ChemComm

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Journal:	*ChemComm*
Manuscript ID:	CC-COM-03-2014-001928.R1
Article Type:	Communication
Date Submitted by the Author:	18-Apr-2014
Complete List of Authors:	Levine, Paul; New York University, Craven, Timothy; New York University, Biology Bonneau, Richard; New York University, Kirshenbaum, Kent; New York University, Department of Chemistry

Journal Name

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

Cite this: DOI: 10.1039/xoxxoooooox

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Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/xoxxoooooox

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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.[1,2] This 'PEGylation' increases the hydrodynamic volume of the product molecules, establishing reduced clearance rates and protection from proteolytic degradation by endogenous enzymes.[3] 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.[4] This polydispersity can engender difficult separation and diminished biological activity for a significant fraction of the product species.[5] For example, PEGylated Interferon α-2a, 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.[6] 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.[7–14] 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.[15] 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.[8,12] In contrast, intrinsic approaches use inherent reactivity to selectively label protein side-chains[13]

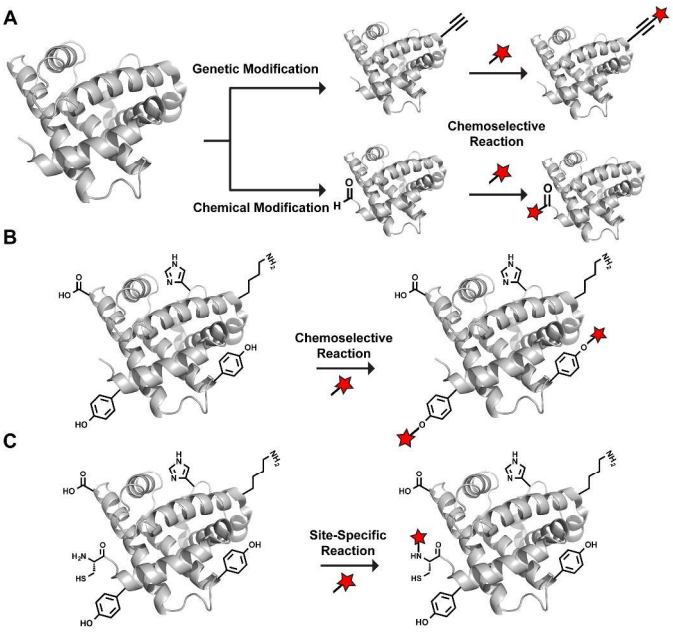

Figure 1. Extrinsic (A) and intrinsic (B and C) bioconjugation strategies. Red star represents molecule of interest to be conjugated to protein architecture.

(Figure 1B) or termini[14] (Figure 1C) in one-step. Although a number of promising intrinsic approaches have been reported, the challenge of selectivity still remains an issue.[16,17] For example, PEGylation of Chymotrypsinogen A using the 'tyrosine click' reaction yields two PEGylated products due to two tyrosine residues located on the external surface of the protein.[16] For this reason, new protocols that enable site-specific PEGylation using intrinsic functionality are needed.

