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Monitoring Protein PEGylation with Ion Exchange Chromatography

Peter Yu, Deanna Hurum, Leo Wang, Terry Zhang, and Jeffrey Rohrer

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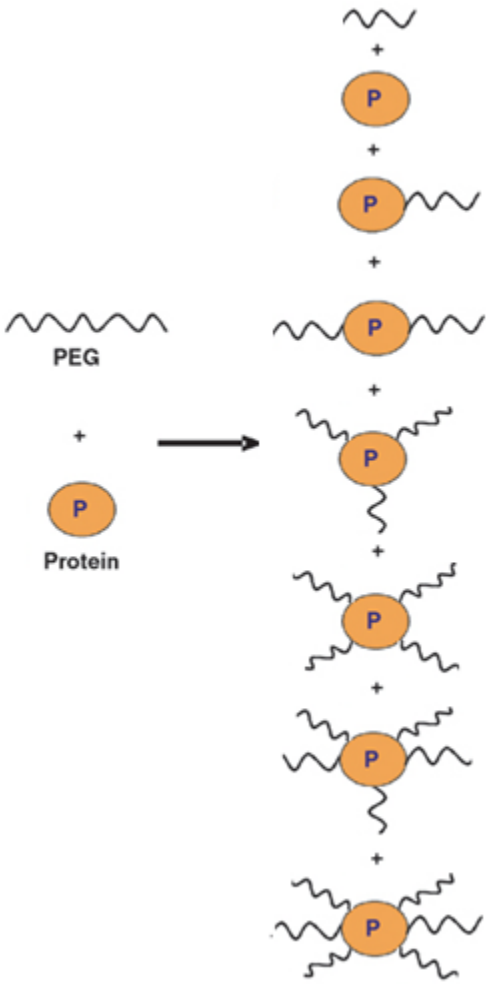
PEGylation of Therapeutic Proteins

- Polyethylene glycol (PEG)ylation is used to modify therapeutic proteins.
  - Improved stability
  - Reduced circulatory clearance
  - Potential for smaller doses/greater time between doses
- It is used in numerous pharmaceutical agents for delivery.
- Most often, *N*-Hydroxysuccinimide (NHS) is used to react with lysine amines to form a covalent attachment with the protein.

Potential Challenges



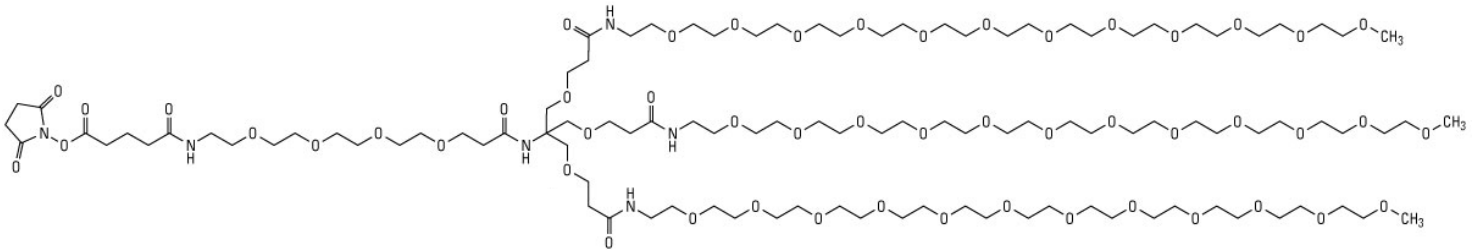
- Positional Isomerism
- Site Specification
- Separation of Complex Mixtures

Ion Exchange and PEGylated proteins

- Two model proteins:
  - Lysozyme
  - RNase A
- A small but commercially available PEG was used to modify these model proteins.
- The reaction conditions, varying both time and the reactant ratio, were investigated.

Reagents

- Branched Amine-Reactive PEG



TMS(PEG)$_{12}$
(Methyl-PEG$_{12}$)$_3$-PEG$_4$-NHS Ester
MW 2420.80

- Lysozyme—six potential lysine sites, 14.3 kD
- RNase A—ten potential lysine sites, 13.7 kD

Sample Preparation

- Dissolve 1 mg protein in 1 mL of phosphate-buffered saline.
- Dispense appropriate volume of PEGylation reagent into protein solution to achieve the desired reactant molar ratios.
- Incubate at room temperature for 30 min.
- Desalt using 0.5 mL Thermo Scientific™ Zeba™ Spin Desalting Columns to remove buffers and unreacted PEGylation reagent.

