WHITEPAPER

The Utility of a Cation Exchange Column for Successful Charge-Variant Analysis of PEGylated Proteins

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Polyethylene glycol (PEG) is a biocompatible compound with extensive use in a wide variety of medical, biopharmaceutical, and industrial applications. It is a popular compound for modifying peptides, proteins, and other biologics through covalent-bond attachment for therapeutic drug applications. PEGylation is the process of attaching PEG chains to a protein drug to shield it from proteolytic enzymes and improve pharmacokinetic properties. However, analytically characterizing PEGylated proteins can be a complex process, and many different methodologies are used.

Protein drugs are PEGylated for many reasons. “PEG has a number of unique properties: It’s a water-soluble polymer, bioinert, and non-immunogenic,” said Athena Papasodero, associate scientist, analytical development at the Pelican Expression Technology™ business unit of Ligand® Pharmaceuticals Inc. “When PEG is conjugated to a protein, it can provide many positive advantages that the native molecule lacks.”

Papasodero explained it increases half-life, reducing dosage frequency and extending the circulation lifetime in the human body. And because PEG is hydrophilic, when it attaches to proteins and other biomolecules, it decreases aggregation and increases solubility. PEG can lower toxicity, immunogenicity, and antigenicity and can also improve stability and decrease degradation.[1-5]

PEG conjugation masks the protein surface and increases the molecular size of the protein. It is transparent and nonfluorescent and therefore non-detectable by itself.

Current Methodologies

Currently, there are three major techniques for determining charge-variant analysis of proteins: ion exchange (IEX) chromatography, imaged capillary isoelectric focusing (iCIEF), and capillary zone electrophoresis (CZE). All three high-resolution techniques separate biomolecules based on differences in the net surface charge and enable for charge-variant characterization of both PEGylated and non-PEGylated proteins.

According to a 2020 study, “iCIEF has become the current industry standard for isoelectric-point (pI) determination and charged-variant quantification of proteins and antibodies.”[6]

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“Variants that can be separated and analyzed using IEX chromatography, iCIEF, and CZE include post-translational modifications such as glycosylation, as well as other modifications including deamidation and fragmentation of the protein,” Papasodero said. “For IEX chromatography, these types of separations are carried out using one of two gradient techniques: salt gradients or pH gradients.”

- Salt gradients have the advantage of historical use; therefore, they are more common and more familiar to scientists in the bioanalytical lab.
- pH gradients are a newer development and offer certain advantages, including the ability to be mass-spectrometry friendly when compared to salt gradients.

However, there are challenges with charge-variant analysis for PEGylated proteins. “The main challenge in characterizing charge variants of PEGylated proteins is the charge-shielding effect[7] in which the steric-bulk of the PEG residue hinders the electrostatic interactions between the amino acid residues of the protein and the charges surface of the stationary phase of the IEX column,” Papasodero explained. “For lysine conjugated PEGylated proteins, the net cationic charge of the protein also decreases as the number of PEGylated lysine residues increase, which can alter selectivity and hinder resolution.”

As researchers from the department of biologics process development at Bristol-Myers Squibb discovered, iCIEF separation of PEGylated proteins creates broad peaks. “Charged variants of PEGylated proteins merge into one broad peak during iCIEF, most likely due to the masking of proteins by the surrounding PEG chain and the increased hydrodynamic volume cause by PEGylation.”[8]

Papasodero explained it also produces distorted peak shapes and modification of pharmalyte contents, methylcellulose concentration, protein concentration, focusing time, and other additives do not yield reasonable peak shapes for iCIEF methods.

The Experiment

To address these challenges, the Pelican Expression Technology team assessed the ability of the YMC BioPro SF column to analyze PEGylated protein charge variants reproducibly.

“We developed a characterization method for PEGylated biotherapeutic proteins that can provide increased information without compromising peak shape and resolution,” Papasodero said, which, as seen above, are two factors that at times are sacrificed when characterizing PEGylated proteins.

The stability indicating method developed by the Pelican Expression Technology team created exceptional separation of charge-variant constituents using YMC BioPro SF. The method can enable others who have similar separation requirements to develop methodology from a proven and effective protocol.

Materials and Reagents

Name	Brand	Catalog #	Description
Sodium Chloride	JT Baker	3628	(≥99.0%), mw 58.44
MES Hydrate	Sigma Aldrich	PHG0003	195.24 (anhydrous basis)
Ammonium Hydroxide	Sigma Aldrich	338818	28% NH_3 in H_2O, ≥99.99% trace metals basis

Experimental Conditions

Sample Preparation: A sample of PEGylated protein was diluted to 10 mg/mL using Milli-Q water.

