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APPLICATIONS

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Isolating PEGylated Proteins Using BioSep™ GFC Columns

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Recent improvements in the manufacturing of BioSep GFC columns have led to a significant improvement in the efficiency, reproducibility, and inertness of the product line. While data has been shown previously for isolating PEGylated proteins from their unmodified protein precursors, recent improvements further increase the purity, loading, and recovery of the different isolated PEGylated forms. Such data show the utility of BioSep in isolating and quantitating PEGylated proteins.

Introduction

Over the last twenty years, the most common intentional modification of protein therapeutics has been the covalent attachment of polyethylene glycol (PEG) moieties to protein therapeutics to increase the pharmacokinetics properties of a protein. Proteins and peptides smaller than 60 kDa molecular weight are predominantly eliminated from the body by the kidneys through dialysis. Any method that increases the overall size of a protein in solution will generally result in an improved half-life in vivo. Because PEG is highly polar and heterogeneous it can be difficult to separate PEGylated proteins from their unmodified precursors; separating proteins by their degree of PEGylation can be even more challenging by common separation modes. Because Gel Filtration Chromatography (GFC) separates compounds based on their relative size in solution, GFC is ideal for separating PEGylated proteins based on their degree of PEGylation. In this technical note several examples of separating PEGylated protein are demonstrated to show the utility of using BioSep GFC columns for the purification of PEGylated proteins as well as monitoring the reaction kinetics of a PEGylation.

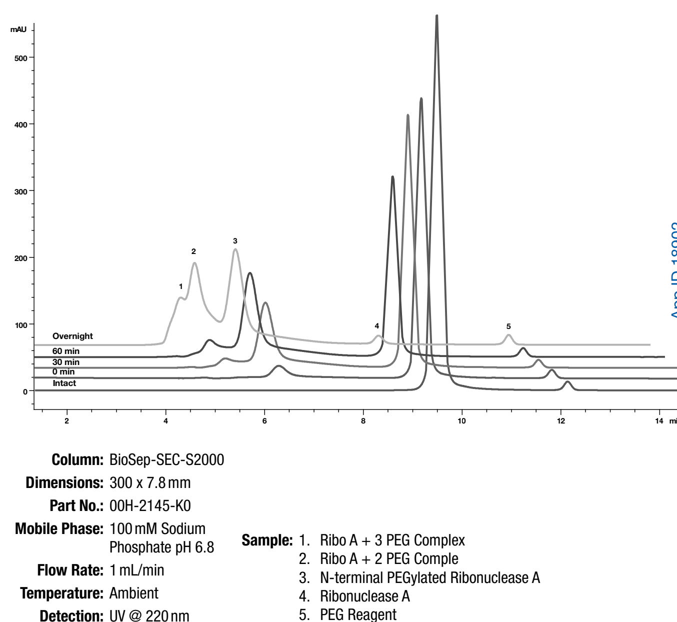
Materials and Methods

PEGylation reagents were obtained from Pierce (Rockford, IL). All other reagents were obtained from Sigma (St. Louis, MO) and solvents were obtained from EMD (San Diego, CA). Chromatography was performed on an Agilent HP1100 HPLC system equipped with an autosampler and UV detector utilizing Agilent ChemStation™ software, and a BioSep-SEC-S2000 GFC column, 300 x 7.8 mm (Phenomenex, Torrance, CA, USA) was used for all PEG/ Protein separations. Mobile phase used for all separations was 100 mM sodium phosphate pH 6.8 with a 1.0 mL/min flow rate. Protein elution was monitored at 220 nm. Proteins were PEGylated using an N-terminal modifying PEGylation reagent (Methoxy PEG propionaldehyde) using recommended modifying conditions. Time points for each reaction were quenched and then run on the BioSep-SEC-S2000 column to determine the degree of PEGylation of each protein tested over the time course.