Instrumentation

- Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS System consisting of:
  - SRD-3600 Integrated Solvent and Degasser Rack
  - DGP-3600RS Biocompatible Dual-Gradient Rapid Separation Pump
  - TCC-3000SD Thermostatted Column Compartment
  - WPS-3000TBRS Thermostatted Biocompatible Rapid Separation Autosampler
  - VWD-3400 Variable Wavelength Detector

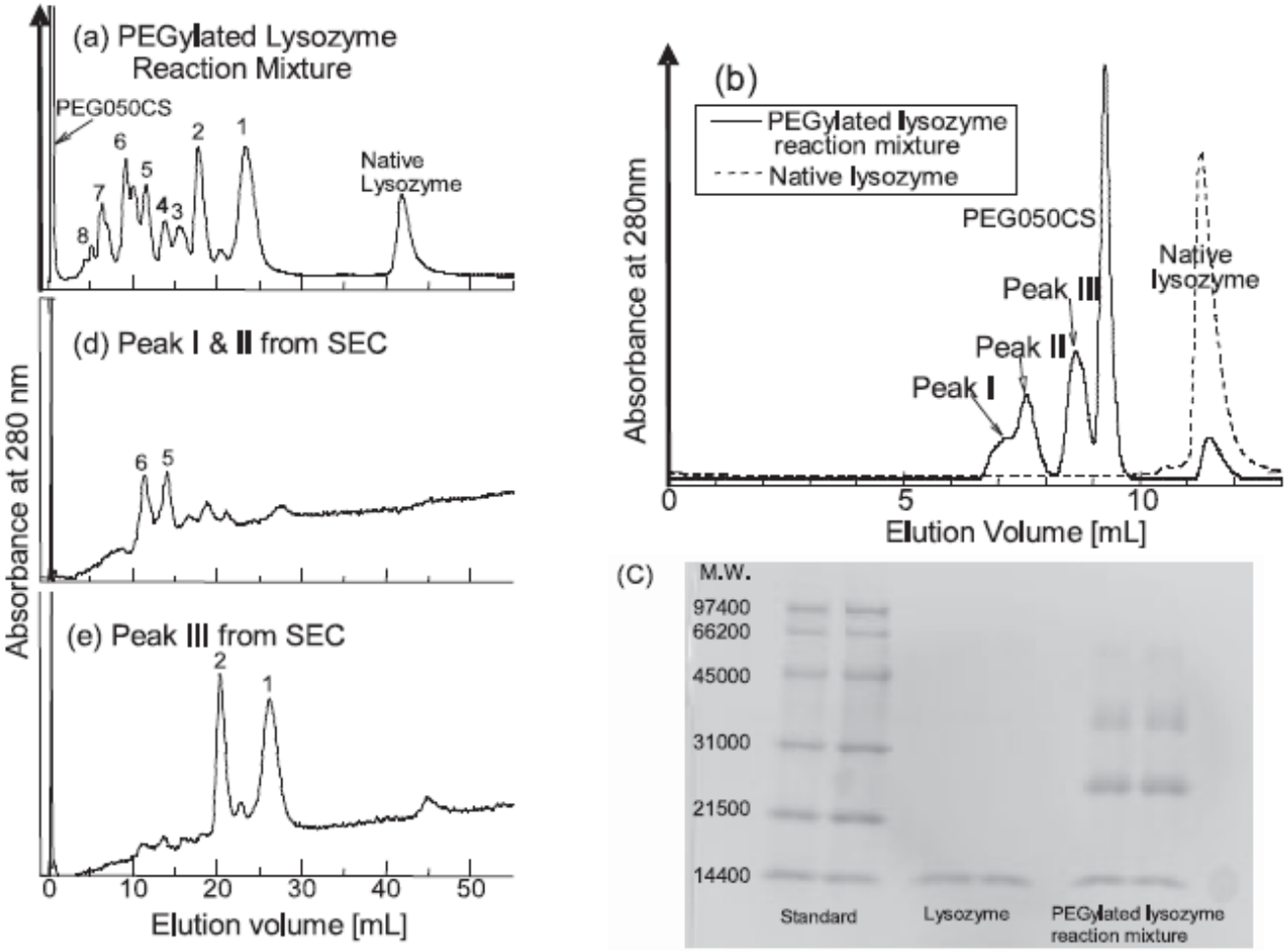
Chromatographic Method

- 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 to 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