Degraded Sample Preparation: A sample of PEGylated protein was diluted to 10 mg/mL in formulation buffer. The sample was held at 40° C for 30 days.

Column Specifications: BioPro IEX SF; particle size 5 µm; column size length X.I.D. (mm) 100 x 4.6; flow rate 0.2 - 0.8 mL/min; pH range: 2 - 12; temp. range 4 - 60° C; column material PEEK.

Chromatographic Conditions

LC System	Thermo Scientific Vanquish UPLC
Detector	Vanquish VF-P20-A
Autosampler	Vanquish VF-A10-A
Column Compartment	Vanquish VH-C10-A
Pump	Vanquish VF-P20-A
Viper Fitting	MP35N 0.18 x 350 mm
Column	YMC BioPro IEX SF 100 x 4.6 mm I.D. 5 µm P/N:SF00S05-1046WP
Mobile phase A*	20 mM MES, pH 5.9
Mobile Phase B*	25 mM MES, 0.5 M NaCl, pH 5.9
Column temp.	35 °C
UV Wavelength	280 nm (reference OFF/16nm-bandwidths)

* Use ammonium hydroxide to adjust pH

Gradient Table

Time	Flow [mL/min]	%A	%B
0.00	0.750	100.0	0.0
7.00	0.750	100.0	0.0
14.00	0.750	70.0	30.0
15.00	0.750	50.0	50.0
16.00	0.750	50.0	50.0
16.50	0.750	100.0	0.0
20.00	0.750	100.0	0.0

The Results

The effect of pH on charge variant analysis of a PEGylated protein is shown in **Figure 1**. The panels show UV traces at 280 nm for (A) full-sized chromatograms of PEGylated protein during the mobile phase pH screen and (B) expanded chromatograms of PEGylated protein during the mobile phase pH screen.

Figure 1

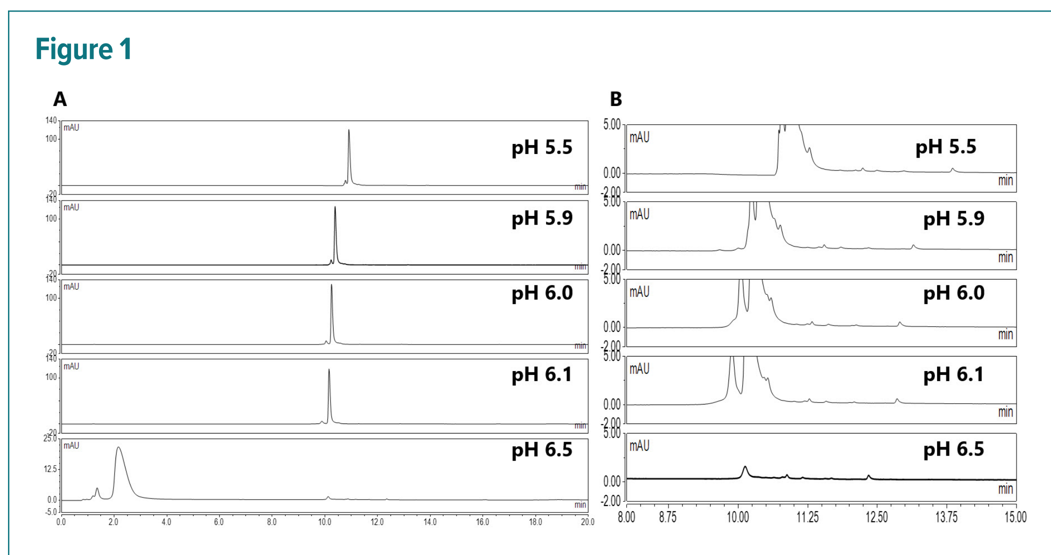


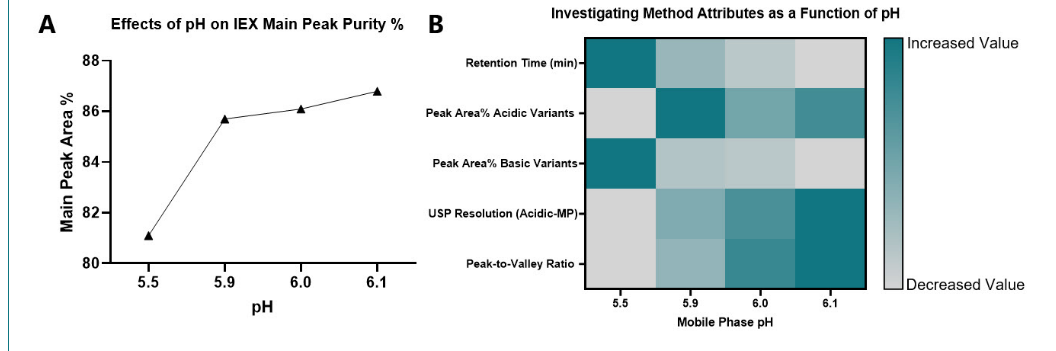
Table 1 reports the sum of the acidic variants, the peak area percentage for the main peak (MP), and the sum of the basic variants. Resolution (R) between the acidic residues and main peak and the ratio of peak height to valley height (End p/v) for the main peak are also reported.

