

## Investigations into Improving the Separation of PEGylated Proteins

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Reversed phase separations were performed on PEGylated and non-PEGylated forms of proteins in an attempt to better understand the various parameters that affect separations. Different proteins were evaluated, as were peptides, in an effort to derive more universal separations of PEGylated proteins from native proteins. HPLC column chemistries were evaluated as well as mobile phase organic composition, temperature, gradient conditions, and pH. Unlike gel filtration, reversed phase separations demonstrate excellent selectivity for proteins based on the specific site of PEGylation. Based on the experiments performed, the best resolution of PEGylated proteins was obtained using a Jupiter® 300 C4 column with acetonitrile being used as the organic mobile phase. Increased temperature does slightly improve resolution of different PEGylated forms, but not as dramatically as is often observed for other protein separations. Other experimental column chemistries using polar-selective media were evaluated, as well as C18 chemistries; such materials did not appear to offer any advantage over the Jupiter 300 C4 chemistry for this application.

### Introduction

Historically, a major disadvantage in the use of proteins and especially peptides as therapeutic drugs has been the short half-life that many of these drugs demonstrate *in vivo*. Small proteins and peptides are rapidly excreted in urine; many studies have found that by adding carbohydrate or other groups the half-life of a protein in serum can be dramatically increased.<sup>1</sup> A common practice in the development of protein and peptide therapeutics is to attach polyethylene glycol (PEG) groups to a protein to increase its serum half-life.<sup>2</sup> While the addition of such PEG groups to a protein or peptide improves its usefulness as a therapeutic drug, the addition also complicates both the characterization and purification of such PEG/protein conjugates away from the “non-PEGylated” protein species.<sup>3</sup>

Many researchers have used size exclusion/gel filtration chromatography as a means of separating PEGylated proteins from their native precursors. Such chromatography is low resolution, low capacity, and has difficulty distinguishing between different sites of PEGylation on a protein.<sup>4</sup> Ion exchange is sometimes used for separating modified proteins, but is also limited by resolution issues. Reversed phase chromatography is often used for characterization of PEGylated species due to its high resolution and ability to be coupled to a mass spectrometer.<sup>5</sup>

Use of reversed phase chromatography for separation of PEGylated proteins is not without its difficulties. PEGylated proteins often demonstrate poor peak shapes and resolution between different PEGylated forms so that isolating proteins modified at a specific site can be challenging. Investigations were

undertaken to try to improve chromatography conditions used for separating PEGylated proteins. Several different proteins were used to try to assure that studies determined the most universal conditions that applied best for all proteins, not just conditions that work for a specific protein. Proteins were PEGylated with commercial modification reagents and focus was placed on determining which chromatography media worked best for such separations. Parameters such as temperature, gradients, and mobile phase composition were all evaluated. While such conditions are definitely not ideal for all proteins, they might be considered starting points for such separations.

### Materials and Methods

Analyses were performed using a HP® 1100 LC system (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with a UV detector. Various HPLC columns were used for evaluations including Jupiter 300 C4, Jupiter 300 C18, and two additional experimental 300 Å medias, EXP1 and EXP2, (all 150 x 4.6 mm) (Phenomenex, Inc., Torrance, CA, USA). Unmodified proteins were purchased from Sigma-Aldrich Biotechnology (St. Louis, MO, USA) and PEGylation reagents to modify proteins were obtained from Pierce Chemicals (Rockford, IL, USA). Solvents were purchased from Fisher Scientific (Fairlawn, NJ, USA).

Protein PEGylation was performed using Methyl-PEO<sub>12</sub>-NHS Ester (P/N 22685), which were reacted similar to the protocol recommended in product literature. Briefly, a 4 mg/mL protein solution in PBS (pH 7.4) was reacted with a 20-fold excess of the PEGylation reagent dissolved in DMSO (20x molar excess to protein, not to number of free amines). The reaction mixture was incubated in an ice bucket for up to two hours (different time points were taken for some experiments). Reaction mixture was quenched with an equal volume of 50 mM Tris/1 % trifluoroacetic acid (TFA) (pH~2). Aliquots of 8 µg of protein (4 µL) were injected on HPLC for analysis.