Mass Spectrometry

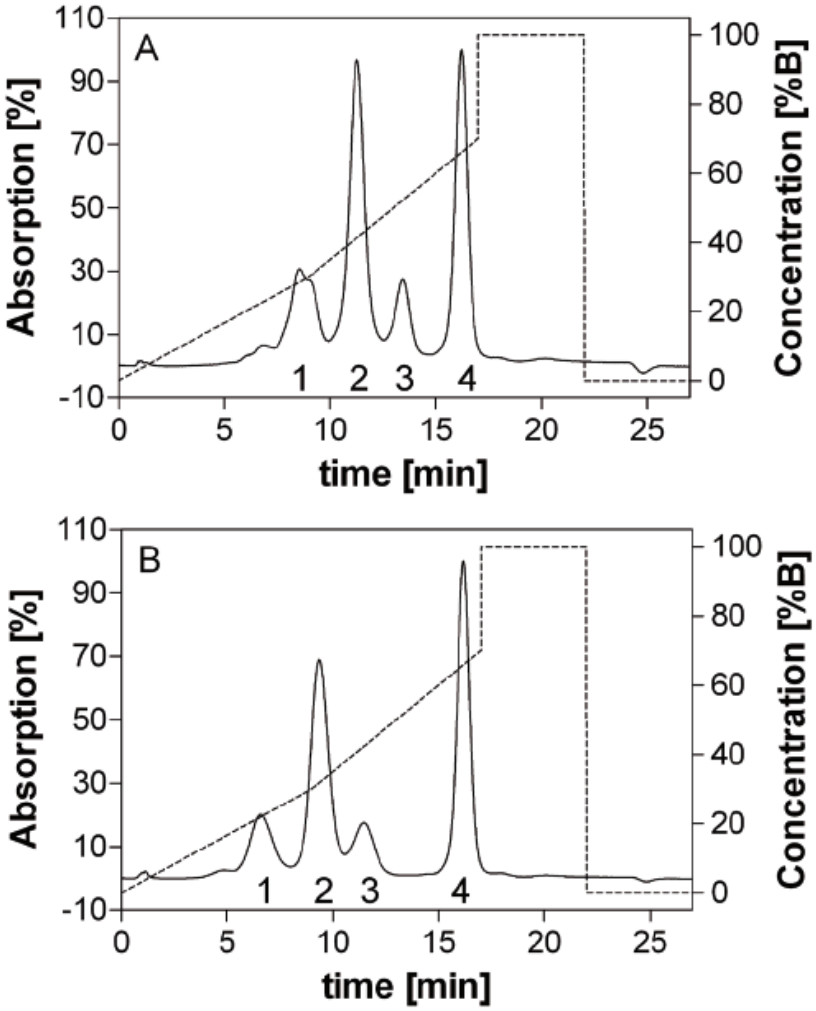
- The PEGylation reaction mixture was desalted and injected on a Thermo Scientific™ BioBasic™ 8 HPLC Column (5 µm, 100 × 1.0 mm).
- MS confirmation was performed on a Thermo Scientific™ Q Exactive™ Orbitrap™ mass spectrometer with 140,000 mass resolution. The Q Exactive was operated in positive full-scan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

Literature Separation of PEGylated Lysozyme



Biotechnol. J. **2010**, *5*, 477–483.