Table 1

pH	Retention Time	Peak area acidic variants (%)	Peak area main peak (%)	Peak area basic variants (%)	USP Resolution (Acidic-MP)	Peak-to-Valley Ratio
5.5	10.910	6.8	81.1	12.1	1.1	2.7
5.9	10.387	7.2	85.7	7.1	1.4	3.7
6.0	10.253	7.0	86.1	6.9	1.6	5.1
6.1	10.160	7.1	86.8	6.1	1.8	5.7

Figure 2 displays the effect of pH on IEX of a PEGylated protein. **Chart A** shows the pH effects on the main peak area percentage. **Chart B** shows a heat map visualization of the method attributes: area percent of the acidic and basic charge variants, retention time, USP resolution between the major acidic peak and the main peak, and the peak-to-valley ratio between the major acidic peak and the main peak as a function of mobile phase pH.

Figure 2



In **Figure 3,** the IEX of a PEGylated protein characterized with the YMC BioPro SF column is shown. The panels show UV traces at 280 nm for (A) full-sized and expanded trace of mobile phase A blank, (B) full-sized and expanded trace PEGylated protein, and (C) full-sized and expanded trace degraded PEGylated protein. The optimized cation-exchange (CEX) high-performance liquid chromatography (HPLC) chromatogram for the PEGylated protein is shown in **Figure 3B**. One major acidic peak with a slight shoulder is observed in the pre-peak region of the chromatogram. Two small peaks are observed in the post main peak region. Six small basic peaks are eluting between 11-14 minutes, and one large basic peak eluting at 13.3 minutes. Over 100 injections were performed in a single run with no loss of resolution or peak shape of the PEGylated protein and charge variants. **Figure 3C** also shows the chromatogram following heat degradation of the PEGylated protein. The effects of heat degradation on the sample are clear: The dominant major acidic peak, the two smaller post main peaks, and the major basic peak increase following heat degradation.

Figure 3

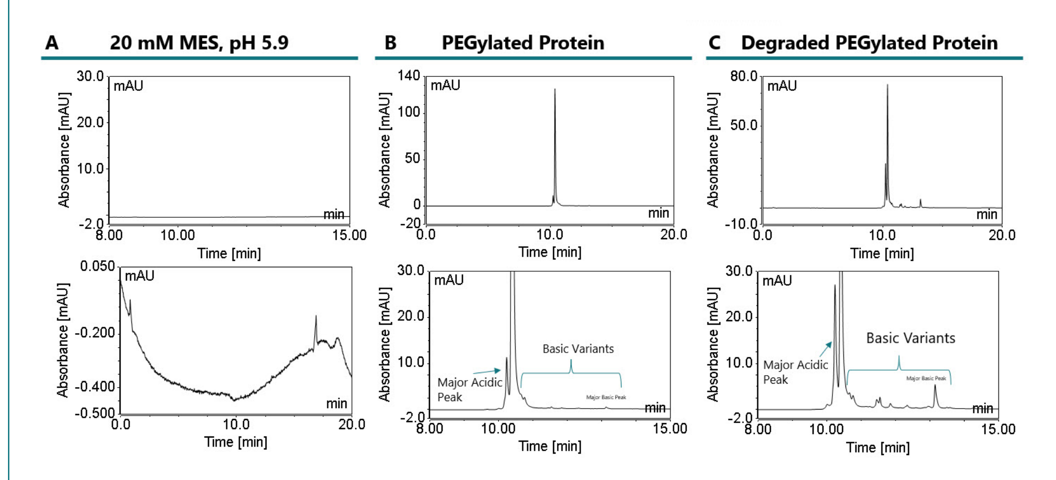
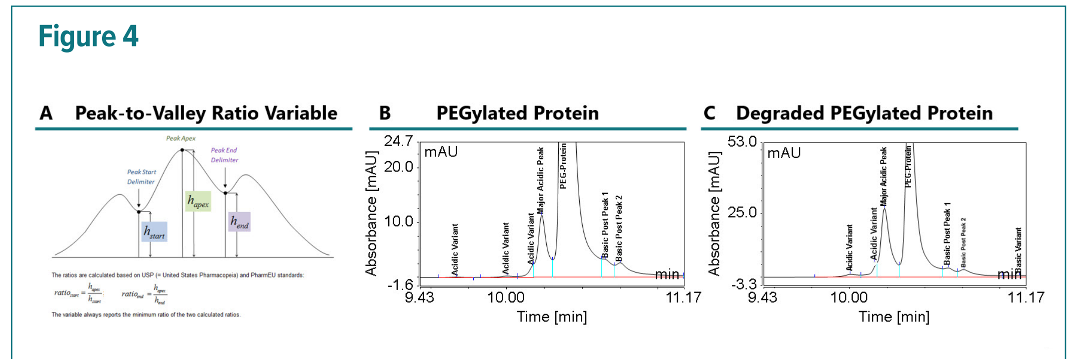