Aqueous mobile phase used for most experiments was 0.1 % TFA and 2 % acetonitrile in water. In some cases 10 mM NH<sub>4</sub>HCO<sub>3</sub> (pH 10.5) or 10 mM triethylamine phosphate (pH 2.3) were also evaluated. For the organic mobile phase, 90 % acetonitrile/0.08 % TFA in water was predominantly used; however, for some experiments 70 % acetonitrile/20 % isopropanol/0.08 % TFA was used. In addition, 70 % acetonitrile/20 % TFA/0.08 % TFA was also evaluated. Gradient conditions varied; for most experiments a gradient from 20 % B to 55 % B in 25 minutes was used. All runs incorporated a 5 minute flush with 90 % B followed by at least a 10 minute reequilibration at 20 % B. The column temperature used for most experiments was 45 °C, though other conditions were evaluated. Flow rate for all analyses was 1 mL/min and protein elution was monitored by UV at 214 nm.



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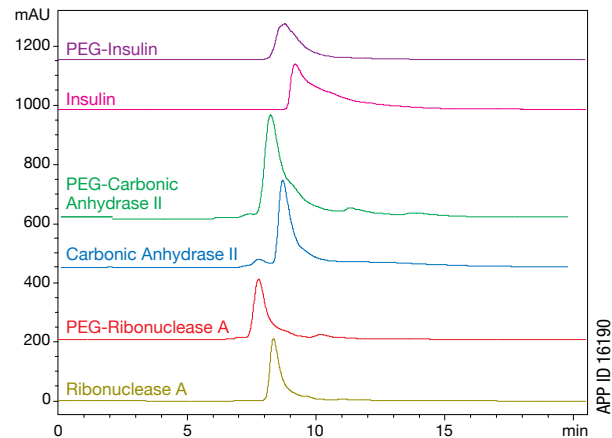
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### Results and Discussion

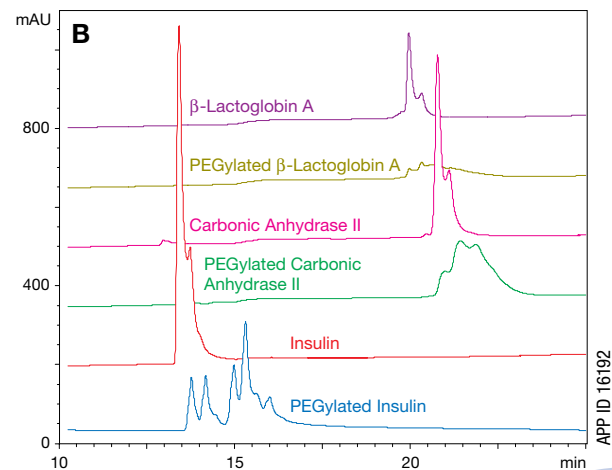
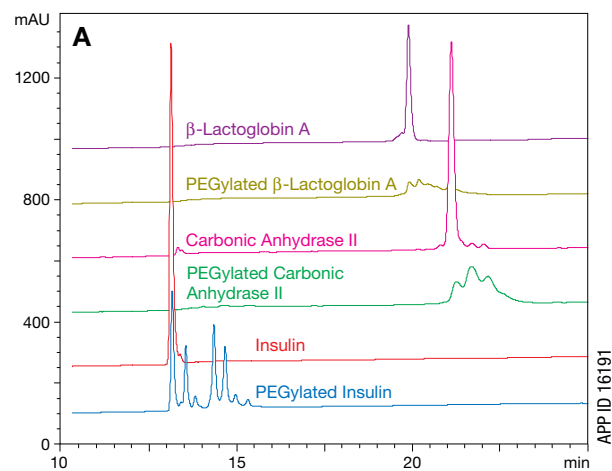
Investigations were undertaken to develop better conditions for separating PEGylated from non-PEGylated proteins by reversed phase chromatography, as well as achieve the best resolution possible for the various PEGylated forms that occur when N-hydroxysuccinimide (NHS) type chemistry is used to modify proteins. The 12-mer length PEG reagent was used as a "worst case scenario" for addressing chromatography difficulties that occur with PEG modifications. The NHS-PEG chemistry typically modifies surface accessible primary amines on proteins (lysine and N-terminal residues), and can also modify single or multiple distinct sites on a protein, resulting in significant heterogeneity between PEGylated forms. While gel filtration chromatography can differentiate between the degrees of polymerization (**Figure 1**) it cannot discriminate between proteins with different modification sites. Baseline separation of specific PEGylated protein forms is very difficult (or impossible in the case of large proteins), and efforts were taken to maximize separation of different PEGylated forms by reversed phase chromatography. Several different proteins were used to develop some level of universality so that conditions developed could be applied to a majority of PEGylated proteins and not just a specific type of protein. As such, six different proteins were initially investigated: insulin, albumin, carbonic anhydrase II,  $\beta$ -lactoglobulin A, myoglobin, and ribonuclease A.

