Monitoring Protein PEGylation with Ion Exchange Chromatography

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Overview

Purpose: To develop a method to characterize a mixture of proteins after branched polyethylene glycol (PEG)ylation.

Methods: Cation-exchange chromatography was used in connection with mass spectrometry (MS) to evaluate the degree of PEGylation of model proteins and to separate mixed isomers present under different PEGylation conditions.

Results: The reaction of lysozyme with a branched PEG was monitored, showing the progression of singly modified proteins at low reactant molar ratios to mixtures of >25 resolved isomers and up to five PEGylation sites at a 10:1 PEG/lysozyme ratio. PEGylation of ribonuclease A, with a total of ten potential lysine modification sites, showed PEG variants of up to six modifications with multiple isomers. This method shows good separation between the native unmodified protein and positional isomers, allowing detailed analysis during reaction optimization and final product characterization.

Introduction

Protein PEGylation has been used as a strategy to extend serum half-life and reduce immunogenicity and toxicity, which are appealing attributes for therapeutic proteins.[1-2] This covalent modification, commonly targeting the ε-amino group of exposed lysines, has been applied in approved PEGylated protein drugs, with many others in development. Monitoring protein PEGylation provides unique analytical challenges. The final therapeutic protein heterogeneity is increased by the potential for multiple individual PEGylation sites, depending on the amino acid sequence, as well as the extent of PEGylation, which depends on reaction conditions. Cation-exchange chromatography can be a useful tool for evaluating the PEGylation degree and the presence of positional isomers.[3] In this work, a method was developed to monitor the PEGylation of the model proteins lysozyme, with 6 potential reaction sites, and ribonuclease A (RNase A), with 10 potential reaction sites, as a function of PEGylation reaction time and reactant molar ratio. In conjunction with MS, the degree of PEGylation as well as the number of isomers can be elucidated.

Methods

Sample Preparation

Proteins were modified with Thermo Scientific™ Pierce™ TMS$(PEG)_{12}$ following the supplied instructions. In short, a 1 mL aliquot of 10 mg/mL protein solution in phosphate buffered saline was reacted with the required amount of 250 mM Pierce TMS$(PEG)_{12}$ in DMSO to achieve the desired molar reactant ratio. The reaction was allowed to proceed at room temperature for 30 min before the samples were desalted using Thermo Scientific™ Zeba™ Spin Desalting Columns. Samples were diluted fourfold with DI water before injection.

Liquid Chromatography

Thermo Scientific™ Dionex™ UltiMate™ 3000 Titanium System* consisting of:

- SRD-3600 Integrated Solvent and Degasser Rack
- DGP-3600BM Biocompatible Dual-Gradient Micro Pump
- TCC-3000SD Thermostatted Column Compartment
- WPS-3000TBFC Thermostatted Biocompatible Pulled-Loop Well Plate Autosampler with Integrated Fraction Collection
- VWD-3400 Variable Wavelength Detector

Column:	Thermo Scientific™ ProPac™ SCX-10G Guard (2 × 50 mm) ProPac SCX-10 Analytical (2 × 250 mm)
Mobile Phases:	A) 20 mM MES, pH 6.1 B) 20 mM MES, 1M NaCl, pH 6.1
Gradient:	0–30% B from 0–30 min, Equilibration at 0% B for 10 min before injection.
Flow Rate:	0.25 mL/min
Inj. Volume:	10 µL
Detection:	UV, 280 nm
Noise:	0.09 mAU
System Backpressure:	90 bar (1300 psi)

* A Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS System can also be used for this application.

Mass Spectrometry

The PEGylation reaction mixture was desalted and injected on a Thermo Scientific™ BioBasic™ 8 HPLC Column (5 µm, 100 × 1.0 mm) for separation and the MS confirmation was performed on a Thermo Scientific™ Q Exactive™ Orbitrap™ mass spectrometer with 140,000 mass resolution. The Q Exactive was operated in positive full scan mode with scan range from 1000 to 3000 m/z.

Data Analysis

Thermo Scientific™ Dionex™ Chromeleon™ 7.1 Chromatography Data System software was used for chromatographic data collection and processing.

Mass spectra deconvolution was accomplished using the Xtract Algorithm of the Thermo Scientific™ Xcalibur™ software.

