspace in the tube, the reactions with minimal air had a very small headspace in the tube, and the “refreshed” reaction was opened after 43 min of reaction time, exposed to air, and then closed again. The mmol of O₂ present at the beginning of the reaction was calculated based on the volume of air present in the headspace and the theoretical content of saturated dissolved oxygen in the liquid.⁶³

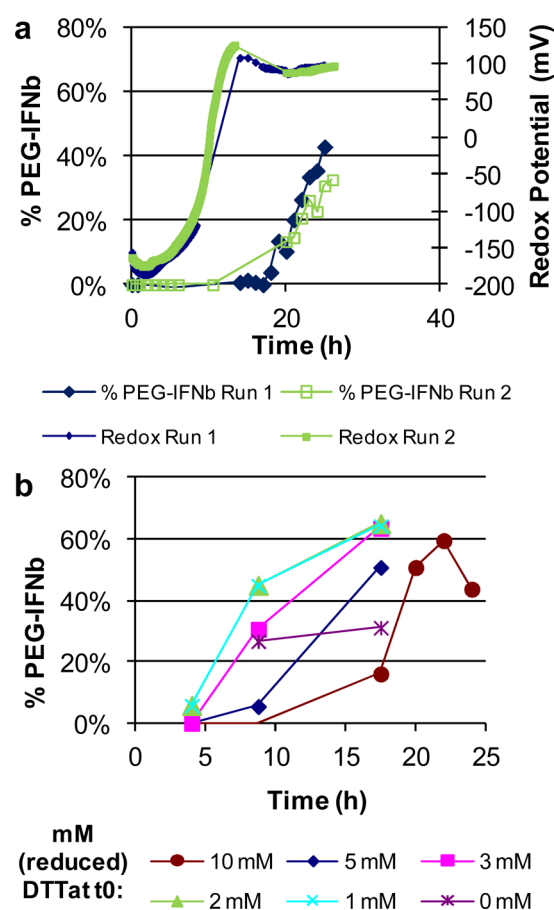


Figure 5. Reaction kinetics versus redox state. Aha-IFNb (0.1 mM, 2 mg/mL) was reacted with mPEG-PA 20 kDa (1 mM, 2 wt %) in the presence of 2 mM TBTA and 1 mM CuSO₄. Reactions were run with a large air headspace (more than 2 molar excess O₂ in headspace versus DTT in reaction). (a) Time course of redox potential and PEGylation reaction, with 10 mM (reduced) DTT at t0, SDS at 0.5 wt %; run in duplicate. (b) PEGylation kinetics with a total of 10 mM (reduced + oxidized) DTT at t0, but varying initial ratios between reduced DTT and oxidized DTT, SDS at 2 wt %.

ratio of PEG to IFNb rather than the molar ratio of reactive groups. Postulating that the PEG itself may be involved in interactions in the reaction mixture, the addition of unreactive PEG diol to the reactions was investigated. In Figure 6 series B, the total wt % of PEG in the reaction mixtures was held constant while varying the content of reactive PEG-alkyne; the PEG balance was provided by PEG diol. With this approach, the reaction efficiency was constant with a 2–10 molar ratio of

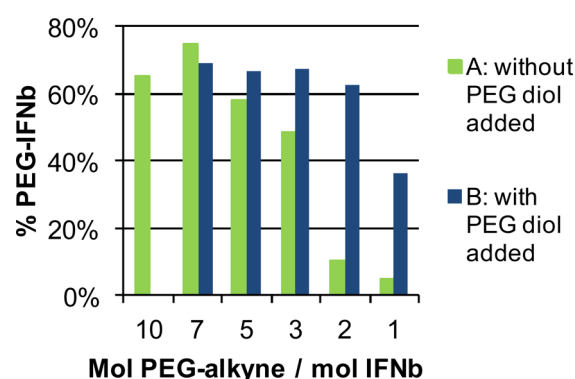


Figure 6. Effect of reactive and unreactive PEG on reaction yields. Aha-IFNb (0.05 mM, 1 mg/mL) was reacted with 40 kDa mPEG₂-PA_c in the presence of 3 mM TBTA, 1.5 mM CuSO₄, 2 wt % SDS, and 2 mM DTT. PEGylation yields were determined at various molar ratios of PEG-alkyne (mPEG₂-PA_c) to IFNb. In series A, as PEG-alkyne levels were reduced, the total wt % of PEG in the reactions reduced from 2 wt % to 0.2 wt %. In series B, as PEG-alkyne was reduced, unreactive 30 kDa PEG diol was added to maintain a constant 2 wt % of PEG in each reaction.