An overlay of PEGylated and non-PEGylated proteins is shown on the Jupiter 300 C4 (**Figure 2A**) and Jupiter 300 C18 (**Figure 2B**) columns. Previous results suggested that the Jupiter 300 C18, with the highest methylene selectivity, was the superior phase for PEGylated protein separations; **Figure 2A** suggests the converse is true in that the Jupiter 300 C4 phase appeared to deliver better resolution between the different PEGylated and native proteins. Such results suggest that various interactions are involved in the separation of PEGylated proteins and that hydrophobicity or shape selectivity may be secondary to polar interactions. Investigations with polar functional experimental phases further complicate these findings as shown in **Figure 3**. These experimental phases both contain polar functional groups with different bonding chemistries; results indicate that the experimental 2 column demonstrates lower resolution than the Jupiter 300 C4 column, while the experimental 1 column closely resembles the Jupiter 300 C4 column. Such results suggest that multiple retention factors are involved in the separation of PEGylated proteins.

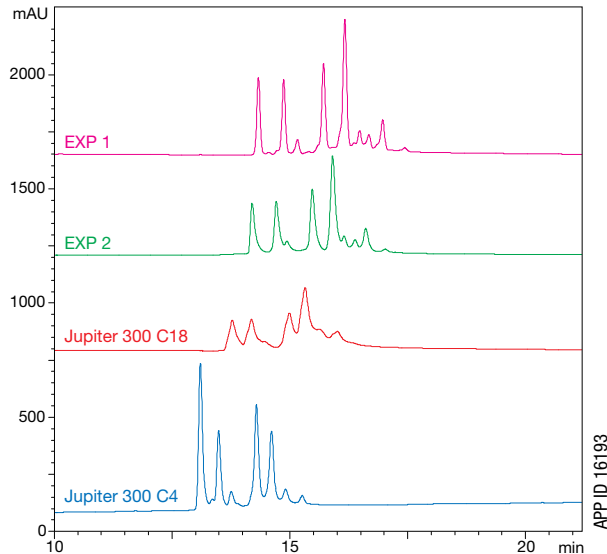
The effects of other chromatography parameters like mobile phase, temperature, and organic gradient were investigated. In previous reports<sup>6</sup>, the influence of adding isopropanol to the organic mobile phase for improving peak shape of proteins was reported; the effects of adding isopropanol to organic mobile phase is shown in **Figure 4**. Isopropanol increases the hydrophobicity of the organic mobile phase as well as having different solvation effects on analytes. Based on results in **Figure 4**, PEGylated proteins work best when acetonitrile is used alone as the organic solvent. Other alcohol/acetonitrile mixtures were investigated and demonstrated a decreased resolution of different PEGylated forms (data not shown). The use of tetrahydrofuran in the organic mobile phase was investigated and delivered no perceptible improvement in the separation of PEGylated forms (**Figure 5**).



