Additional Studies in the Separation of PEGylated Proteins by Reversed Phase Chromatography

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Abstract
Additional studies were undertaken to better understand the chromatographic behavior of PEGylated proteins in an effort to improve purification and characterization techniques of such proteins. In previous studies[1], proteins were modified with relatively small polyethylene glycol moieties (PEG). However, in this study proteins were PEGylated using larger (20 kDa and 40 kDa) PEGylation reagents that are commonly used in pharmaceutical drug development. Generated PEGylated proteins were separated from unmodified proteins using different reversed phase medias (Jupiter® C4 and Jupiter C18). Similar to previous results, reversed phase media can separate PEGylated proteins based on both their degree of modification as well as the site of PEG modification; GFC medias can only isolate proteins based on degree of modification. Unlike previous studies where it was found that the Jupiter C4 medias generated the best separation of PEGylated proteins, in this study it was found that the Jupiter C18 media provided the best separation of PEGylated proteins from their unmodified counterparts. Such results further clarify good method starting points for developing analytical and preparative separations of PEGylated proteins.

Introduction
The benefits of covalently conjugated PEG molecules have been well studied in their application to therapeutic protein & oligonucleotide drugs, hormone factors, lipids, and nano-particles. PEGylation increases the size of the molecule and protects it from proteolysis and enzymatic degradation in biological fluids, thus extending the duration of its efficacy in the body. [1] PEGylating proteins is considered the standard method for improving the pharmacokinetics of protein therapeutics currently approved for a variety of diseases including cancer, renal failure, and autoimmunity.[2]

PEGylation is the chemical process of covalently attaching Polyethylene glycol (PEG) chains to specific amino acid residues of polypeptides or other molecules. It increases the surface polarity of a bio-polymer while increasing the overall stokes radius of the complex (resulting in an overall increase in net hydrophobicity). Depending on the desired site of modification, different attachment chemistries can be used to attach single or multiple PEG moieties to a protein, most commonly used are chemistries that attach to primary amines on proteins (either the N-terminus or any Lysine residue) or to a free sulfhydral (cysteine residues) moiety.

A previous study[3] using low molecular weight PEGs focused on improving reversed phase chromatography conditions for separating different sites of PEGylation on various proteins. Numerous parameters including temperature, gradients, and mobile phase composition were evaluated; such results suggested that elevated temperature, acetonitrile mobile phases and C4 based medias (Jupiter C4) provided the best resolution of different sites of PEGylation as well as optimal separation between a PEGylated and unmodified protein.

Additional studies were initiated to determine if similar chromatography is observed when larger PEG forms were used. Such PEGs (20 kDa and 40 kDa PEGs with varying chemistries) are more typically used in pharmaceutical drug development and thus might give more relevant information for those developing PEGylated protein therapeutics.

Materials and Methods
Analyses were performed using a HP 1100 LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a DAD detector. Various HPLC columns were used for evaluations including Jupiter® 300 5 µm C4, Jupiter® 300 3 µm C18 and 5 µm C18 and (all 150 x 4.6 mm, Phenomenex, Torrance, CA, USA). Native proteins were purchased from Sigma Chemicals (St. Louis, MO, USA) and PEGylation reagents to modify proteins were obtained from Jenkem Technology (Beijing, China). Solvents were purchased from Fisher Scientific (Fairlawn, NJ, USA).

Protein PEGylation was performed using two PEG N-hydroxysuccinimide (NHS) esters derivatives including Methoxy PEG Suiccinimidyl Carboxy Methyl Ester, MW 20 kDa (M-SCM-20K) and Methyl-PEO12-NHS Ester; Methoxy PEG Propionaldehyde, MW 20 kDa (M-ALD-20K), and Y-shape PEG Aldehyde, MW 40 kDa (Y-ALD-40K). Native proteins were dissolved in phosphate buffer pH 7.8, M-SCM-20K were dissolved in dry water-miscible DMSO; protein PEGylation reaction was done with 6-fold molar excess of M-SCM-20K for protein solution concentration of 40 mg/mL. Another set of native proteins and PEG substances (M-ALD-20K and Y-ALD-40K) were dissolved all in phosphate buffer pH 6.5 with 20 mM of sodium cyanoborohydrate; the reaction was done with 8-fold molar excess of M-SCM-20K for protein solution concentration of 40 mg/mL.

The reaction mixture was incubated in an ice bucket for up to two hours (different time-points were taken for some experiments). Reaction mixture is quenched with an equal volume of 50 mM Tris/ 1 % TFA (pH~2). Diluted in mobile phase, aliquots of 10-15 µg of protein (4 µL) were injected in HPLC for analysis.

