Analytical Methods to Qualify and Quantify PEG and PEGylated Biopharmaceuticals

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Key Words
Polyethylene glycol (PEG), size-exclusion column, charged aerosol detection, HPLC, proteins

Goal
To develop and evaluate a two-dimensional LC system with charged aerosol detection for the simultaneous characterization of protein PEGylation and unreacted PEGylation reagent.

Introduction
The use of polyethylene glycol (PEG) as a safe and low cost additive for the pharmaceutical and cosmetic industries is common practice. Over the last decade the field of biopharmaceuticals has begun to use the process of covalently bonding PEG to their active peptides or proteins (PEGylation) to improve bioavailability and reduce immunogenicity along with several other benefits.[1] Typical PEGylation reagents often contain heterogeneous mixtures of different PEG chain lengths. This is in contrast to Thermo Scientific™ Pierce™ PEGylation Reagents, which are homogeneous compounds of defined molecular weight and spacer arm length. The PEGylation reagents are chosen according to the reaction specificity of their terminal groups, as well as the length and degree of chain branching desired. The Thermo Scientific MS(PEG)$_n$ series consists of N-hydroxysuccinimide (NHS) esters, which react at pH 7-9 with primary amine groups by nucleophilic attack, forming amide bonds and releasing the NHS (Figure 1). The molar ratio of reagent-to-protein used in the reaction affects the number of amine groups modified. Optimizing this ratio and the reaction conditions provides the specific level of modification needed for an application.

While the characterization of proteins is often accomplished by a combination of HPLC with UV and MS detection, the quantification of PEG and PEG reagents is more challenging as they do not contain sufficiently active chromophores for UV characterization.[2] Such limitations are readily overcome by charged aerosol detection.[3] If this analysis was performed using a simple one-dimensional LC system with size exclusion chromatography (SEC), data quality would suffer due to the low MW of the PEGylation reagent and the large amount of non-volatile buffer salt present in the reaction. Therefore, two-dimensional (2D) analysis was examined. A two-dimensional LC simply means that the sample is fractionated using one mode (e.g. SEC) and then transferred and reanalyzed using a second mode with sufficiently different (orthogonal) selectivity (e.g. reversed-phase C8). Two-dimensional LC can be categorized as to whether or not the transfer is done online by the instrument and how this is actually accomplished. In this work, an online 2D LC system was developed using a large sample loop to trap the PEGylation reagent after elution from the SEC column with the sample's buffer matrix and then transferring this to the C8 chemistry. Online reaction monitoring of the PEGylation process was also accomplished using customized user defined autosampler program methods. These methods and results for all techniques examined will be discussed.

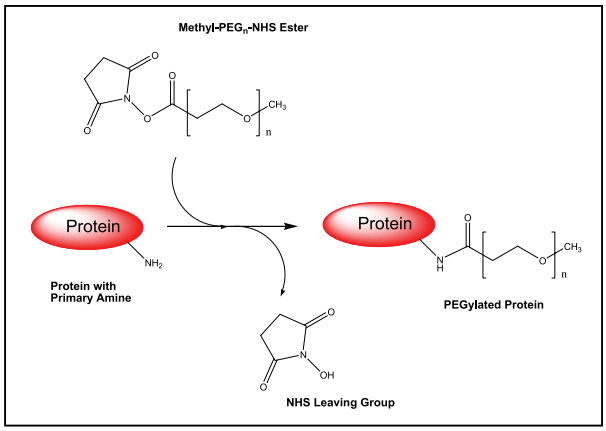

Figure 1. Schematic of protein PEGylation with MS(PEG)$_n$. Proteins are many times larger than the PEGylation reagent and usually contain several amine groups, each of which could be labeled.

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Experimental

An amine reactive PEGylation reagent was first used with a traditional preparation method and then secondly with the reaction carried out on the autosampler tray. The LC method used a specialty size-exclusion column for the first dimension, which trapped the PEG in a 500 µL loop. The contents of the loop were then loaded onto an analytical C8 column using gradient elution in the second dimension. A diode array detector along with charged aerosol detection was used throughout these experiments.