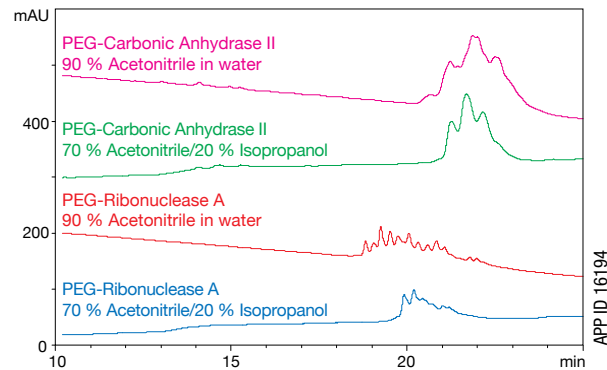
**Figure 1.** GFC runs of PEGylated and native proteins on a BioSep® 2000 column. Note the molecular weight shift due to PEGylation. Only differences in level of PEGylation can be resolved.



**Figure 2.** A) Reversed phase of PEGylated and native proteins runs on a Jupiter 300 C4 column. Note the good resolution of multiple PEGylated forms for all proteins tested. B) Reversed phase of PEGylated and native proteins runs on a Jupiter 300 C18 column. Note the decrease in resolution of PEGylated forms versus the C4 column in 2A.



**Figure 3.** Reversed phase of PEGylated insulin runs on various columns. Note the reduced resolution for the Jupiter 300 C18 and EXP 2 columns when compared to the Jupiter 300 C4 column. Although it is difficult to visualize, the Jupiter 300 C4 actually has better resolution of PEGylated forms than the EXP1 column.

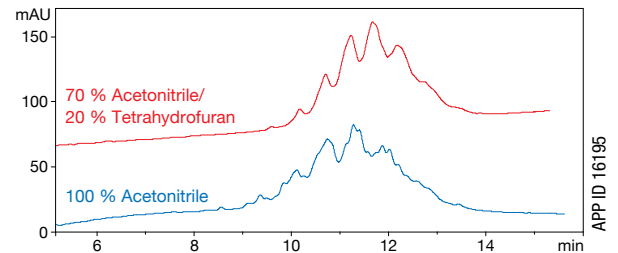


**Figure 4.** Reversed phase runs of PEGylated carbonic anhydrase II and PEGylated ribonuclease A using different organic mobile phase compositions. Unlike other applications, the addition of isopropanol does not improve resolution of different PEGylated forms.

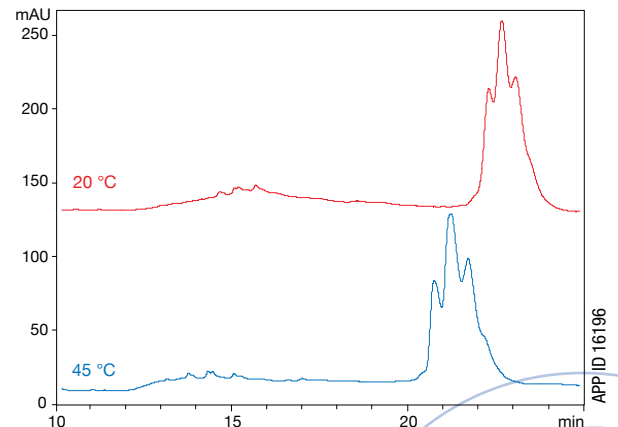
The effect of temperature was evaluated (**Figure 6**) and higher temperature was found to slightly improve the resolution of PEGylated forms. The improvement dictated the use of 45 °C for additional studies (little utility was seen in using temperatures above 45 °C, as further temperature increases did not provide any dramatic improvement in efficiency or resolution). Next, changing both initial organic mobile phase percentage as well as the gradient slope was investigated (**Figure 7**). Decreasing the slope of the gradient does appear to improve the resolution of different PEGylated protein forms, but also increases the peak width, thus limiting the utility of increasing run time to improve separations. Initial organic mobile phase percentage had little effect at low percentage B, but resulted in decreased loading and reduced peak efficiency and resolution when initial mobile phase percent was raised above 25 % B. This is most likely due to incomplete binding of the protein to the stationary phase at high organic mobile phase resulting in the protein elution band being more diffuse. Based on such results, our preferred gradient using