Aqueous mobile phase used in reverse phase experiments was 0.1 % TFA and 2 % ACN in water and gradient from 20 % to 65 % B of organic mobile phase, 90 % acetonitrile/0.085 % TFA in water in 25 min was used. After each run the column had a 5 minutes flush with 90 % B followed by re-equilibration at 20 % B. The column temperature used was 45 °C. Flow rate for all analyses was 1 mL/min and UV monitored protein elution was at 220 nm (such conditions were indicated to give the best results for PEGylated proteins based on the previous study).

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Results and Discussions

Previous results had shown that reversed phase chromatography (RP) provided equal or superior separation of PEGylated proteins when compared to gel filtration chromatography (GFC). Unlike GFC, which can only separate PEGylated proteins based on degrees of polymerization, RP chromatography can also separate PEGylated species based on site of PEG attachment. Method development starting parameters such as mobile phase composition (Water/ TFA and Acetonitrile), temperature (45 °C), gradient (20-65 %) and RP media (Jupiter® 5 µm C4) were determined. This study was initiated to see if such parameters applied to larger PEG species as well as determine if a new wide pore media (Jupiter 3 µm C18) was useful for separation of PEGylated proteins.

Various PEG derivatives with different PEG chain length, shape, and molecular weight were chosen to monitor heterogeneity and complexity of PEGylated proteins/peptides. Two N-terminal PEGylation reagents were chosen, Methoxy PEG aldehyde, MW 20 kDa (PEG3) and Y-shape 40 kDa PEG aldehyde (PEG 4) which undergo reductive amination reactions with primary amines in presence of cyanoborohydride pH 6.5 [to take advantage of the lower pK_a of the N-terminal amine (pK_a~8) compared to amino acid side chains, such conditions result in selective modification of the N-terminus].[4]

Two other amine PEG reagents, Methoxy PEG NHS-ester, MW 20 kDa (PEG 1) and 12-mer Methoxy PEG NHS-ester, MW 1 kDa (PEG 2) react efficiently with primary amino groups (-NH2) at higher 7.8 pH forming amide bonds at each lysine (K) residue as well as the N-terminal amine.

Reverse phase separation data completed in this study demonstrate a distribution of PEG-protein conjugations. **Figure 1** shows chromatographic differences in the PEGylation reaction of alpha-Chymotrypsinogen A protein using the two different 20 kDa PEGylation reagents. As it was described, one PEGylation reagent reacts with both the Lysine side chain and N-terminus of Chymotrypsinogen (PEG 1, **Figure 1A**); the other PEGylation reagent reacts only with N-terminus (PEG 3, **Figure 1B**). From the results it appears that the reaction occurs at different rates and generates different species that can be separated chromatographically. As expected, PEG 1 quickly modifies multiple sites that can be separated chromatographically; despite claims of specific modification it appears that PEG 3 reacts with side chains, albeit at a lower reaction rate than PEG 1.

Based on experimental data of reverse phase separation, the PEGylation reaction could be done in one hour for some proteins, and others requires longer time. **Figure 1A and 2** show the PEGylation process (using PEG 1) for alpha-chymotrypsinogen A completed in 60 minutes and for ribonuclease A the reaction can take more than 24 hours to reach completion. PEGylation of Substance P peptide with 1 kDa PEG molecule Methoxy PEG NHS-ester, presumably reacts efficiently with primary amino groups (-NH2) at higher 7.8 pH, separating as one peak on RP media (PEG 2, **Figure 3**).

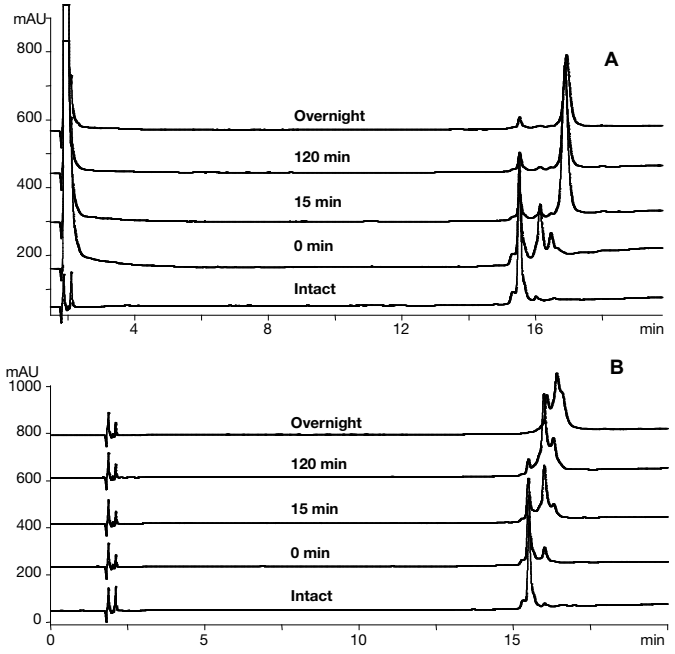

Figure 1.
Reverse phase separation on Jupiter 300 C18 3 µm particles column. Dynamics of PEGylation reaction at different time-points of alpha-Chymotrypsinogen A are shown with (A) Amine 20 kDa Methoxy PEG NHS-ester at pH 7.8 (PEG 1) and (B) with N-Terminus 20 kDa Methoxy PEG aldehyde at pH 6.5 (PEG 3).

