A Stability-Indicating Method for Separation of Charge Variant Impurities of a PEGylated Protein by CEX-HPLC

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Introduction

PEGylation of a biologic is the process of attaching polyethylene glycol (PEG) chains to a peptide or protein to shield from proteolytic enzymes and improve pharmacokinetics and pharmacodynamics of the therapeutic. However, PEG conjugation is a complex process that often imparts heterogeneity to the protein, and as a result requires detailed analytical characterization to understand the effects of the process. In this study, we investigated the role of mobile phase pH on the analysis of a PEGylated protein expressed in the Pelican Expression Technology™ platform (PET), a microbial expression system based on the Gram-negative bacterium Pseudomonas fluorescens. The platform design enables rapid, robust, and cost-effective production of proteins, antibodies, and peptides for use in human therapeutics, vaccines, and other applications. First, we developed a CEX method that enabled for the separation and resolution of charge variants of the PEGylated protein and then, to determine the identity of the charge variants, we conducted a forced degradation study to understand the product degradation pathways and to establish a stability-indicating method. We demonstrate that buffer pH plays a critical role in counteracting the charge shield effect of PEG conjugation in allowing for electrostatic interaction of the protein with the IEX resin.

Theory

Conjugation of PEG to a protein results in both an increase in the hydrodynamic radius of the protein and a change to the surface charge accessibility of the protein or peptide. This phenomenon known as charge shielding, makes the characterization of charge variant species extremely challenging. The level of shielding and surface amino acid accessibility can alter the elution times of the protein or peptide. As a result, buffer composition and pH become critical factors when developing IEX methods for PEGylated proteins because small changes to the composition can have dramatic effects on the surface charge of the protein and its capability to interact with the IEX resin.

Objective of This Work

At present, imaged capillary isoelectric focusing (iCIEF) has become the industry standard for determining charged variant forms of protein therapeutics. However, PEGylation causes broad peak formation hindering characterization and therefore renders iCIEF unsuitable. Here we report our novel method with the use of the YMC BioPro SF column with UV detection which significantly improved the separation of charge variants in PEGylated proteins that were produced in *Pseudomonas fluorescens*, which is the foundational basis of the Pelican Expression Technology™ platform.

Experimental

Reagents and Chemicals			
Name	**Brand**	**Catalog #**	**Description**
Sodium Chloride	JT Baker	3628	(≥99.0%), mw 58.44
MES Hydrate	Sigma Aldrich	PHG0003	mw 195.24 (anhydrous basis)
Ammonium Hydroxide	Sigma Aldrich	338818	28% NH3 in H2O, ≥99.99% trace metals basis
Purified water suitable for HPLC analysis	--	--	(18.2 MOhm resistivity, ≤ 5 ppb TOC, filtered through a 0.2 µm filter-sterilization apparatus)

LC Conditions: 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
Column	YMC BioPro IEX SF 100 x 4.6 mmI.D. 5µm P/N:SF00S05-1046WP
Mobile Phase A	20mM MES, pH 5.9
Mobile Phase B	25 mM MES, 0.5 M NaCl, pH 5.9
Column Temperature	35 ° C
UV Wavelength	280 nm (reference OFF/16nm-bandwidths)
Flow Rate	0.75 mL/minute
Gradient	0 to 7 minutes, 0% B; 7 to 14 minutes, 0 to 30% MP-B; 15 to 16 minutes, 50% MP-B; 16 to 16.5 minutes, 50% to 0%B; 16.5 minutes to 20 minutes, 0% MP-B

Sample Preparation	
Control	PEGylated Protein at a concentration of 10 mg/mL.
Heat degraded	A sample of PEGylated protein at 10 mg/mL in formulation buffer was held at 40° C for 30 days. The sample was diluted to 0.25 mg/mL with purified water prior to analysis.
Low pH stress	The pH of the PEGylated protein sample was adjusted to pH 3.2 with 0.1N HCl. The sample was then incubated for 7 days at 25 ° C before being neutralized to pH 6.9 with 20X PBS.
High pH stress	The pH of the PEGylated protein sample was adjusted to pH 10.2 with ammonium hydroxide. The sample was then incubated for 7 days at 25 ° C before being neutralized to pH 6.9 with 20X PBS.
Photo-stress	A sample of Pegylated protein at a concentration of 0.25 mg/mL was placed in a photostability chamber (3600 klux.hr, ~4 days and 600 Watt.hr/m²) for 24 hours.
iCIEF	PEGylated protein at a concentration of 1 mg/mL was diluted to 0.2 mg/mL in iCIEF sample matrix (% 3-10 ampholyte, 0.35% (w:v) methyl cellulose, 10 mM iminodiacetic acid, 10 mM Arginine, and 8 M urea). Sample focusing was performed at 1500 V for 1.0 min and 3000V for 5.0 min on the Maurice Protein Simple.