PEGylated Lysozyme Separation from a Vendor's Application Note



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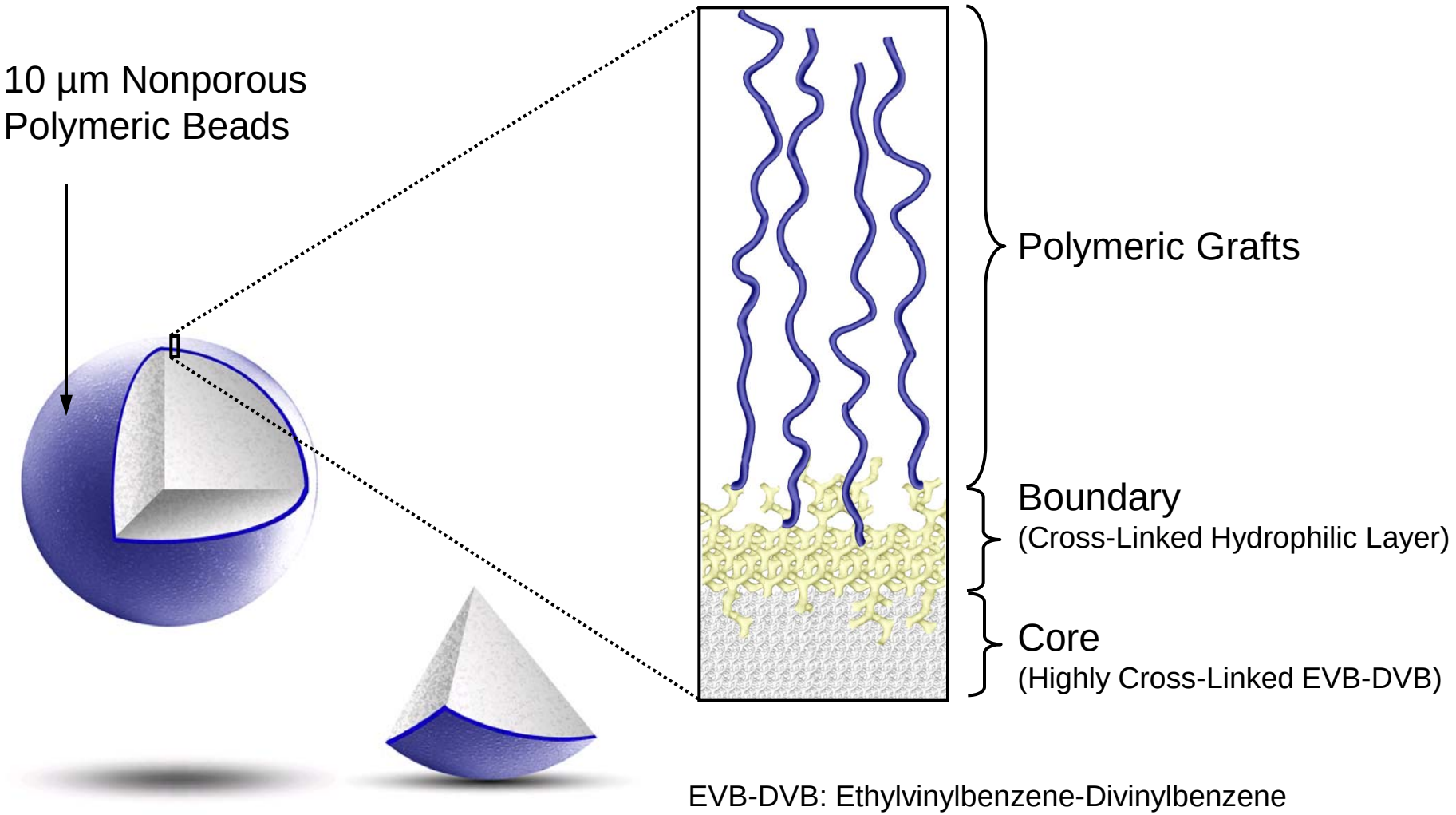
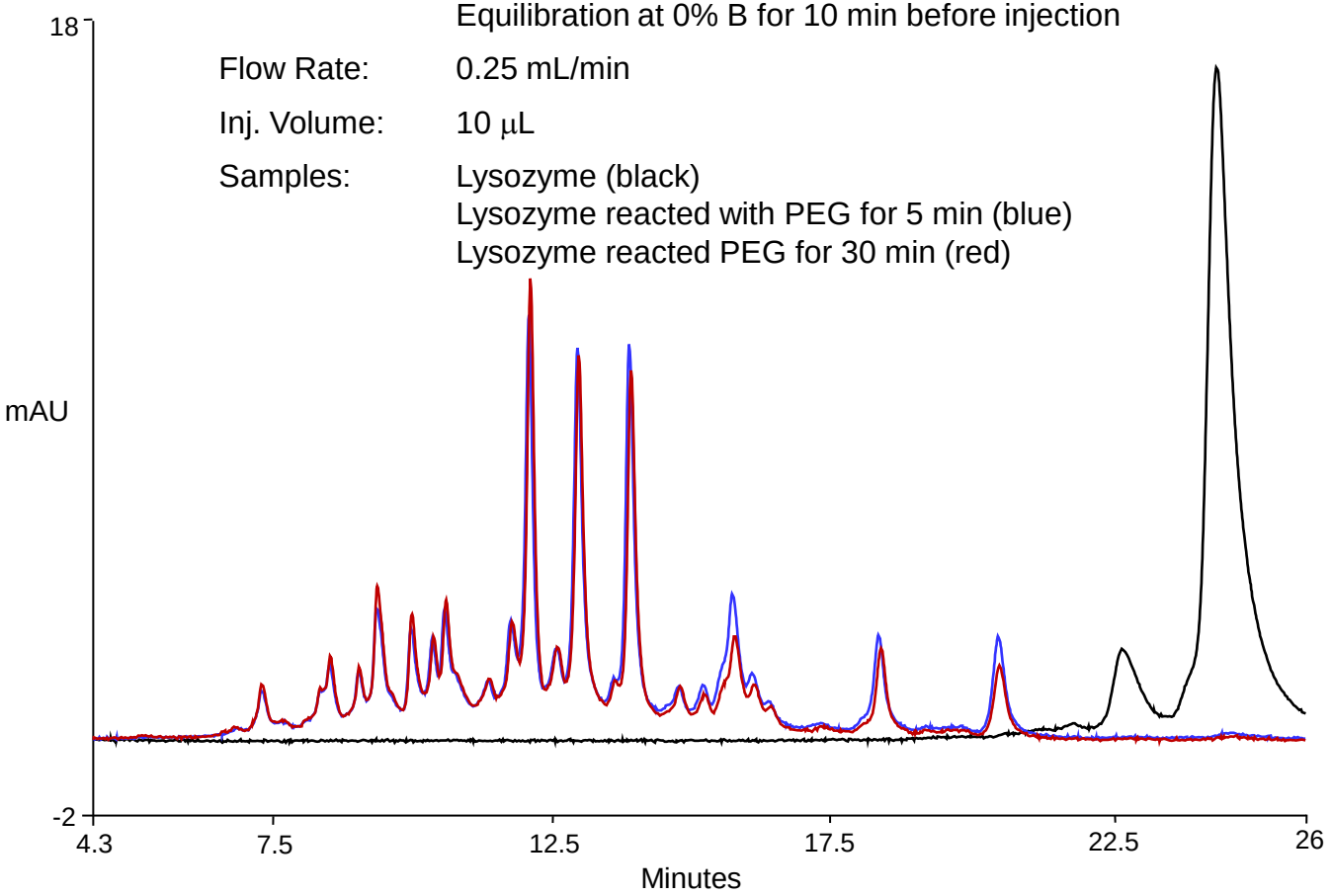
ProPac IEX Resin with Grafted Polymeric Functionalized Chains



EVB-DVB: Ethylvinylbenzene-Divinylbenzene

Changes in Lysozyme PEGylation with Reaction Time

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 to 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)