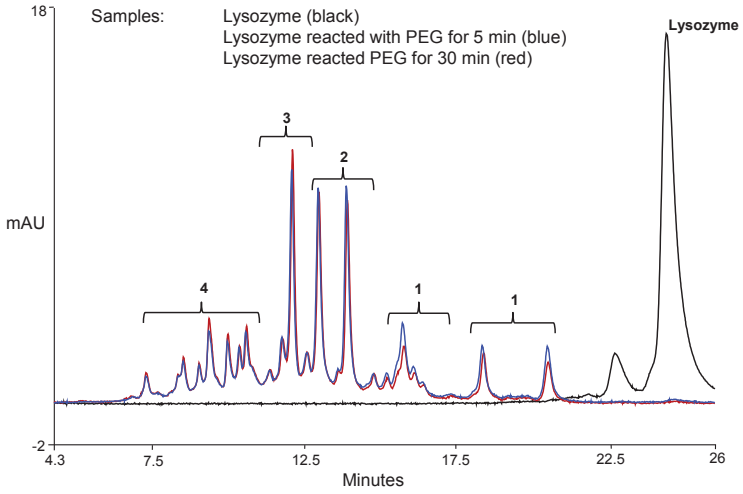
Results

Monitoring PEGylation by Strong Cation-Exchange Chromatography

N-Hydroxysuccinimide esters are common reactive groups used for PEGylation. These reagents readily react with primary amino groups. This reaction changes both the hydrophobicity and the overall charge of the protein. Using the model protein lysozyme, these changes can be evaluated by strong cation-exchange (SCX) chromatography as a function of time as shown in Figure 1.

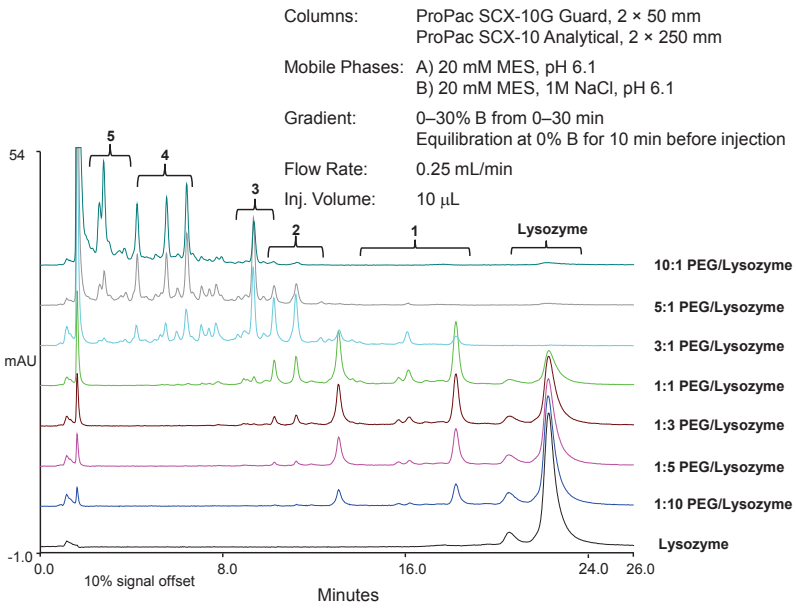
FIGURE 1. PEGylated lysozyme separated on the ProPac SCX-10 Analytical Column (2 × 250 mm). Subtle differences in the extent of PEGylation are apparent for reaction of a 3:1 molar ratio PEG/lysozyme for 5 min (blue) and 30 min (red). Unmodified lysozyme is shown for reference (black). Peaks are marked by the degree of PEGylation.

Columns:	ProPac SCX-10G Guard, 2 × 50 mm ProPac SCX-10 Analytical, 2 × 250 mm
Mobile Phases:	A) 20 mM MES, pH 6.1 B) 20 mM MES, 1M NaCl, pH 6.1
Gradient:	0–30% B from 0–30 min Equilibration at 0% B for 10 min before injection
Flow Rate:	0.25 mL/min
Inj. Volume:	10 µL
Samples:	Lysozyme (black) Lysozyme reacted with PEG for 5 min (blue) Lysozyme reacted PEG for 30 min (red)



When lysozyme is modified at different reactant molar ratios, the changes in PEGylation are more dramatic, as shown in Figure 2. With increasing amounts of the PEG reagent, the native lysozyme protein is lost and numerous modified proteins are detected.