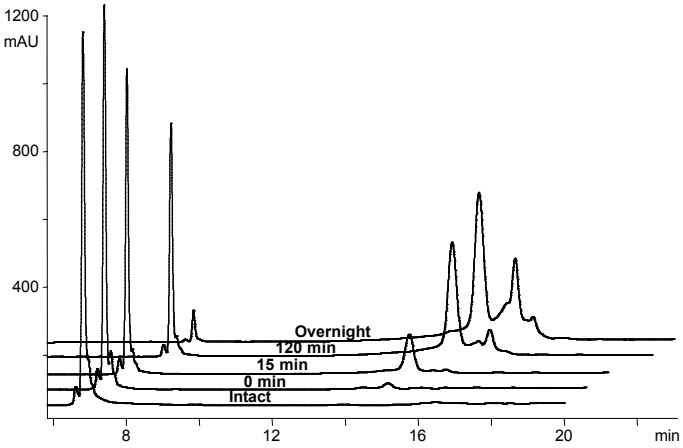

Figure 2.
Separation of N-Terminus PEGylated Ribonuclease A with 20 kDa Methoxy PEG aldehyde (PEG 1) on Jupiter 300 3 µm C18.

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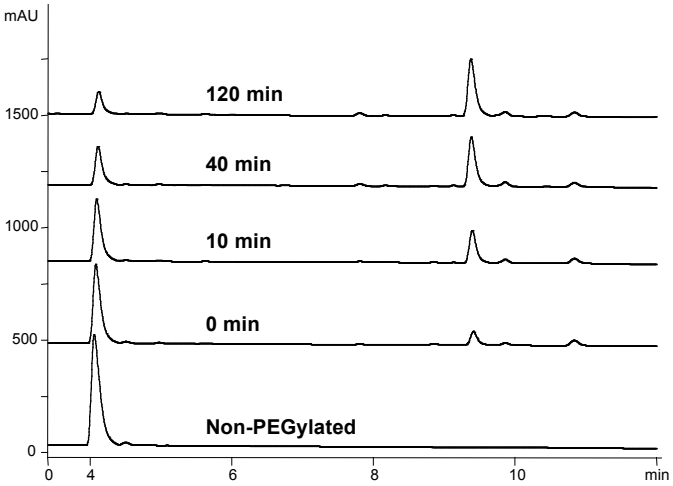

Figure 3.
Separation of Amine PEGylated 1-7 fragment peptide Substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe) with 1 kDa Methoxy PEG NHS-ester (PEG 2) on Jupiter® 300 5 µm C4.

Some PEG derivatives separate/elute with longer time on RP, creating difficulties of separation/purification of PEGylated products from unreacted PEG molecules. For some proteins, the conjugated proteins and PEG moiety elute with similar retention times on RP and GFC medias. Selected proteins in this study could still be separated on Jupiter® C18 3 µm and PEGylation reaction process are well presented (**Figure 4**, separation of N-Terminus PEGylated beta-lactoglobulin A with 40 kDa Y-shape Methoxy PEG aldehyde). While retention of analytes are similar, a further modification of separation method may be considered to improve separation.

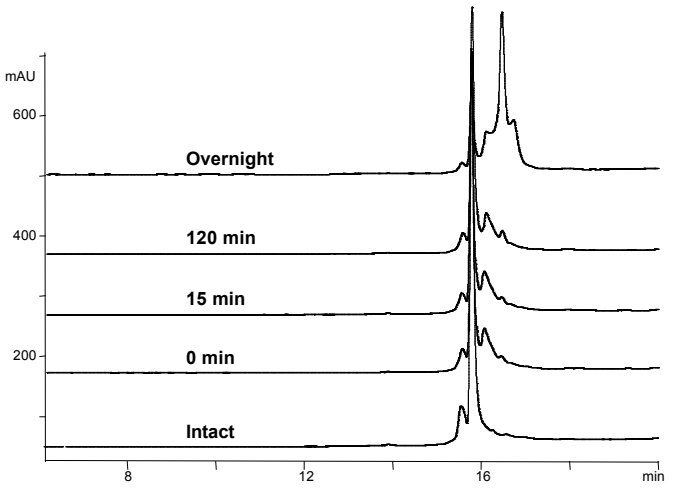

Figure 4.
Separation of N-Terminus PEGylated beta-Lactoglobulin A with 40 kDa Y-shape Methoxy PEG aldehyde (PEG 4) at pH 6.5 on Jupiter C18 3 µm.