Results and Discussion

Unlike other separation modes where chromatography is based on interactions between a stationary phase and an analyte, in gel filtration chromatography (GFC), separations are based solely on the size in solution of an analyte and its ability to penetrate the pores of a polar-functionalized media. This gives GFC some unique advantages in separating PEGylated proteins, because modified proteins can be isolated based solely on their degree of modification (1x, 2x, etc. of PEG groups attached to a protein). This differs from reversed phase separations of PEGylated proteins where the site of modification can also have an influence on the separation of PEGylated proteins. When one looks at a time course study of a PEGylation reaction, GFC fractionation allows one to assess the progress of the reaction to determine an appropriate endpoint. An example of such reaction monitoring is shown in **Figure 1** where ribonuclease A is reacted with a 20 kDa PEG. The time course study shows significant amounts of unreacted protein at the 60 minute time point versus the overnight time point, where little unmodified protein remains. However, there is a significant amount of multi-PEG species observed in the reaction suggesting a shorter reaction time to maximize mono-PEG modified protein.

Figure 1.
Time course study of the PEGylation reaction of Ribonuclease A with a 20 kDa PEG. Note the increase of the singly-PEGylated protein in the later time points with a corresponding decrease in unmodified protein. BioSep 2000 separates the different protein species based on the degree of PEGylation.



While the PEG reagent used in this experiment is supposedly exclusive to the N-terminus of the protein alone, several reactions performed with this reagent (data not shown) has found multiple modifications to occur with different reaction kinetics based on the protein used; such variability is demonstrated in **Figure 2** as a time course study using chymotrypsinogen A. In the example using chymotrypsinogen A, the singly-PEGylated protein is the most predominant species present even after 30 minutes reaction time, confirming previous observations about reaction kinetics. Such results suggest an optimal incubation time between 30-60 minutes for this protein to maximize the singly-modified PEGylated protein while minimizing proteins with multiple modifications. Such results show the utility of using GFC with the BioSep 2000 column for monitoring the PEGylation reaction as well as purifying PEGylated proteins.

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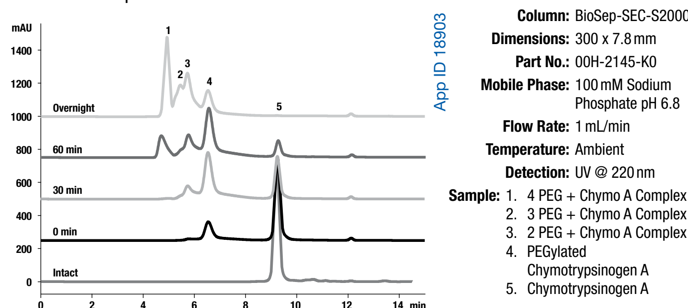
Figure 2.
PEGylation reaction of Chymotrypsinogen A monitored using BioSep 2000. Note the different reaction kinetics based on the protein used. In this case, the BioSep column well resolves the singly-modified protein from multimeric species.



Conclusions

These experiments demonstrate the utility of BioSep-SEC-S2000 GFC for monitoring protein PEGylation reactions. The BioSep 2000 column optimally separates proteins below 200 kDa molecular weight, which makes it ideal for monitoring most PEGylation reactions where proteins, PEG, and the resulting conjugates are typically below 200 kDa. The improved efficiency and inertness of the BioSep media also allows for better resolution of different PEGylated species, resulting in better quantitation of reaction kinetics as well as allowing for better purification of individual PEGylated proteins.

BioSep™ Ordering Information

Stainless Steel Columns (mm):	Narrow Bore	Analytical		Preparative
Phases	300 x 4.6	300 x 7.8	600 x 7.8	300 x 21.2
BioSep-SEC-S2000	00H-2145-E0	00H-2145-K0	00K-2145-K0	00H-2145-P0
BioSep-SEC-S3000	00H-2146-E0	00H-2146-K0	00K-2146-K0	00H-2146-P0
BioSep-SEC-S4000	00H-2147-E0	00H-2147-K0	00K-2147-K0	00H-2147-P0

Stainless Steel Guard Columns (mm)	Narrow Bore	Express	Analytical
Phases	30 x 4.6	35 x 7.8	75 x 7.8
BioSep-SEC-S2000	03A-2145-E0	03Q-2145-K0	03C-2145-K0
BioSep-SEC-S3000	03A-2146-E0	03Q-2146-K0	03C-2146-K0
BioSep-SEC-S4000	03A-2147-E0	03Q-2147-K0	03C-2147-K0

If BioSep analytical columns do not provide at least an equivalent separation as any other GFC column of similar porosity, type and dimensions, return the column with comparative data within 45 days for a FULL REFUND.

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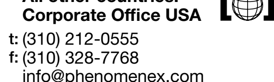

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