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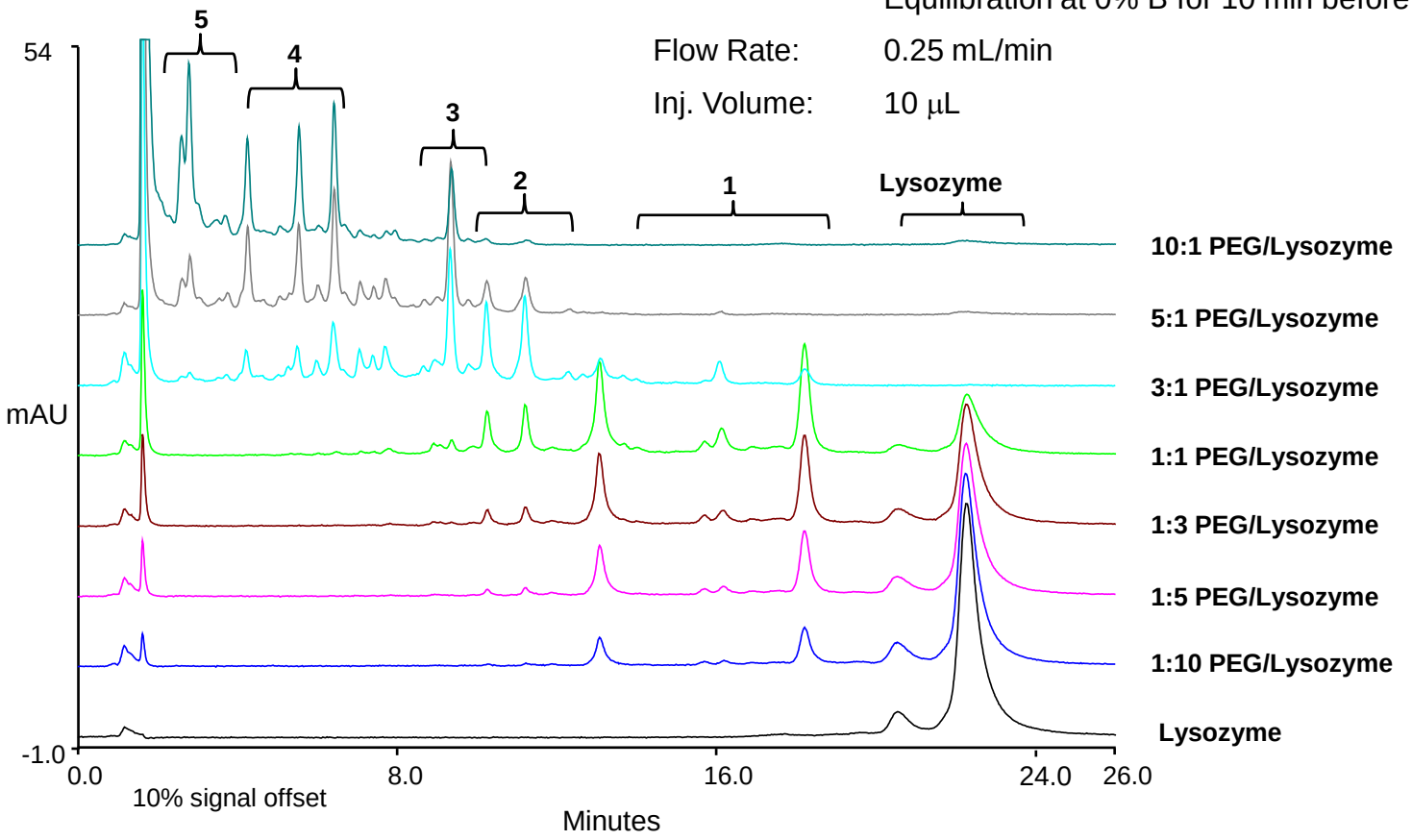
Changes in PEGylation with Reactant Ratios—Lysozyme

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 to 30 min Equilibration at 0% B for 10 min before injection
Flow Rate:	0.25 mL/min
Inj. Volume:	10 µL



