Site-specific PEGylation of interferon-beta by Cu(I)-catalyzed cycloaddition

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Background

- PEGylation of therapeutic proteins can improve properties such as serum half-life, but lack of control over the PEGylation site can lead to reduced bioactivity and inhomogeneity of the product.
- Incorporation of a nonnatural amino acid into a recombinant protein can allow for site-specific conjugation using chemistries that are orthogonal to the natural amino acid functional groups.
- Interferon-beta (IFNb) is used clinically for the treatment of multiple sclerosis and hepatitis C virus.
- We have incorporated azidohomoalanine (AHA) at the N-terminus of IFNb and then conjugated a PEG-alkyne to the protein via a Cu(I)-catalyzed cycloaddition ("click chemistry").

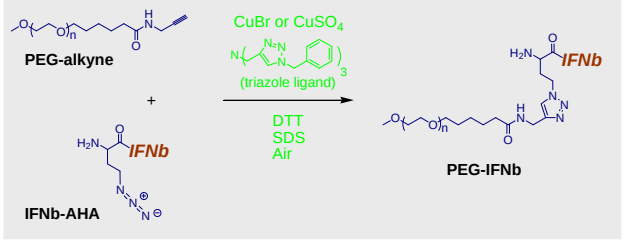
Methods

IFNb-AHA:

- Human IFNb was engineered to remove 3 out of 4 natural Met residues (M36I, M62I, M117T), to maintain bioactivity by modifying 2 other residues that interact with the internal Met (I40F, I44L), and to eliminate methionine aminopeptidase cleavage of the N-terminal AHA by modifying the penultimate residue (S2E).
- The N-terminal codon for Met in the mature protein was left intact.
- The protein was expressed in auxotrophic *E. coli*, in media containing AHA but no Met. The resulting recombinant protein contained AHA at the N-terminus (rather than Met).

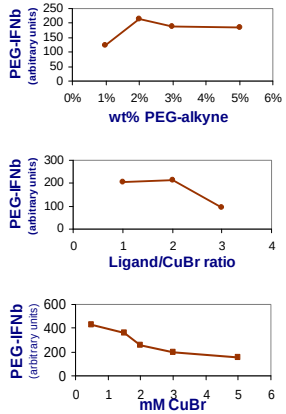
PEGylation:

- PEG-alkyne was reacted with IFNb-AHA while the protein was *denatured* and *reduced.*



Results

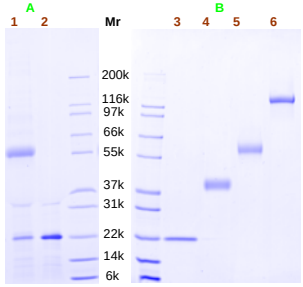
Optimization of reaction conditions



Soluble fractions of the reaction mixtures were separated by SDS-PAGE and the resulting PEG-IFNb bands were quantified by densitometry. Baseline conditions: 2% SDS, 100 mM phosphate, 10 mM DTT, pH 7.55, 0.5 mg/mL IFNb-AHA, 2 wt% PEG-alkyne 10 kDa (2 mM), 1 mM CuBr, triazole ligand at 2X [CuBr], rt, overnight.

- PEGylation was most efficient around 2-3 wt% PEG-alkyne, with higher concentrations likely being limited by viscosity.
- A 1:1 or 2:1 molar ratio of triazole ligand to copper catalyst was most efficient.
- Higher concentrations of CuBr caused the protein (primarily the unPEGylated protein) to precipitate, perhaps by copper-induced oxidation reactions.
- Optimal conditions resulted in >90% of IFNb-AHA being PEGylated.

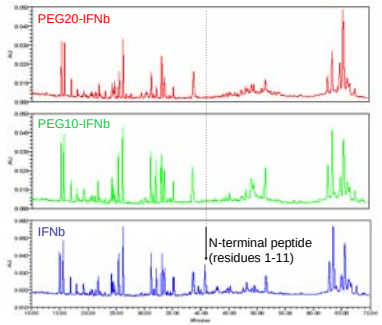
SDS-PAGE



A) Reaction mixture with (1) or without (2) Cu catalyst (excess unreacted PEG skews the gel). B) Purified IFNb-AHA (3), PEG10-IFNb (4), PEG20-IFNb (5), PEG40-IFNb (6).

- Reaction conditions lead to efficient monoPEGylation of IFNb-AHA (the reaction mixture also contains some IFNb-Met which is unreactive).
- PEG 10 kDa (linear), 20 kDa (linear), and 40 kDa (branched) have been conjugated to IFNb-AHA at up to 48 mg scale.

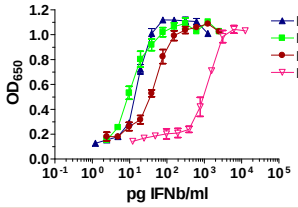
Peptide map



RPLC chromatogram of protein samples following tryptic digest.

- The 1-11 peptide peak is present in the IFNb sample but is absent in the PEG10-IFNb and PEG20-IFNb samples. No other peaks differed significantly between the samples.
- PEGylation was specific to the AHA residue at position 1, with no side reactions to other residues.

Antiviral activity



Bioactivities were assessed *in vitro* as ability to prevent lysis of A549 cells exposed to EMC virus (lower EC_{50} = more potent *in vitro*).

- Betaseron * EC_{50} = 17 pg/mL
- PEG10-IFNb EC_{50} = 11 pg/mL
- PEG20-IFNb EC_{50} = 48 pg/mL
- PEG40-IFNb EC_{50} = 1249 pg/mL
- *in vitro* bioactivities of the PEG-IFNbs were a function of the MW of the conjugated PEG.

* Betaseron is a commercial IFNb

Conclusions

- 10, 20, and 40 kDa PEG-alkynes were conjugated to IFNb-AHA in the presence of SDS and DTT.
- The reaction proceeded with high yield and was specific to the N-terminal AHA residue.
- The resultant conjugates retained *in vitro* bioactivity, with a dependence on the PEG MW. The enhanced residence time of PEG-IFNb expected *in vivo* would thus lead to a significantly more potent therapeutic.
- The process allows PEGylation of recombinant proteins at a single predetermined site, without side reactions to other residues in the protein.