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Intrinsic Bioconjugation for Site-Specific Protein PEGylation at N-Terminal Serine

Paul M. Levine, Timmy W. Craven, Richard Bonneau and Kent Kirshenbaum

Recently developed chemical ligation protocols were elaborated for rapid N-terminal protein PEGylation. We introduce a PEG-salicylaldehyde ester and demonstrate its site-specific ligation to the N-terminus of the RNase S protein and to the therapeutic polypeptide PTH (1-34).

The conjugation of poly(ethylene glycol) to proteins has become one of the most successful and widely utilized approaches to improve the stability and pharmacokinetics of biological therapeutics. This ‘PEGylation’ increases the hydrodynamic volume of the product molecules, establishing reduced clearance rates and protection from proteolytic degradation by endogenous enzymes. Implementation of typical chemical conjugation strategies can result in heterogeneous PEGylated proteins due to the presence of multiple sites of reactivity on the protein surface. This polydispersity can engender difficult separation and diminished biological activity for a significant fraction of the product species. For example, PEGylated Interferon αE2a, a hepatitis C therapeutic, can consist of various positional isomers and retains only a fraction of its activity (7%) in comparison to the original protein. In this study, we introduce a new method for the site-specific introduction of PEG chains onto proteins.

Ongoing studies are developing both extrinsic and intrinsic chemical reactivity for site-specific bioconjugation. Extrinsic bioconjugation is two-step process that requires genetic or chemical modification to incorporate a non-natural reactive moiety into the native protein target (Figure 1A). In the first step, extrinsic chemical reactivity is established by introducing non-natural amino acids or by modifying the protein termini, such as oxidizing the N-terminus to an aldehyde. Extrinsic functionality can also be introduced into proteins via chemo-enzymatic protocols. The second step then selectively conjugates the molecule of interest, such as PEG, to the protein target through a chemoselective process, such as a cross-coupling or ‘click’ reaction. In contrast, intrinsic approaches use inherent reactivity to selectively label protein side-chains

Figure 1. Extrinsic (A) and intrinsic (B and C) bioconjugation strategies. Red star represents molecule of interest to be conjugated to protein architecture.
Ser/Thr ligation, has established a chemoselective reaction between an oligomer fragment bearing a C-terminal salicylaldehyde ester and a fragment containing an N-terminal serine or threonine residue.\textsuperscript{18–21} Reversible imine formation by aldehyde capture in the presence of an N-terminal serine or threonine residue and a subsequent 1,5 O→N acyl shift produces a stable N,O-benzylidene acetal intermediate (Supplementary Figure 2). The stable intermediate is only formed in the presence of a 1-amino-2-hydroxy function, such as present for N-terminal serine or threonine. Cleavage of the acetal with a trifluoroacetic acid solution readily converts the intermediate into a native serine/threonine linkage at the ligation site.

We have previously adopted Ser/Thr ligation to show that peptoid oligomers bearing C-terminal salicylaldehyde esters can be ligated to peptides containing N-terminal serine or threonine residues, establishing native amide linkages at the ligation site.\textsuperscript{22} In addition, Ser/Thr ligation was used for the semi-synthesis of hybrid peptoid-protein conjugates by introducing modifications to current expressed protein ligation techniques.\textsuperscript{23} In this report, we evaluate whether Ser/Thr ligation can be used as an intrinsic reactivity protocol for site-specific N-terminal protein PEGylation in the presence of a PEG-salicylaldehyde.

First, we synthesized monodisperse PEG-salicylaldehyde from commercially available starting material (Figure 2A) and characterized product formation following high-performance liquid chromatography (HPLC) purification by electrospray ionization (ESI) mass spectrometry (calc. \(m/z\): 844.4; obs. \(m/z\): 844.8, Figure 2B). In addition, \(^1\)H-NMR was also used to characterize PEG-salicylaldehyde due to the distinct chemical shifts of the aldehyde and aromatic protons (Figure 2C). Relative to starting material, PEG-salicylaldehyde displays the addition of salicylaldehyde as confirmed by the appearance of a sharp singlet around 10 ppm and peaks between 7-8 ppm in the \(^1\)H-NMR spectrum.

Next, we generated S-protein from bovine pancreatic ribonuclease A (RNase A) by proteolysis. S-protein contains an N-terminal serine residue required for ligation (Figure 3A). The N-terminal portion of RNase A (residues 1-20), termed the S-peptide, can be excised by enzymatic cleavage using subtilisin to provide the S-protein.\textsuperscript{24,25} Following HPLC purification and characterization by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF), the corresponding S-protein was obtained (calc. \(m/z\): 11,534.3; obs. \(m/z\): 11,533.2, Figure 3B).