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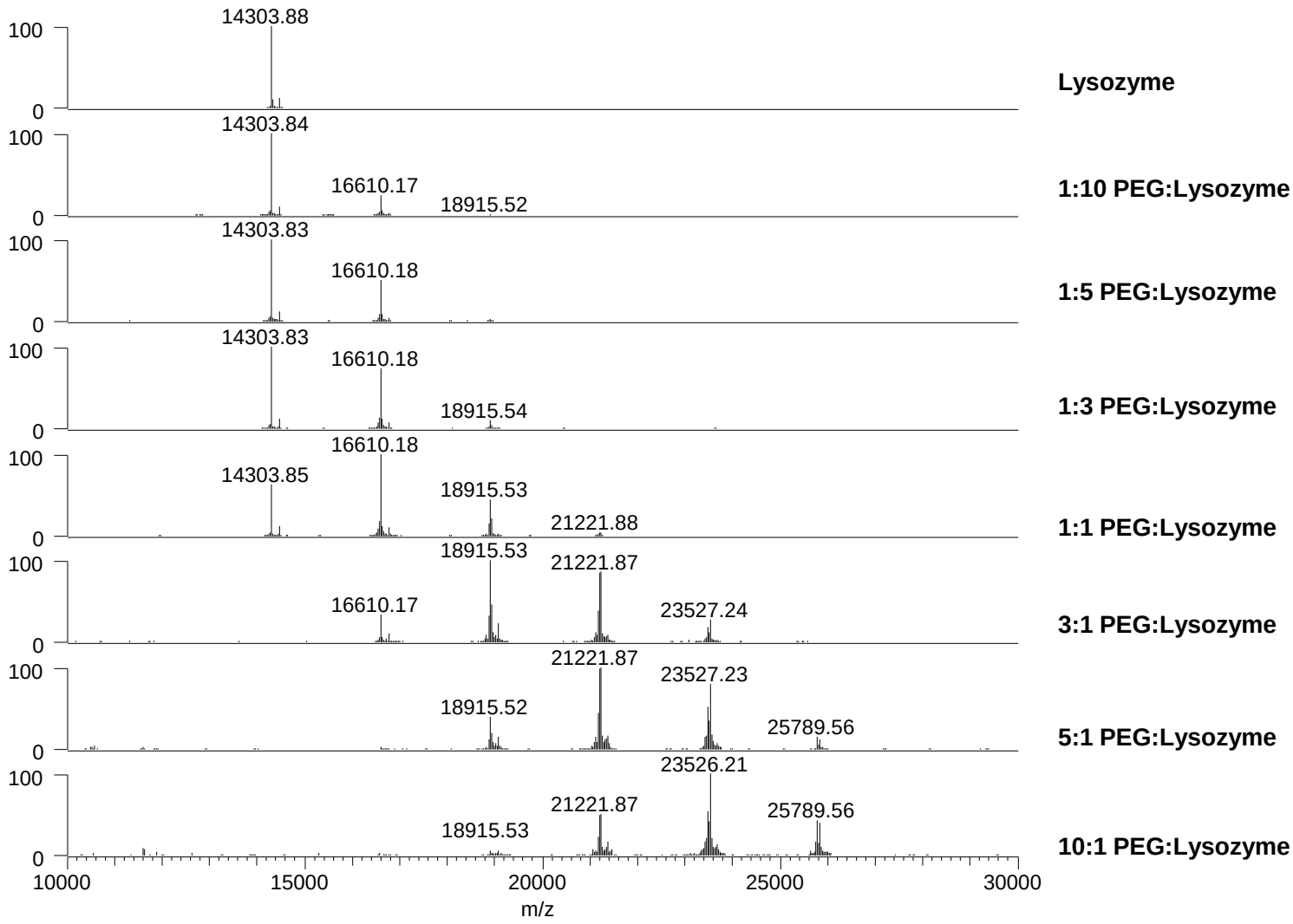
PEGylated Lysozyme—Deconvoluted MS Spectra



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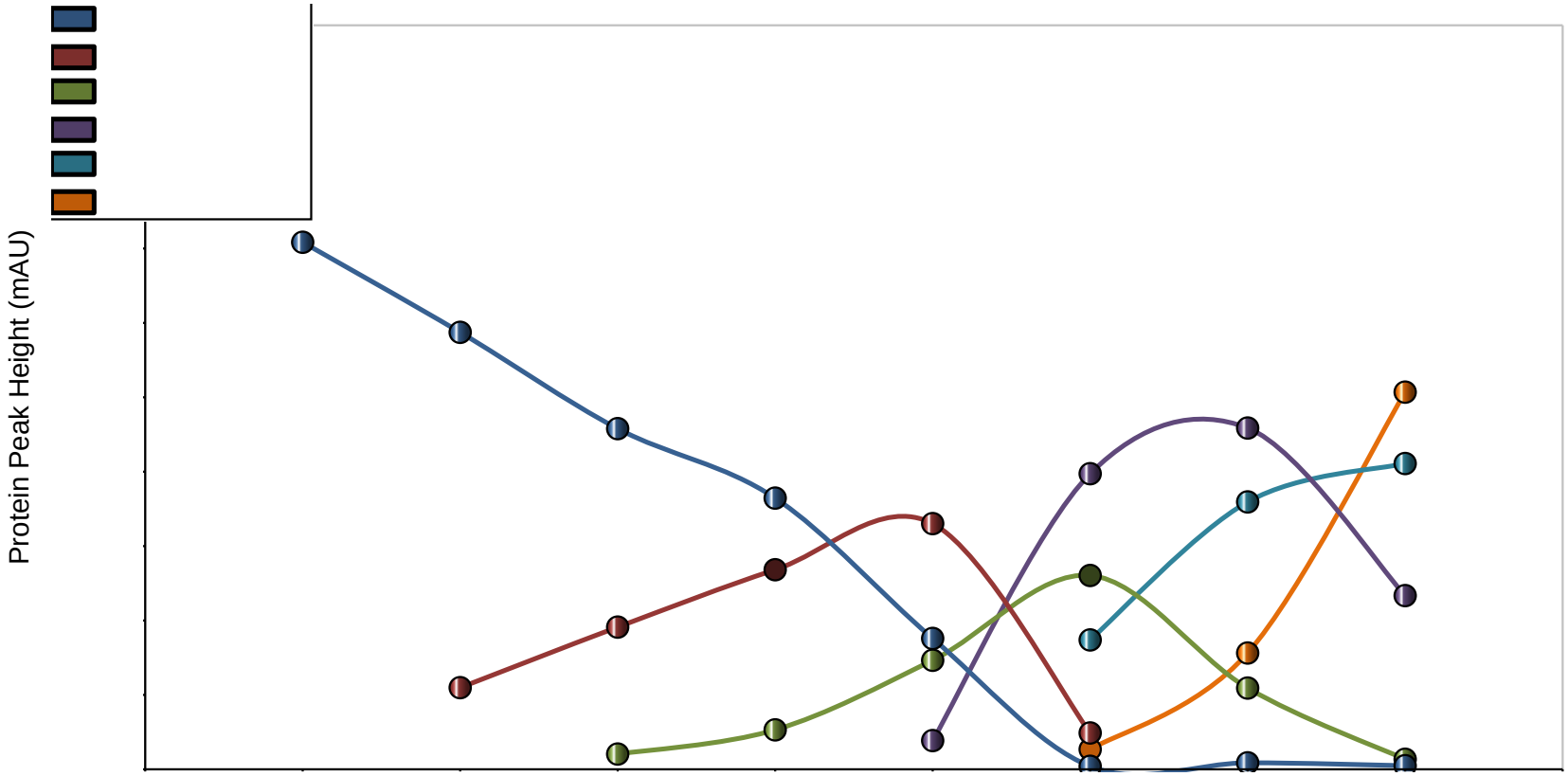
Expected Masses Correlate with Observed Masses