PEG-alkyne to IFNb, whereas without the addition of PEG diol (series A), the efficiency fell off at molar ratios less than 7. This effect has been reproduced with unreactive PEGs of varying molecular weights and end groups.

The optimized reaction conditions as given in Figure 6, 0.05 mM Aha-IFNb, 2 wt % total PEG (balance provided by PEG diol), 3 mM TBTA, 1.5 mM CuSO₄, 2 wt % SDS, and 2 mM DTT, afforded reproducible high reaction yields and quantitative protein recoveries. As the NAA incorporation efficiency varied during fermentation optimization, the content of Aha was measured in order to determine the actual PEGylation efficiency. As an example, by MALDI analysis of a tryptic digest of IFNb, the incorporation of Aha was determined to be 88%. The subsequent reaction with 10 molar excess of 40 kDa branched mPEG₂-PA yielded 85% PEGylation of total IFNb, indicating a PEGylation efficiency of 97% of Aha-containing IFNb. During GMP production with 3 molar excess of mPEG₂-PA, the reaction proceeded with 84–98% PEGylation efficiency.

With the developed conjugation conditions, Aha-IFNb was conjugated to PEGs of varying molecular weights and architectures. IFNb conjugates with linear 10, 20, 30, and 40 kDa mPEG-PA as well as 40 kDa branched mPEG₂-PA structures were refolded, purified, and analyzed for in vitro and in vivo characteristics. For brevity, these will be referred to as PEG-10-IFNb, PEG-20-IFNb, PEG-30-IFNb, PEG-40-IFNb, and

PEG₂-40-IFN β (a subset is shown in Figure 7). Mass units of PEG-IFN β conjugates herein refer to the mass of protein only

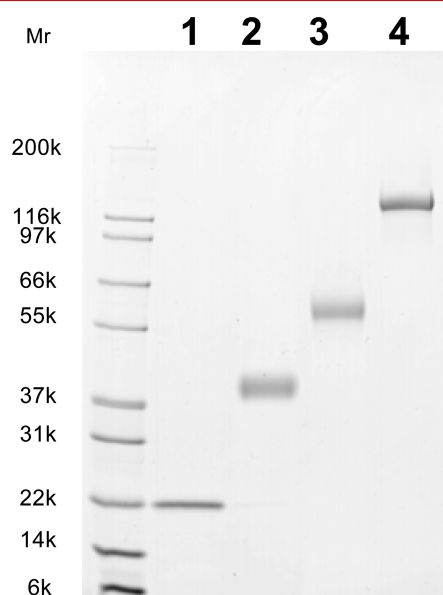


Figure 7. SDS-PAGE analysis of PEG-IFN β conjugates. (1) Aha-IFN β , (2) PEG-10-IFN β , (3) PEG-20-IFN β , (4) PEG₂-40-IFN β .

and do not include the associated mass of PEG. For example, 1 μ g of Betaseron refers to 1 μ g of IFN β protein and 1 μ g of PEG₂-40-IFN β refers to 1 μ g of IFN β protein (combined with 2 μ g of attached PEG).