FIGURE 2. Progression of lysozyme PEGylation as a function of reactant molar ratio monitored by cation-exchange chromatography. Peaks are marked by the degree of PEGylation.



Mass Spectrometry

MS of the PEGylated lysozyme samples was used to confirm that changes in the chromatography correlated with an increasing degree of PEGylation, as shown in Figure 3. Clear increases in mass are observed with average mass differences of 2.3 kDa, the expected mass of the reacted PEG. (Table 1)

FIGURE 3. MS of PEGylated lysozyme. As the concentration of the PEG reagent is increased, clear increases in the mass of proteins in the samples are observed.

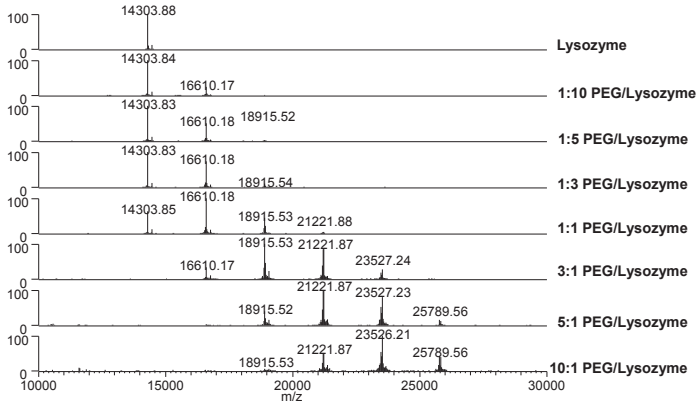


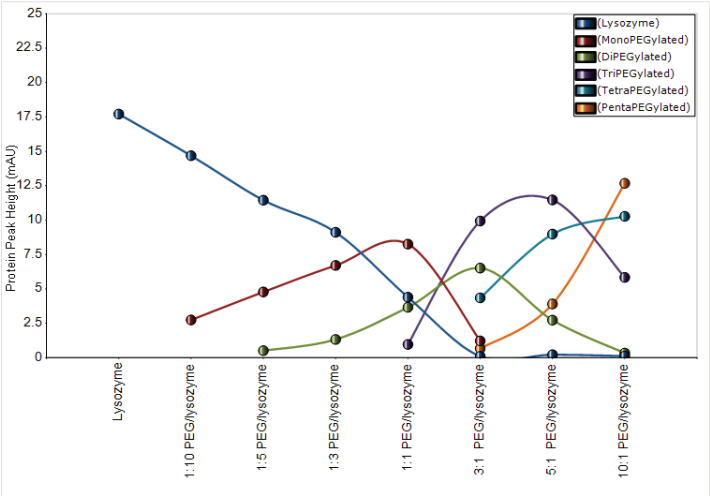
Table 1. Expected and observed masses of modified lysozyme.

Protein	Expected Mass (kDa)	Observed Mass (kDa)
Lysozyme	14.3	14.3
Lysozyme + 1 PEG	16.6	16.6
Lysozyme + 2 PEG	18.9	18.9
Lysozyme + 3 PEG	21.2	21.2
Lysozyme + 4 PEG	23.5	23.5
Lysozyme + 5 PEG	25.8	25.8
Lysozyme + 6 PEG	28.1	Not Detected

Reaction Conditions and Degree of PEGylation

Correlation of mass changes with the changes in chromatography allows the assignment of the PEGylation degree for multiple positional isomers. By tracking a subset of these isomers, as shown in Figure 4, reaction conditions can be chosen to control the degree of protein modification.

FIGURE 4. Changes in lysozyme PEGylation as a function of reaction conditions. Lines serve only as guides to indicate changes.



PEGylation of Ribonuclease A (RNase A)

RNase A was investigated as a model protein that has potentially greater heterogeneity after PEGylation, as demonstrated by a larger number of separated peaks by cation-exchange chromatography.

FIGURE 5. Progression of RNase A PEGylation as a function of reactant molar ratio monitored by cation-exchange chromatography. Greater complexity in the products is observed. Peaks are marked by the degree of PEGylation, which was determined by comparison of chromatography to MS data that follow in Figure 6.