the Jupiter 300 C4 column was from 20-55 % B in 25 minutes. Such conditions seemed to work best for most proteins; obviously more hydrophobic or hydrophilic proteins will require optimized gradients.

In an effort to better understand reaction kinetics as well as general retention behavior of PEGylated proteins, different timepoints of the PEGylation reaction were analyzed. Results for two proteins are shown in **Figures 8** and **9**. In general, the PEGylation reaction concurrently occurs rapidly at several different protein sites in a fixed ratio. As the reaction continues, more heavily PEGylated (and later eluting) forms were observed. In our studies, there seems to be a limit to the degree of modification for some proteins; this is most likely due to inaccessibility of the modifying reagent to react to some free lysines on specific proteins, but in some cases may be related to depletion of reagent with proteins that contain numerous lysines. A minor observation for such studies is that it is critical that a proper quenching reagent is used to stop the PEGylation reaction. In our studies we found that lowering the pH as well as adding an amine scavenger was required to stop the reaction. In many cases improper quenching of the reaction produced confused results for a time course study (data not shown). Regardless, in every protein tested there was always more than one PEGylated protein peak observed by reversed phase HPLC; each seemingly ascribed to a different site of PEG modification. Indeed, from such results it appears that protein modification site influences the reversed phase separation of proteins much more than the degree of modification. In every

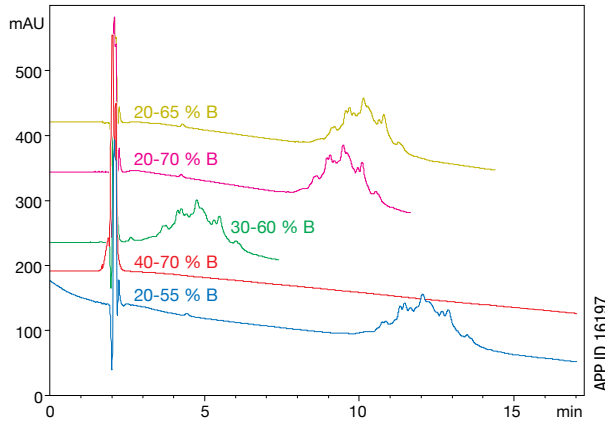


**Figure 5.** Tests of tetrahydrofuran (THF) organic mixtures. Reversed phase runs of PEGylated  $\beta$ -lactoglobulin A using different organic mobile phase compositions on Jupiter 300 C4. 70 % acetonitrile/20 % THF were substituted for the organic mobile phase. For various proteins tested, the addition of THF slightly decreased the resolution of PEGylated forms.

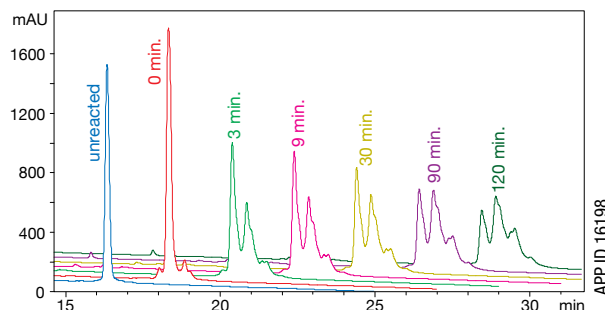


**Figure 6.** Reversed phase runs of PEGylated carbonic anhydrase II at different temperatures. Increasing temperature to 45 °C slightly improves resolution of different PEG forms.