The in vitro potencies of the conjugates with various PEG structures were compared by taking advantage of the antiviral properties of IFN β . The conjugate concentrations which inhibited 50% of Encephalomyocarditis virus lysis of A549 lung carcinoma cells (EC_{50}) are given in Table 2 (a higher EC_{50}

Table 2. In Vitro Potency of IFN β s and PEG-IFN β s as Determined by the Antiviral A549 Cytopathic Effect Assay^a

molecule	N	EC_{50} (pg/mL)
Betaseron	87	21 $\pm$ 17
Met-IFN β	6	12 $\pm$ 8.5
PEG-10-IFN β	10	16 $\pm$ 7.3
PEG-20-IFN β	23	150 $\pm$ 110
PEG-30-IFN β	34	180 $\pm$ 140
PEG-40-IFN β	31	300 $\pm$ 250
PEG ₂ -40-IFN β	114	790 $\pm$ 600

^aData expressed as the mean concentration needed to provide 50% protection from viral-mediated cell lysis (EC_{50}) $\pm$ standard deviation. N represents number of assays completed, PEG₂-40-IFN β was generated using mPEG₂-PA₄.

indicates a lower in vitro potency). The EC_{50} of PEG-10-IFN β was comparable to Betaseron (unPEGylated IFN β -1b). However, as the linear PEG molecular weight increased to 20–40 kDa, the in vitro potency decreased from 14% to 7%, respectively, of Betaseron. Adding a branch to the 40 kDa PEG structure decreased the measured potency further, to 3% of Betaseron.

The in vivo pharmacokinetic behaviors of the conjugates were compared in two species. Table 3 shows the results from subcutaneous (sc) administration in rats. Conjugation of linear 20 kDa PEG to IFN β increased the elimination half-life by more than 3-fold and the AUC by more than 6-fold when compared to

Table 3. Pharmacokinetics of Betaseron and PEG-IFN β s after Subcutaneous Administration in Male Sprague Dawley Rats^a

molecule	dose (mg/kg)	$AUC_{IFN\beta, obs}$ (h·pg/mL)	$t_{1/2}$ (h)
Betaseron	0.5	91000 $\pm$ 47000	1.7 $\pm$ 0.61
PEG-20-IFN β	0.5	570000 $\pm$ 49000	5.3 $\pm$ 0.98
PEG-30-IFN β	0.5	1000000 $\pm$ 420000	6.6 $\pm$ 1.6
PEG-40-IFN β	0.5	670000 $\pm$ 350000	5.1 $\pm$ 1.5

^aData for each molecule represent mean $\pm$ standard deviation calculated from three animals (except PEG-30-IFN β data is from two animals).

Betaseron. Increasing the PEG size to 30–40 kDa did not yield any significant further increase in these parameters in this model (due to material limitations, PEG₂-40-IFN β was not tested in this model). Table 4 and Figure 8 show the results from sc and intravenous (iv) administration in cynomolgus monkeys. This model is more relevant as human IFN β binds to the primate IFN β receptor but not to the rodent IFN β receptor. Here, increasing the linear PEG size from 20 to 40 kDa increased exposure by both routes, with the sc elimination half-life increased by more than 2-fold and the sc AUC increased by almost 5-fold. Adding a branching structure to 40 kDa PEG increased all parameters further, with approximately 2-fold increases in sc half-life and AUC compared to linear 40 kDa PEG. Bioavailability also increased in line with increasing PEG size and branching.

As a measure of the combined effects of potency and pharmacokinetic modifications on in vivo efficacy, various PEG-IFN β conjugates were compared to Betaseron in a tumor xenograft model in SCID mice. While not a model of multiple sclerosis, the model allows for a quantitative measure of biological activity in vivo by utilizing the antiproliferative effects of IFN β on certain tumor cell lines.⁴⁷ Mice were implanted with Daudi cells and then treated with PEG-IFN β or Betaseron for four weeks. Betaseron (3 μ g) was dosed at the typical clinical frequency of three times per week. PEG-20-IFN β and PEG-30-IFN β were administered with a dose equipotent to the 3 μ g Betaseron dose, based on in vitro bioactivity, but only once a week. PEG₂-40-IFN β was administered with a dose equivalent to 1/10 the bioactivity of the Betaseron dose (equivalent to 0.3 μ g Betaseron), once a week. Figure 9a illustrates that the extended in vivo exposure afforded by conjugating 20 kDa PEG to IFN β reduced tumor growth compared to Betaseron treatment in spite of the reduced dosing frequency. The PEG-IFN β s showed progressively more reduction in tumor size as the PEG size increased. PEG₂-40-IFN β , with only 1/10 the bioactivity-based dose and weekly administration, completely prevented tumor growth. Figure 9b illustrates a similar study, wherein equal protein masses (10 μ g of protein) of Betaseron or of PEG₂-40-IFN β were dosed at varying frequencies. PEG₂-40-IFN β dosed every other week was more effective than Betaseron dosed once a week or three times a week.