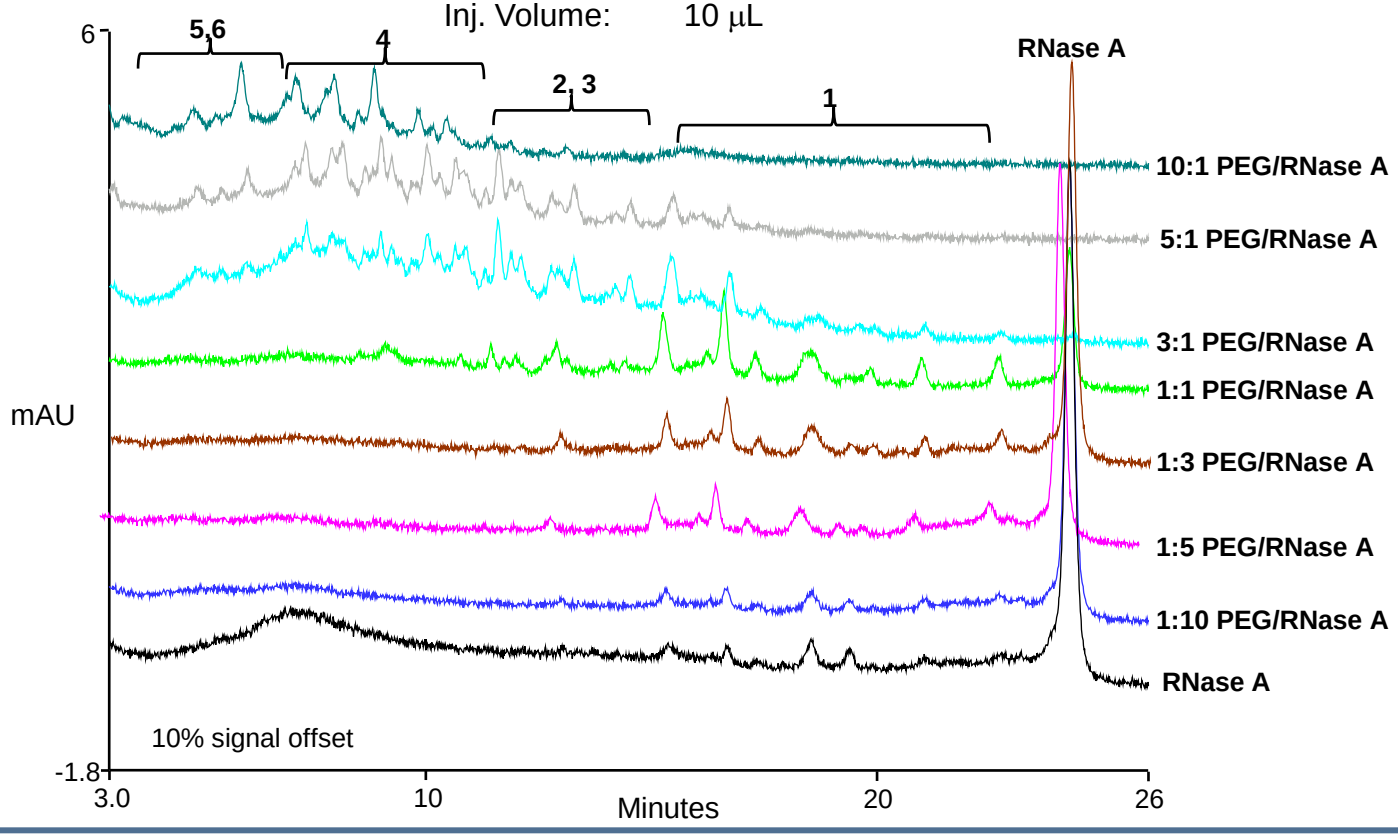
Protein	Expected Mass (kDa)	Observed Mass (kDa)
Lysozyme	14.3	14.3
Lysozyme + 1 PEG	16.6	16.6
Lysozyme + 2 PEG	18.9	18.9
Lysozyme + 3 PEG	21.2	21.2
Lysozyme + 4 PEG	23.5	23.5
Lysozyme + 5 PEG	25.8	25.8
Lysozyme + 6 PEG	28.1	Not Detected