Recent advances in chemical ligation techniques, in particular

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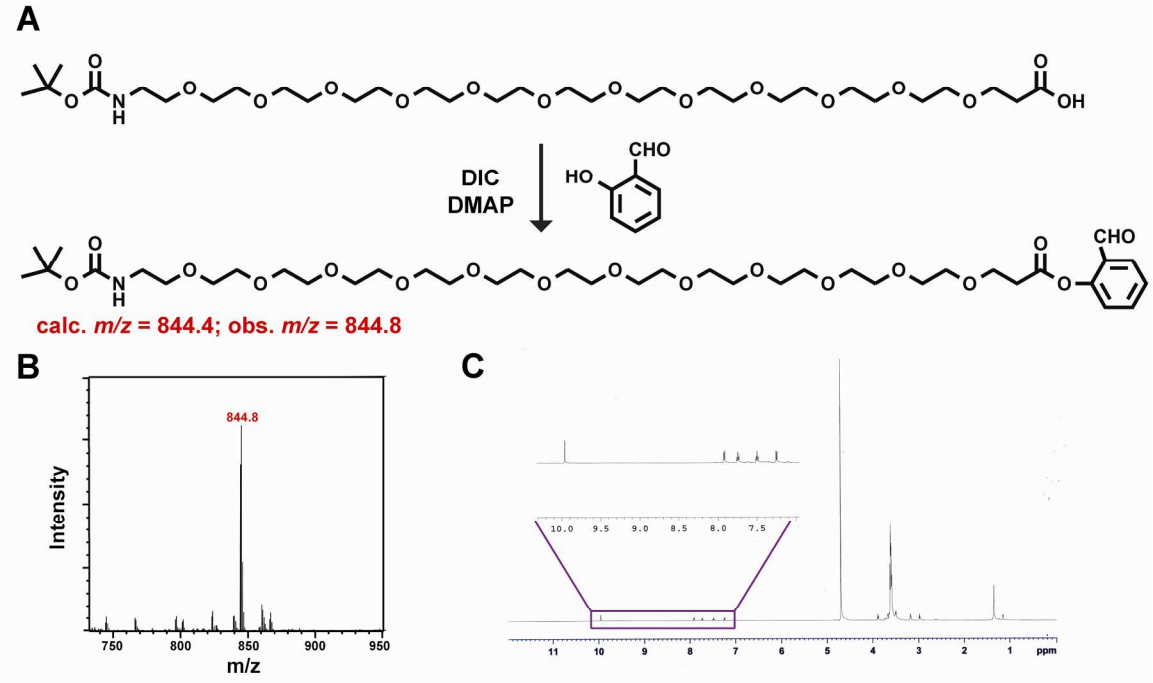

Figure 2. Synthesis and characterization of a monodisperse PEG-salicylaldehyde. A) PEG-salicylaldehyde was synthesized from commercially available starting material. Electrospray ionization (ESI) mass spectrometry (B) and ^{1}H-NMR (C) confirm formation of a monodisperse PEG-salicylaldehyde. DIC: *N,N'*-Diisopropylcarbodiimide; DMAP: 4-Dimethylaminopyridine.

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.[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.[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.[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.[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

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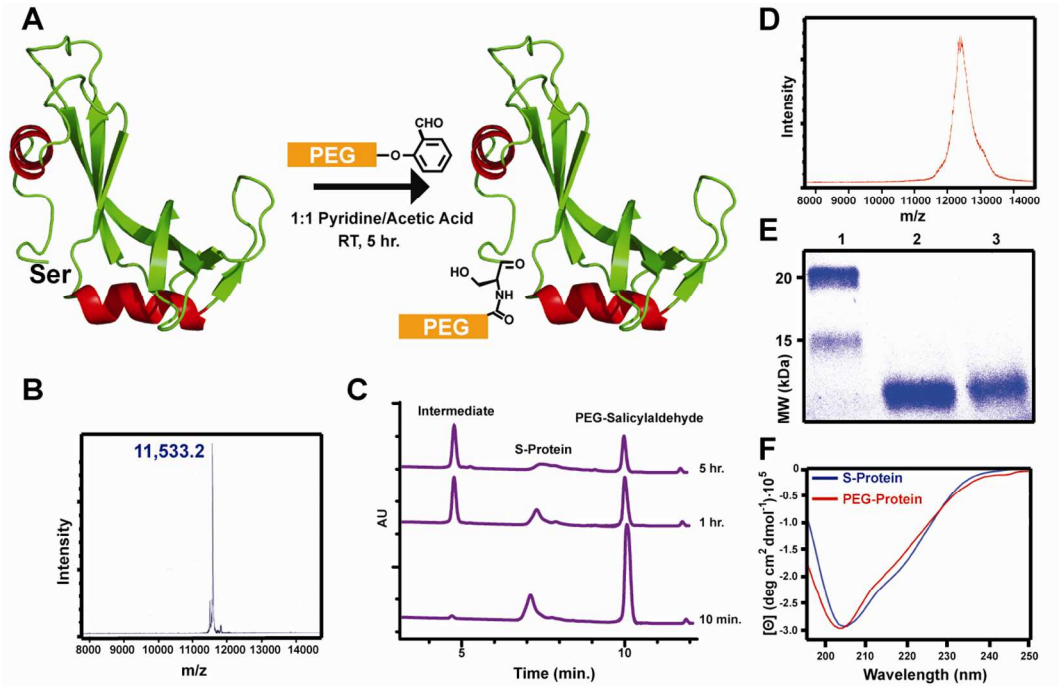

Figure 3. Synthesis of N-terminal PEGylated S-protein by ligation and characterization. A) Schematic diagram of serine ligation between PEG-salicylaldehyde and the S-protein containing an N-terminal serine residue (PDB ID: 1FS3). Final protein concentration = 10 mM. B) MALDI-TOF analysis of purified 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).