DISCUSSION

Many researchers have performed bioconjugations via CuAAC,⁴⁸ but a number of our approaches are unique.⁴⁹ When developing a chemistry step for manufacturing scale, the reactant ratios and reaction yields are critical. Bioconjugations are typically performed at low molar concentrations, adding a challenge for timely reaction yields using high molecular weight/low diffusivity reactants. The 2 molar excess of PEG used here to achieve high

Table 4. Pharmacokinetics of PEG–IFNBs after Subcutaneous or Intravenous Administration in Cynomolgus Monkeys^a

molecule	dose (mg/kg)		AUC _{IFNB-obs} (h·pg/mL)		<i>t</i> _{1/2} (h)		bioavailability (%)
	iv	sc	iv	sc	iv	sc	
PEG-20–IFNB	0.06	0.44	9100000 ± 2700000	7200000 ± 5200000	10 ± 0.63	12 ± 3.5	11
PEG-30–IFNB	0.06	0.44	13000000 ± 3100000	17000000 ± 22000000	13 ± 5.3	14 ± 5.5	18
PEG-40–IFNB	0.06	0.44	31000000 ± 4300000	35000000 ± 12000000	16 ± 1.9	27 ± 12	15
PEG ₂ -40–IFNB	0.06	0.30	35000000 ± 2300000	60000000 ± 7500000	25 ± 3.1	56 ± 24	34

^aData for each PEG–IFNB represent mean and standard deviation calculated from three animals. Bioavailability is calculated using the formula $F = (AUC_{IFNB-sc} \times dose_{iv}) / (AUC_{IFNB-iv} \times dose_{sc})$. PEG₂-40–IFNB was generated using mPEG₂-PA₃.

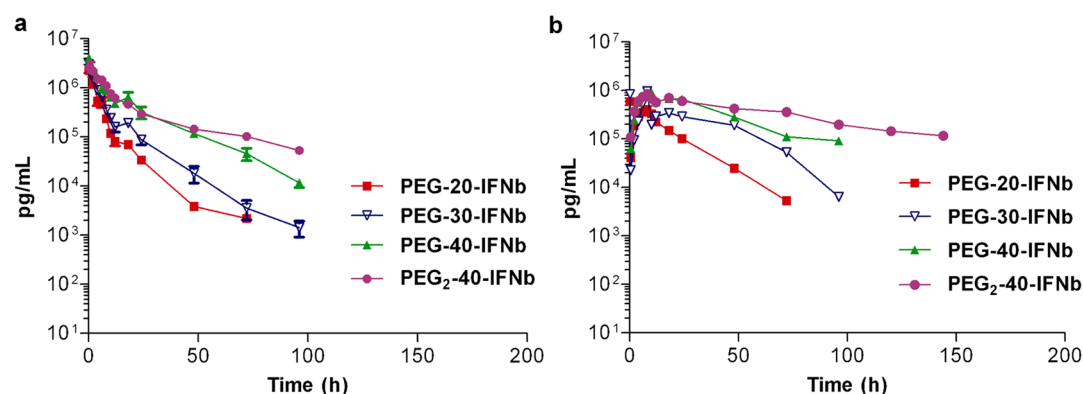


Figure 8. Pharmacokinetic profiles of PEG–IFNBs after subcutaneous or intravenous administration in cynomolgus monkeys. PEG₂-40–IFNB was generated using mPEG₂-PA₃. Plasma samples were collected at times indicated and assayed for serum levels of the conjugate by measuring antiviral activity. (a) Intravenous administration at 0.06 mg/kg. (b) Subcutaneous administration at 0.44 mg/kg (0.30 mg/kg for PEG₂-40–IFNB).