Figure 4 shows peak-to-valley ratios used to judge peak separation for CEX-HPLC chromatograms of a PEGylated protein. The panels show UV traces at 280 nm for (A) an example showing a peak that is integrated as a main peak without baseline contact. It is separated by two neighboring peaks with the peak delimiters being on a valley, (B) expanded trace PEGylated protein, and (C) and expanded trace degraded PEGylated protein.

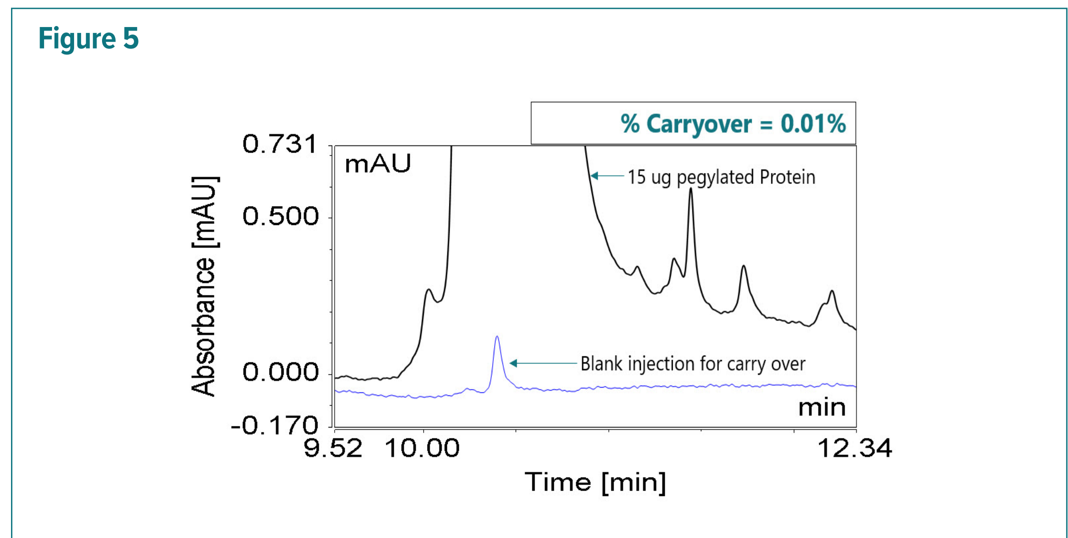
Figure 4



Carryover from injection to injection can be a significant problem in PEGylated-protein analysis. As shown in **Figure 5**, the YMC BioPro SF IEX column minimizes carryover: In the 118th injection using this column, there is minimal carryover. The percentage of carryover was calculated using this equation:

$$\frac{\text{PEGylated protein area in blank injection for carryover}}{\text{PEGylated protein area in sample injection}} \times 100$$

Figure 5



Conclusion

The YMC BioPro SF column provides a tool for the analysis and confirmation of PEGylated protein and PEGylated protein charge variants, increasing mobile phase pH results in earlier retention times. From visual inspection of the data, similar chromatographic profiles are visible; however, a buffer pH 5.9 results in the elution of two small acidic variant species not present when differing pH mobile phases were screened. The column also exhibited good retention time, area, and relative-area reproducibility.

"Conjugation of the PEG group to a protein increases the shielding effect, causing the protein to elute early before the salt or pH gradient begins," Papasodero said. "Our results suggest the combination of YMC BioPro SF column and optimized salt and pH buffer compositions can enhance charge-variant characterization of PEGylated molecules. The difference in profiles between non-PEGylated and PEGylated variants of the protein can offer rapid insight into the degree of PEGylation during PEGylation optimization and development."

Overall, this method is superior to current charge-variant analyses of PEGylated proteins because it can be easily replicated and yield repeatable results; it also has little carryover and the potential to be used as a platform method for charge-variant characterization of PEGylated proteins, lowering both cost and the development time required.

The BioPro SF column provides an illuminating analytical approach for charge-variant drug characterization.

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