Results

Initial attempts at analyzing the charge variance of the PEGylated protein and a thermally degraded sample using iCIEF resulted in complex profiles, with broad peaks and poor resolution (**Figure 1**).

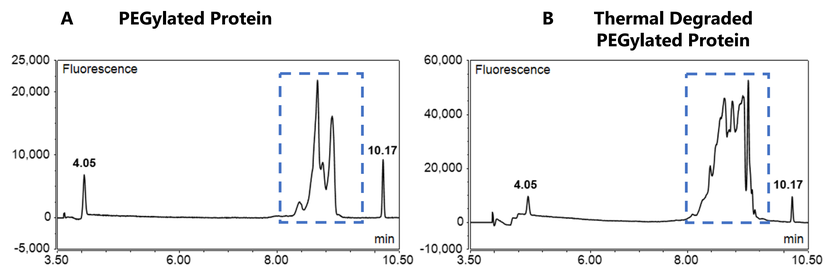

Figure 1. Electropherograms of PEGylated protein with 8 M urea. Sample focusing was 1500 V for 1.0 min and 3000V for 5.0 min. (A) PEGylated protein, and (B) thermal degraded PEGylated protein. The complex profile of the protein is highlighted in the blue box.

Switching to CEX chromatography enabled for an improvement in peak shape and resolution of impurities. Screening mobile phase buffer pH highlighted the importance of pH for binding of the PEGylated protein to the column and improving resolution of major impurities (**Figure 2**).

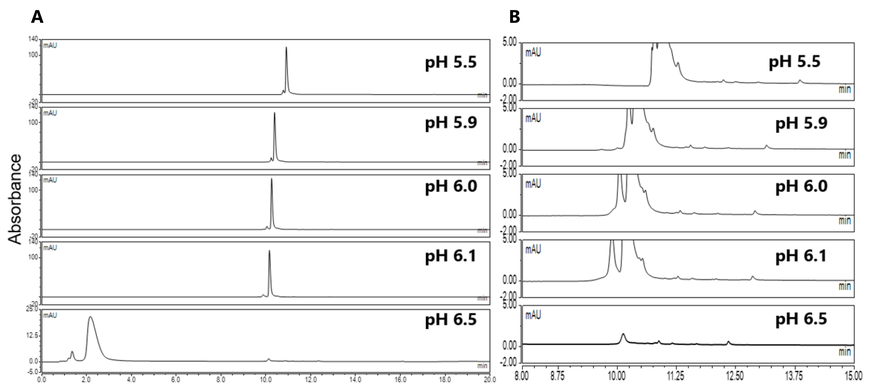

Figure 2. Effect of pH on charge variant analysis of PEGylated Protein. Panels show UV trace at 280 nm; (A) Full sized chromatograms of PEGylated protein during mobile phase pH screen; (B) Expanded chromatograms of PEGylated protein during mobile phase pH screen.

The main peak % area of acidic and basic variant % areas were determined using the optimum pH 5.9 mobile phase (**Table 1** and **Figure 3**).

Table 1 reports peak area percentage for the main peak (MP), sum of the acidic variants, and sum of the basic variants.

Mobile Phase pH	Retention Time	Peak Area Acidic Variants (%)	Peak Area Main Peak (%)	Peak Area Basic Variants (%)
5.5	10.910	6.8	81.1	12.1
5.9	10.387	7.2	85.7	7.1
6.0	10.253	7.0	86.1	6.9
6.1	10.16	7.1	86.8	6.1
6.5	----	----	----	----