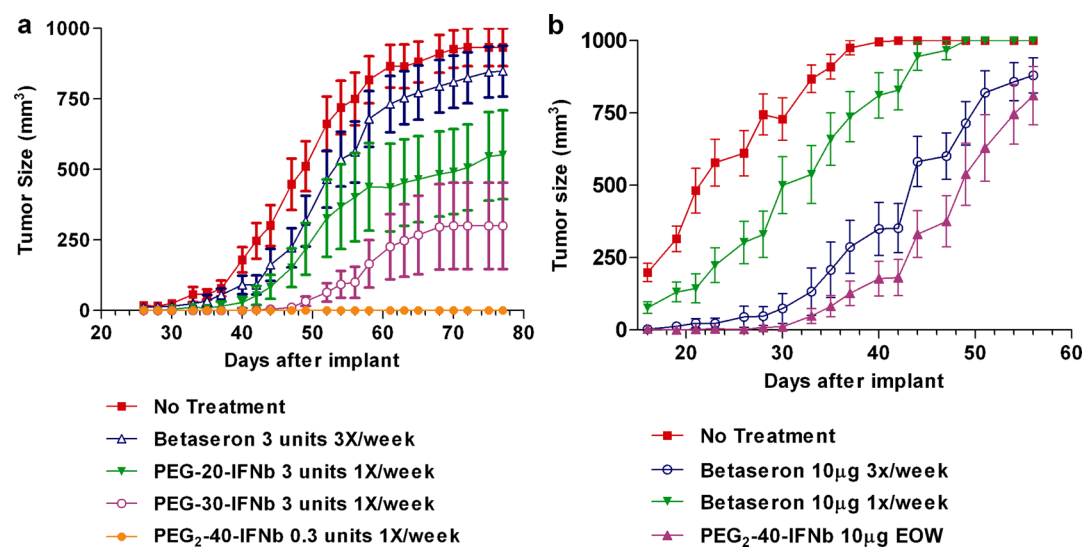


Figure 9. Growth of Daudi tumors in CB.17 SCID mice. Daudi cells were implanted subcutaneously on day 0. Mice were treated as indicated for four weeks. Data for each group represent the mean and standard error of the mean from 12 mice. Two experiments are shown, with IFNB dosing based on equivalent in vitro bioactivity or equivalent protein mass. (a) Bioactivity-matched dosing. Betaseron was administered at 3 μ g (3 arbitrary units) per dose subcutaneously, three times a week. PEG-20–IFNB and PEG-30–IFNB treated mice were administered a dose with bioactivity equivalent to 3 μ g of Betaseron (based on antiviral assay), once a week. PEG₂-40–IFNB (generated from mPEG₂-PA₃) treated mice were administered a dose with bioactivity equivalent to 0.3 μ g (0.3 arbitrary units) of Betaseron, once a week. (b) Mass-matched dosing: 10 μ g of Betaseron was dosed three times a week or once a week, and 10 μ g of PEG₂-40–IFNB (based on protein mass; generated from mPEG₂-PA₃) was dosed every other week.

reaction yields with Aha-containing IFNB compares favorably to 5–200+ molar excesses reported by others for CuAAC bioconjugations.^{37,38,50–52} The copper is present in up to 15 molar excess over the acetylide group, providing reasonable kinetics without problematic copper-induced degradation. Finn and co-workers have postulated that the low molarity aqueous conditions in bioconjugations necessitate a weakly complexing ligand in order to avoid strong inhibitory copper chelates.⁴⁶ Ours

is the first report we are aware of to optimize DTT as the reducing agent for CuAAC, perhaps due to concerns that the complexation of copper by thiol groups would similarly impede the reaction. Once the necessary air exposure conditions were determined, we were able to reproducibly achieve good yields with this convenient DTT/CuSO₄/air system. This system also avoids the requirement for rigorous exclusion of air when using the bathophenanthroline disulfonic acid ligand,⁵¹ avoids potential

degradation products from using ascorbic acid as the reducing agent,⁵³ and has no requirement for special equipment.⁵⁴ The TBTA ligand has low water solubility and works effectively as a solid in the heterogeneous system. It is convenient as it can be removed by filtration following reaction and worked at least as effectively as more water-soluble ligands when tested in our system. Similar methods to those developed here have also been successfully used to PEGylate potent bivalent scFv molecules.⁵⁵