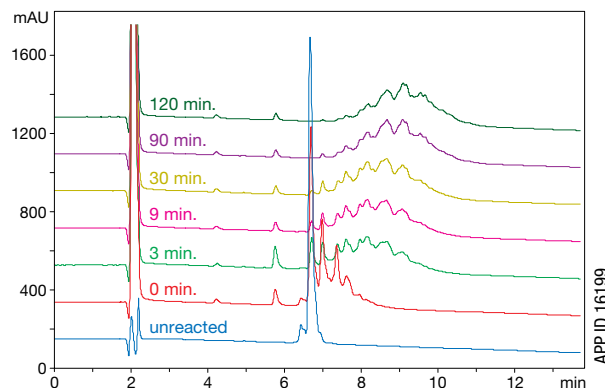
case tested, the PEGylated protein can be baseline separated from unmodified protein, but with larger proteins that contain numerous potential PEGylation sites separating individual PEG forms is not always possible. This is in contrast to gel filtration where only a few peaks are observed that correspond only to the degree of modification with no resolution of individual modification sites.



**Figure 7.** Reversed phase runs of PEGylated ribonuclease A using different gradient conditions on a Jupiter 300 C4 column (gradient length 25 min). Note that if initial % B is increased much above 25 % then retention and resolution of PEGylated forms are lost. Gradient slopes between 1-2 %/min appear to be a good compromise between resolution and run time.



**Figure 8.** PEGylation time course study of carbonic anhydrase II. Different time points from a PEGylation reaction of carbonic anhydrase II were taken: unreacted, 0 min., 3 min., 9 min., 30 min., 90 min., and 120 min. Note the increase of greater retaining PEGylated forms over time.



**Figure 9.** PEGylation time course study of ribonuclease A. Different time points from a PEGylation reaction of ribonuclease A were taken: unreacted, 0 min., 3 min., 9 min., 30 min., 90 min., and 120 min. Note the increase of greater retaining PEGylated forms over time.

## Conclusions

This study was focused on improving the reversed phase chromatography of PEGylated proteins as well as observing the effects of PEGylation. In these studies the most optimal conditions for most proteins tested were:

- The use of a C4 column, like the Jupiter 300 C4, versus C18 columns.
- The use of a standard 0.1 % TFA/acetonitrile gradient method versus using alcohols or other organic mobile phase modifiers.
- The use of moderately shallow gradient conditions (1-2 %/min) with low initial organic concentration.
- Moderate elevated column temperatures of around 45 °C for improved peak shape and resolution.

In every case tested, reversed phase chromatography gave improved resolution of PEGylated and unmodified proteins versus gel filtration chromatography. Reversed phase chromatography using the Jupiter 300 C4 column provides separation of specific PEGylated forms in most cases. In addition, reversed phase chromatography of PEGylated protein can be scaled to preparative and process-scale separations or can be connected to a mass spectrometer for more detailed identity information (formic acid buffer can be used instead of TFA for LC/MS applications with only a slight loss in resolution). Retention is mostly influenced by modification site, but more modified proteins generally elute later under reversed phase conditions. While such observations are only based on a few proteins using a specific PEGylation reagent, such observations should be a good starting point for other applications of separating PEGylated proteins.

If you would like more information on these columns or any of the applications listed, please contact Phenomenex. Also, if you are new to protein and peptide HPLC or are doing method development work, call us today to request your FREE copy of our 75-page, "User's Guide – Introduction to Peptide and Protein HPLC."

## ORDERING INFORMATION

Part No.	Description
00F-4167-E0-TN	Jupiter 300 Å C4, 5 µm, 150 x 4.6 mm
00F-4053-E0-TN	Jupiter 300 Å C18, 5 µm, 150 x 4.6 mm

## References

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