Degree of PEGylation Tracked by Cation Exchange



Changes in PEGylation with Reactant Ratios—RNase A

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 to 30 min Equilibration at 0% B for 10 min before injection
Flow Rate:	0.25 mL/min
Inj. Volume:	10 µL



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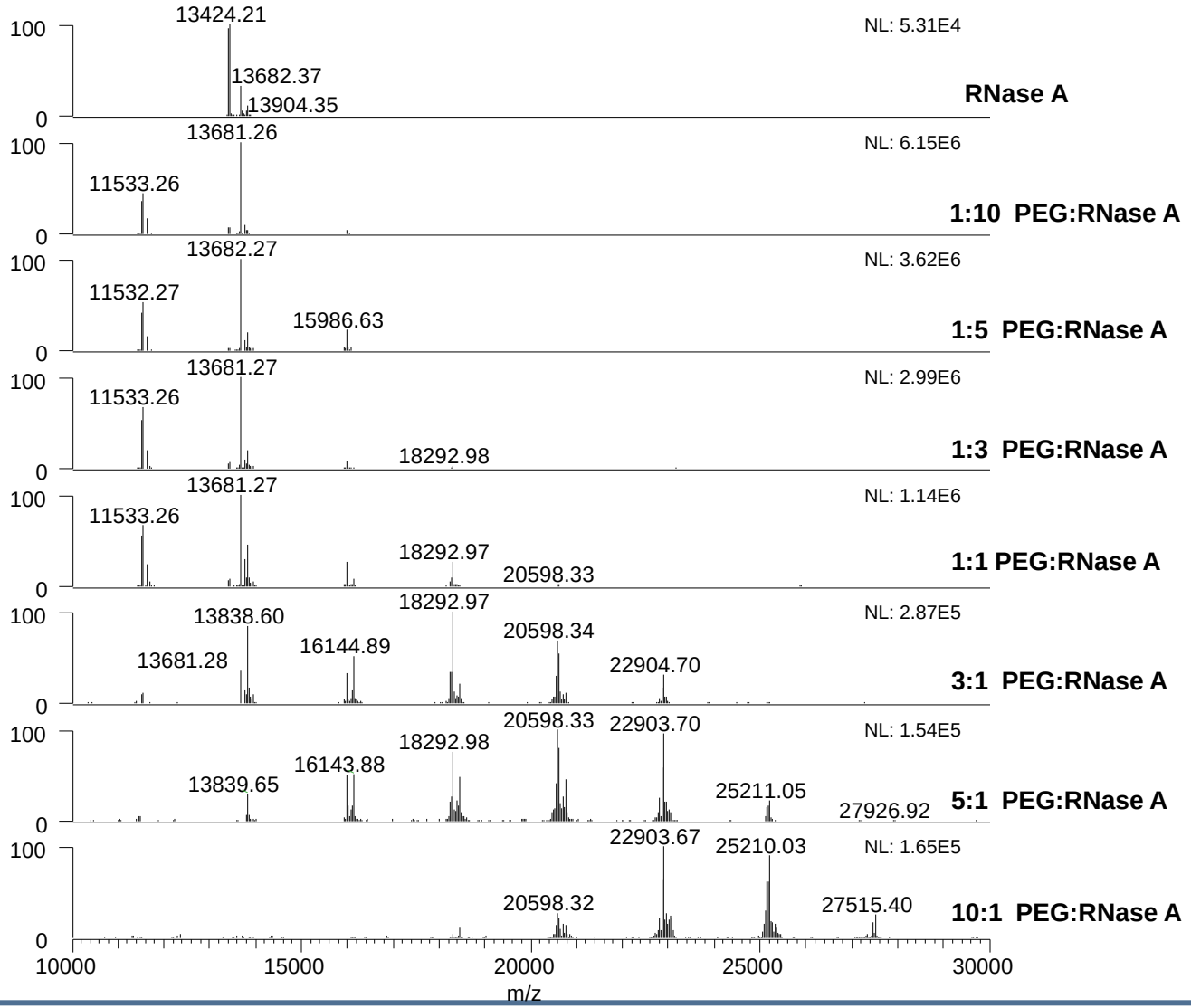
PEGylated RNase A – Deconvoluted MS Spectra



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Expected Masses Correlate with Observed Masses

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

Conclusions

- 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 with multiple positional isomers.

Thank you for your attention!