Columns:	ProPac SCX-10G Guard, 2 × 50 mm ProPac SCX-10 Analytical, 2 × 250 mm
Mobile Phases:	A) 20 mM MES, pH 6.1 B) 20 mM MES, 1M NaCl, pH 6.1
Gradient:	0–30% B from 0–30 min Equilibration at 0% B for 10 min before injection
Flow Rate:	0.25 mL/min
Inj. Volume:	10 µL

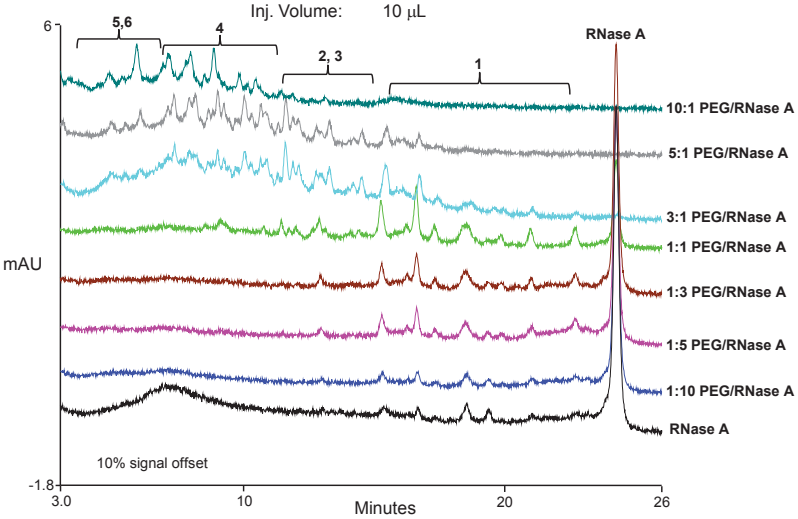


FIGURE 6. MS of PEGylated Rnase A. As the concentration of the PEG reagent is increased, clear increases in the mass of proteins are observed, indicating up to six PEGylation sites.

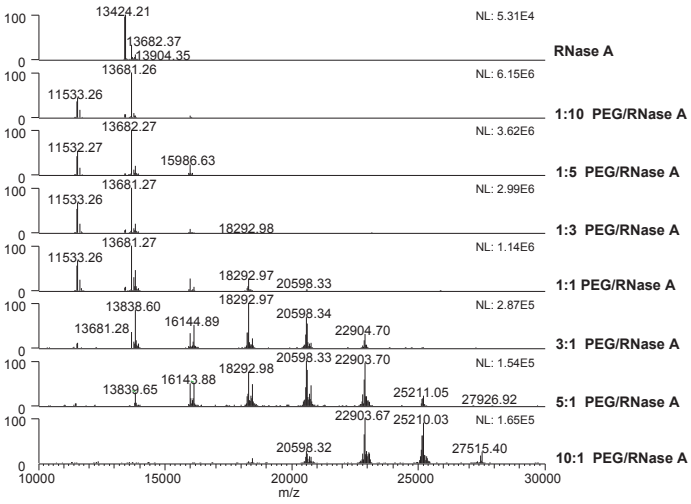


Table 2. Expected and observed masses of modified RNase A.

Protein	Expected Mass (kDa)	Observed Mass (kDa)
Ribonuclease A	13.7	13.4, 13.7
Ribonuclease A + 1 PEG	16.0	16.0
Ribonuclease A + 2 PEG	18.3	18.3
Ribonuclease A + 3 PEG	20.6	20.6
Ribonuclease A + 4 PEG	22.9	22.9
Ribonuclease A + 5 PEG	25.2	25.2
Ribonuclease A + 6 PEG	27.5	27.5
Ribonuclease A + 7 PEG	29.8	Not Detected

Conclusion

- PEGylated proteins were separated using SCX chromatography, revealing numerous isomers.
- These isomers were separated by the degree of PEGylation as confirmed by MS. PEGylation can be monitored as a function of reaction conditions, allowing reaction optimization for the desired product.
- SCX chromatography is shown to be a powerful method for characterizing modified protein mixtures.

References

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2. Jevsevar, S.; Kunstelj, M.; Porekar, V.G. PEGylation of Therapeutic Proteins, *Biotechnol. J.* **2010**, *5*, 113–128.
3. Abe, M.; Akbarzaderaleh, P.; Hamachi, M.; Yoshimoto, N.; Yamamoto, S. Interaction Mechanism of Mono-PEGylated Proteins in Electrostatic Interaction Chromatography, *Biotechnol. J.* **2010**, *5*, 477–483.

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