Our PEGylation conditions are also distinctive, though not unprecedented,^{56–59} for being performed on a reduced, denatured protein prior to refolding. IFN β is notoriously hydrophobic and difficult to maintain in solution;¹⁴ all of the initial IFN β recombinant products were formulated with human serum albumin in order to maintain solubility and reduce noncovalent and disulfide-linked aggregates. Our approach avoids potential issues with IFN β precipitation and disulfide cross-linking during the reaction while demonstrating that the protein could be refolded after PEGylation. This approach may not be generalizable to all proteins but has been useful in many cases, especially with hydrophobic proteins expressed as inclusion bodies.

The reaction dependence on total PEG content, rather than solely the molar ratio of reactive alkyne to protein, may be related to interactions between the main PEG chains and SDS. Associations between PEG and SDS have been well studied and are complex.⁴² Above the critical aggregation concentration, SDS micelles can encapsulate the PEG backbone inside the micelles' hydrophobic core. At higher SDS concentrations, the PEG can move back out of the micelles and wrap around the outside of the micelles in a necklace-like complex. The phase diagram for these systems is determined by the concentrations of both PEG and SDS. It may be that a minimum amount of PEG is required to achieve the optimal configuration for reactivity between the PEG end group and Aha-IFN β . It appears the majority of the necessary PEG can be provided as inexpensive PEG diol.

Similar methods to those reported here have been used to manufacture PEG₂-40-IFN β under GMP at >800 L fermentation scale for clinical testing.¹⁶ Titers of 0.6 g/L were achieved with 81–85% incorporation of Aha; subsequent fermentation and codon improvements have increased titers to greater than 3 g/L. The reaction proceeded reproducibly with 84–98% PEGylation efficiency and conjugation detected solely at the Aha residue. The lack of multi-PEGylated or mis-PEGylated species allowed purification post-PEGylation to focus merely on reaction cleanup and polish rather than removal of undesired reaction products. Residual copper levels in the drug substance were <0.1 ppm with no special efforts for removal. After 18 months at 5 °C, no unPEGylated IFN β was detectable in the drug product, indicating the triazole linkage is stable.

PEGylation of IFN β demonstrated classical effects on the biological properties of the molecule.^{10,25} As the PEG molecular weight increased, the *in vitro* bioactivity began to decrease, presumably due to increased steric hindrance and concomitant reduction in the k_{on} resulting from an increased frequency of a hydrated PEG “cloud” blocking interactions between IFN β and its receptor.⁶⁰ The effect was magnified with a branched PEG, as there is increased likelihood of one of the PEG branches being located in the binding zone at a given point in time. It is interesting to note the varying magnitude of *in vitro* bioactivity decreases upon PEGylation, even at the same site on similar proteins. Reported IFN β bioactivities (as % antiviral activity of the unPEGylated protein) after conjugating 20 kDa PEG to the

N-terminus by reductive alkylation include 48% for 20 kDa mPEG-O-2-methylpropionaldehyde conjugated to IFN β -1a,²⁸ 100% for 20 kDa mPEG-propionaldehyde to IFN β -1a,³² 20% for the same PEG conjugated to murine IFN β ,³² and 66% for 20 kDa mPEG-butyraldehyde conjugated to IFN β -1b;¹⁴ for 40 kDa PEG, values include 19–25% for branched PEGs conjugated to IFN β -1b¹⁴ and “inactive” for a conjugate with IFN β -1a.³² Given the wide range in reported results for N-terminal reductive alkylation of IFN β , it is difficult to quantitatively compare the effect of N-terminal PEGylation of Aha via CuAAC but it does appear that the *in vitro* bioactivity effects are of a similar order of magnitude.