To consider the complexity of PEGylated product separation, several reverse phase columns were selected for this test and several protein sample fractions at different PEGylation reaction time-points were analyzed (**Figure 5**). Comparing data on Jupiter 300 C18 3 µm, Jupiter 300 C18 5 µm, and Jupiter 300 C4 5 µm for separation of large 20 kDa (**Figure 5A and B**) and 40 kDa (**Figure 5C**) PEG molecules conjugated with proteins, the C18 delivers better resolution compared to Jupiter C4. However, for separation of PEGylated proteins and peptides with small PEG molecules less than 1 kDa, the C4 media gives better resolution performance[3] (data not shown here). Furthermore, separation on smaller particles (Jupiter C18 3 µm column) gives an advantage in separation of big PEGylated molecules compared to the Jupiter 5 µm C18 column. This is clearly observed based on the separation of some selected fractions of PEGylated alpha-chymotrypsinogen A and beta-lactoglobulin A proteins.

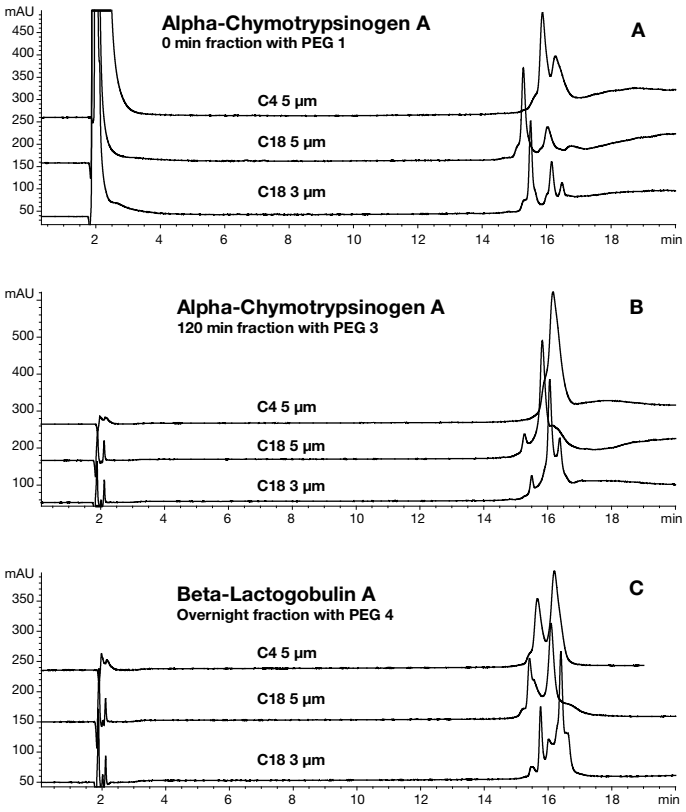

Figure 5.
Reversed phase separations of different PEGylated and intact proteins selected at different reaction time-points on three different columns. **(A)** PEGylated alpha-Chymotrypsinogen A with PEG 1 Amine 20 kDa Methoxy PEG NHS-ester, 0 min fraction; **(B)** PEGylated alpha-Chymotrypsinogen A with PEG 3 N-Terminus 20 kDa Methoxy PEG aldehyde, 120 minute fraction; **(C)** PEGylated beta-Lactoglobulin A with PEG 4 N-Terminus 40 kDa Y-shape Methoxy PEG aldehyde, overnight fraction. Good resolution was achieved on Jupiter 3 µm C18 small particles versus larger Jupiter 5 µm C18 particles or Jupiter C4 bonded media.

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Conclusion

This technical note shows that the evaluated Jupiter® RP media is a good analytical tool to analyze and characterize protein PEGylation processes. Additional useful applications for RP chromatography include defining sufficient excess of PEG moiety for reaction with proteins of interest, monitoring PEG conjugation process for native bio-molecules, and to purify their PEGylated products. We have shown further improvements and advantages for separation of large PEGylated conjugates and unreacted molecules using smaller particle reverse phase Jupiter 300 3 µm C18 media versus Jupiter 300 5 µm C18, and advantage of separation of smaller PEGylated conjugates on reverse phase Jupiter 300 5 µm C4 media.

References

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Ordering Information

Part No.	Description	Unit
00F-4263-E0	Jupiter 300 3 µm C18, 150 x 4.6	ea
00F-4167-E0	Jupiter 300 5 µm C4, 150 x 4.6	ea
00F-4053-E0	Jupiter 300 5 µm C18, 150 x 4.6	ea

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