We then evaluated if PEG-salicylaldehyde can be selectively and directly ligated through a native amide linkage to the N-terminus of the S-protein (Figure 3A). First, PEG-salicylaldehyde was coupled to the S-protein in the presence of 1:1 pyridine/acetic acid, forming the corresponding N,O-benzylidene acetal intermediate. This reaction was monitored by analytical HPLC, which established that coupling was complete after 5 hours (Figure 3C). Following completion of the reaction (consumption of the S-protein), the intermediate was cleaved with TFA to afford the PEGylated protein conjugated through a native amide bond. MALDI-TOF analysis of the crude reaction confirmed formation of PEGylated S-protein (Figure 3D). Following lyophilization of the aqueous solution, the crude PEGylated protein was assessed for homogeneity by gel
Figure 3. Synthesis of N-terminal PEGylated S-protein by ligation and characterization. A) Schematic diagram of serine ligation between PEG-salicylaldehyde and the S-protein containing an N-terminal serine residue (PDB ID: 1FS3). Final protein concentration = 10 mM. B) MALDI-TOF analysis of purified S-protein. C) Analytical HPLC analysis of the ligation reaction. D) MALDI-TOF analysis of the crude ligation reaction after cleavage, confirming formation of PEGylated S-protein. E) SDS-PAGE analysis of crude ligation reaction after cleavage (lane 1, marker; lane 2, WT S-protein; lane 3, PEGylated S-protein). F) Circular dichroism spectra of S-protein (blue) and N-terminal PEGylated S-protein (red). Scans were performed at 25 °C in 10 mM PBS buffer (pH 7.5).

Figure 4. Synthesis of N-terminal PEGylated PTH (1-34) A) Schematic diagram of ligation between PEG-salicylaldehyde and PTH (1-34) containing an N-terminal serine residue (PDB ID: 1ET1). Final protein concentration = 10 mM. B) Analytical HPLC analysis of the ligation reaction. C) MALDI-TOF analysis of purified PTH (1-34) (blue) and the crude ligation reaction after cleavage (red). D) Circular dichroism spectra of PTH (1-34) (blue) and N-terminal PEGylated PTH (1-34) (red). Scans were performed at 25 °C in 10 mM PBS buffer (pH 7.5).

electrophoresis (Figure 3E). As expected, Coomassie staining revealed a distinct band corresponding to a discrete mono-PEGylated S-protein. Despite containing seven lysine residues within the S-protein, all characterization data is consistent with site-specific modification at the N-terminus. To evaluate any conformational perturbations associated with N-terminal PEGylation of the S-protein, far-UV circular dichroism (CD) spectroscopy was used. The CD signature of N-terminal
PEGylated S-protein was comparable to that of the wild-type S-protein, with a characteristic minimum observed at ~204 nm (Figure 3F). These results suggest that N-terminal PEGylation of the S-protein has negligible impact on the overall structure of the protein.

Next, we used serine ligation to generate an N-terminal PEGylated variant of parathyroid hormone (1-34) or PTH (1-34) (Figure 4A). PTH (1-34) is a therapeutic polypeptide that agonizes the class B G-protein-coupled receptor PTHr1 and is currently marketed (as Fortas®) to enhance bone density and formation in patients diagnosed with osteoporosis. PTH (1-34) exhibits a poor pharmacological attributes.

PEG-salicilaldehyde was therefore ligated to the N-terminal serine residue of PTH (1-34). The reaction was monitored by analytical HPLC, which determined that coupling was complete after 1 hour (Figures 4B). Following completion (consumption of PTH 1-34), the intermediate was cleaved with TFA to afford the PEGylated peptide, as determined by MALDI-TOF (calc. m/z: 4,736.5; obs. m/z: 4,736.4, Figure 4C).

The helical character at the C-terminus of PTH (1-34) has been shown to be critical for recognition by the PTHr1 receptor. In this case, the CD spectrum of PEGylated PTH (1-34) displayed a loss of secondary structure relative to the unmodified polypeptide. These results suggest that this modification at the N-terminus of PTH (1-34) can have a significant impact on the structure of the therapeutic peptide. Recent evidence has shown that N-terminal PEGylation of small helical peptides can effect peptide helicity.

The ideal chemoselective conjugation reaction would enable rapid covalent modification under biocompatible conditions. Although we have demonstrated that bioconjugation via serine ligation can be conducted rapidly at the protein level, the reaction conditions (i.e. using pyridine/acetate acid as a solvent followed by subsequent TFA cleavage) may not be suitable for many proteins. Issues pertaining to solubility or protein denaturing/refolding will have to be addressed on a case-to-case basis, as protein stability in these conditions will vary. Future efforts will focus on investigating ligation under other solution conditions.

Conclusions

The studies described herein establish a robust synthetic route for rapidly and site-specifically introducing PEG chains onto peptides and proteins through native amide linkages at N-terminal serine residues. Chemoselective fragment condensation reactions were conducted using either the S-protein or PTH (1-34) and homogenous PEG-salicilaldehyde. We anticipate this methodology will be used to site-specifically bioconjugate PEG chains onto the N-terminus of various peptide and protein therapeutics to potentially enhance their pharmacological attributes.

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Notes and references

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A rapid and site-specific method to introduce PEG chains onto the N-terminus of peptides and proteins through native amide linkages at serine is described.