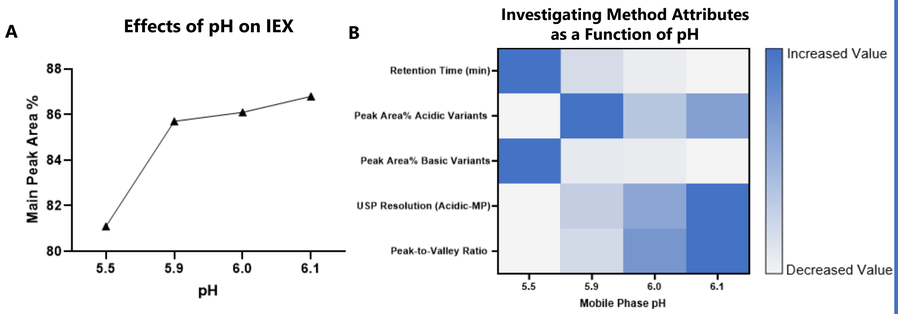

Figure 3. Evaluating the effect of pH on IEX of PEGylated Protein. (A) The left panel shows the pH effects on main peak Area %. (B) The right panel shows the area percent of the acidic and basic charge variant species, retention time, USP resolution between the major acidic peak and the main peak, and the peak-to-valley ratio between the major acidic peak and the main peak.

Results Continued

The final CEX method showed no interference of the mobile phase, and the ability to resolve new peaks formed through thermal degradation of the protein (**Figure 4**).

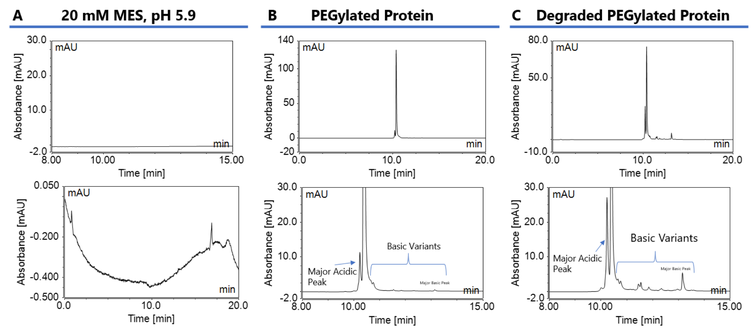

Figure 4. CEX analysis of the PEGylated protein. Panels show UV trace at 280 nm for (A) Full sized and expanded trace of Mobile Phase A blank, (B) Full sized and expanded trace PEGylated protein, (C) Full sized and expanded trace of thermal degraded PEGylated protein.

118 consecutive injections of the PEGylated protein followed by a mobile phase blank injection demonstrated both the reproducibility of the method and the limited carryover using a YMC BioPro SF IEX Column (**Figure 5**).

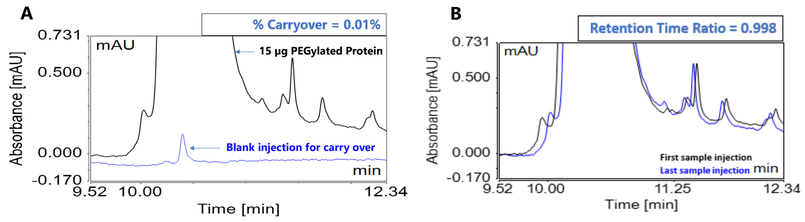

Figure 5: IEX of PEGylated protein. Panels show UV trace 280 nm for (A) expanded trace of PEGylated protein and blank, (B) expanded trace of first and last injection of PEGylated protein, 118 injections in sequence.

Analysis of force degraded PEGylated protein material by CEX identified that the method was stability-indicating and could be used to monitor changes to charge variance during development (Figure 6).

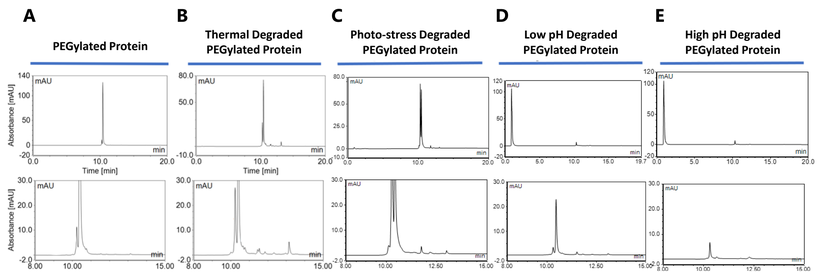

Figure 6. IEX of PEGylated protein. Panels show UV trace at 280 nm for (A) Full sized and expanded trace of PEGylated protein, (B) Full sized and expanded trace thermal degraded PEGylated protein, (C) Full sized and expanded trace of photo-stressed degraded PEGylated protein, (D) Full sized and expanded trace of low pH stressed degraded PEGylated protein, and (E) Full sized and expanded trace of high pH stressed degraded PEGylated protein.

Conclusion

The YMC BioPro SF column provides a valuable tool for the analysis and confirmation of PEGylated protein and PEGylated protein charge variants. 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.

Outlook

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

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