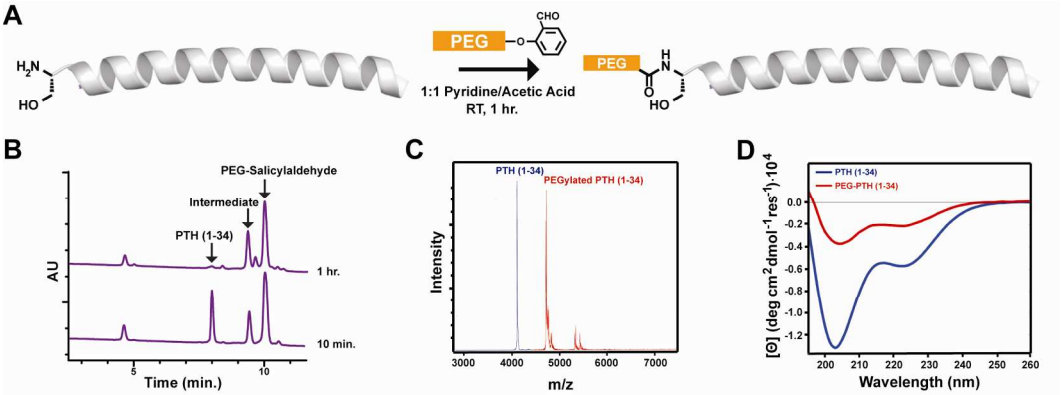

Figure 4. Synthesis of N-terminal PEGylated PTH (1-34) A) Schematic diagram of ligation between PEG-salicylaldehyde and PTH (1-34) containing an N-terminal serine residue (PDB ID: 1ET1). Final protein concentration = 10 mM. B) Analytical HPLC analysis of the ligation reaction. C) MALDI-TOF analysis of 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).[25] 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 Forteo®) to enhance bone density and formation in patients diagnosed with osteoporosis. PTH (1-34) exhibits a poor half-life in serum (~5 min), making it an attractive target for PEGylation to potentially enhance proteolytic stability.[26] PEG-salicylaldehyde 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 and 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.[27] In this case, the CD spectrum of PEGylated PTH (1-34) displayed a loss of secondary structure relative to the unmodified polypeptide.[28] 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.[29]

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-salicylaldehyde. 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.

Acknowledgements

This work was supported by the NSF (Award CHE-1152317 to K.K. and IOS-1126971 to R.B.) and the NIH (Award GM 32877-21/22 to R.B.). P.M.L thanks New York University for a Dean's Dissertation Fellowship.

Notes and references

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Electronic Supplementary Information (ESI) available: General ligation procedures. See DOI: 10.1039/c000000x/

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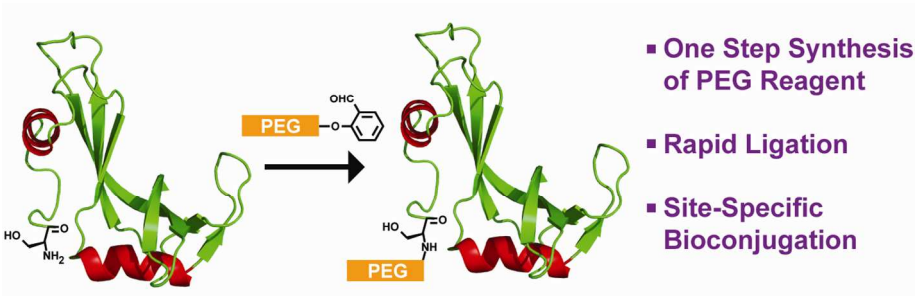

A rapid and site-specific method to introduce PEG chains onto the N-terminus of peptides and proteins through native amide linkages at serine is described.