As expected, PEGylation increased the total exposure and the elimination half-life of IFN β when administered *in vivo*. Increasing PEG molecular weight or branching increased these parameters further, although not for 30–40 kDa linear PEGs in rats. The magnitude of increases in IFN β AUC and $t_{1/2}$ upon conjugation of 20 kDa PEG (sc administration in rats), or further increase when increasing PEG from 20 kDa linear to 40 kDa branched (sc administration in monkeys), were in accord with values reported in the literature, although the reported range is wide and convoluted by differences in administered dose, animal model, route, and bioanalytical methods.^{14,26,28,32,61}

Given that *in vitro* activity of PEGylated proteins is not predictive of *in vivo* efficacy,¹⁰ the tumor xenograft model provides a comprehensive evaluation of the therapeutic effect of the various physicochemical changes to IFN β upon PEGylation. Even though PEG₂-40-IFN β had the lowest *in vitro* bioactivity (3% of Betaseron) and was dosed at 10% of the activity units only once a week, it was strikingly the most effective molecule by completely preventing tumor growth. The results here are similar to the revelations in the development of Pegasys. Pegasys is the conjugate of a branched 40 kDa PEG with interferon α -2a and is indicated for chronic hepatitis C and B treatment. The *in vitro* antiviral activity of Pegasys is 7% of the parent protein yet demonstrates much more powerful *in vivo* antitumor activity than the parent protein.¹² Clinical trials showed weekly administration of Pegasys is a significantly more effective regimen for hepatitis C than thrice weekly interferon α -2a and also increases patient compliance.⁶² In particular, the nearly constant blood concentrations of Pegasys avoided the cyclic trough levels of interferon α -2a below efficacious levels. Likewise, we anticipate that PEG₂-40-IFN β may represent a significant therapeutic improvement over unPEGylated IFN β s with reduced dosing frequency in the treatment of multiple sclerosis. Clinical results of PEG₂-40-IFN β administration will be reported elsewhere.

■ CONCLUSIONS

We report here the practical application of global NNAA substitution to generate a PEGylated protein with absolute site-specificity through bioorthogonal conjugation chemistry. CuAAC was optimized for scalable high efficiency manufacturing, using DTT as the reducing agent in the presence of SDS. The PEGylated IFN β conjugates generated herein demonstrated increased *in vivo* efficacy, in spite of reduced *in vitro* potency, due to greatly improved pharmacokinetic properties. While the N-terminus was the chosen conjugation site for the current study, this method could be applied to any site in a protein which permits substitution of an appropriate natural amino acid. Our approach eliminates positional isomers and multiconjugated species and allows optimal choice of conjugation site for desired physicochemical and biological properties. This site-specific

technology can advance the development of many classes of engineered biomolecules such as polymer–protein conjugates, antibody–drug conjugates, and bispecific protein–protein conjugates.

■ ASSOCIATED CONTENT

■ Supporting Information

IFN β sequence, reaction of PEG-alkynes with Aha, SDS-PAGE stained for PEG and for protein, and titration curves of PEG-IFN β antiviral activity. This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: 206-518-5724. Fax: 206-518-5799. E-mail: nnairn@allozyne.com.

Present Address

†Altravax, Sunnyvale, California, United States.

Notes

The authors declare the following competing financial interest(s): All authors are, or have been, employed by Allozyne.

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■ ABBREVIATIONS USED

Aha, azidohomoalanine; AUC, area under curve; BPA, bathophenanthrolinedisulfonic acid disodium salt; CuAAC, copper-catalyzed azide–alkyne cycloaddition; DTT, DL-dithiothreitol; IFN β , interferon β ; iv, intravenous; NNAA, nonnatural amino acid; sc, subcutaneous; PEG, polyethylene glycol; TBTA, tris(benzyltriazolylmethyl)amine; TCEP, tris(2-carboxyethyl)-phosphine hydrochloride

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