Sample Preparation

The PEGylation reagent used was Thermo Scientific $MS(PEG)_8$. Sample preparation followed the procedure outlined in the reagent instruction sheet.[4] The reagent was reconstituted to 250 mM in DMSO and then diluted to 10 mM in phosphate buffered saline (PBS) for the immunoglobulin G (IgG) study. Since the online reaction was spread over several hours, the second dilution used DMSO for this process. It should be noted that when performing online preparations with DMSO, this reagent has a freezing point of 19 °C so tray cooling should be avoided.

Two proteins were evaluated in these experiments. The first protein, rabbit serum IgG (2 mg/mL), was prepared manually by hand pipetting the buffer, protein and PEGylation reagent (Table 1, volume in µL) into vials, waiting 30 min and then quenching the reaction with 5 µL additions of 1 M Tris HCl at pH 7.5. The second protein, bovine serum albumin (BSA) (2 mg/mL, Sigma), was prepared robotically by adding similar volumes of each reagent as the IgG sample set, but using individual autosampler injection programs to perform reagent additions, waiting and finally injecting the sample. Standards were also prepared online according to the volumes shown in Table 1.

Table 1. Buffer, protein and PEGylation reagent volumes (in µL) for the preparation of the rabbit serum IgG sample set (2 mg/mL)

	PEG 0x	PEG 5x	PEG 10x	PEG 20x	PEG 40x
Volume PBS	82.90	82.24	81.57	80.24	77.58
Volume Protein	17.10	17.10	17.10	17.10	17.10
Volume 10 mM $MS(PEG)_8$	0.00	0.67	1.33	2.66	5.32

	PEG Std 1	PEG Std 2	PEG Std 3	PEG Std 4	PEG Std 5
Volume PBS	94.68	97.34	98.67	99.34	199.34
Volume Protein	0.00	0.00	0.00	0.00	0.00
Volume 10 mM $MS(PEG)_8$	5.32	2.66	1.33	0.67	0.67

Liquid Chromatography

The Thermo Scientific Dionex™ UltiMate™ 3000 x2 Dual RSLC system was equipped with a DPG-3600RS dual-gradient rapid separation pump, a WPS-3000TRS autosampler, a TCC-3000RS rapid separation thermostatted column compartment, a 10-port 2-position high pressure valve and a 6-port 2-position high pressure valve, a DAD-3000RS rapid separation diode array detector, and a Thermo Scientific Dionex Corona™ ultra RS™ Charged Aerosol Detector.

Column 1:	Thermo Scientific MAbPac™ SEC-1 5 µm, 300 Å, 4 × 300 mm
Column 2:	Thermo Scientific Acclaim™ C8 3 µm, 3.0 x 150 mm
Mobile phase A:	100 mM ammonium acetate, pH 6.7
Mobile phase B:	Acetonitrile
Mobile phase C:	Deionized water
Gradient:	Table 2
Pump Right flow rate:	0.400 mL/min
Pump Left flow rate:	1 mL/min
DAD settings:	UV_1: 214 nm UV_ 2: 280 nm, 3D Field on (200-300 nm)
Corona ultra RS settings:	Filter setting: 4 Nebulizer temp: 15 °C Power function: 1.0

Pump Right was used for the SEC analysis and ran isocratically with 10 mM ammonium acetate, pH 6.7 with 5% acetonitrile. Pump Left was used for the reversed phase chromatographic analysis. The system configuration is shown in Figure 2.

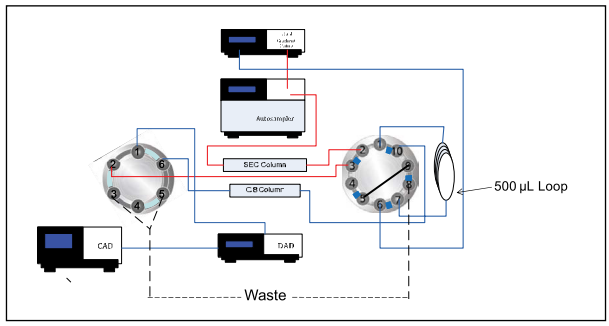

Figure 2. Schematic of 2D LC system configuration

Table 2. Timing of the gradient and loop fill

Time	Pump Left (C8)				Valve Position	
	% A	% B	% C	Curve	Left	Right
0	5	10	85	5	1_2	10_1
6.5					1_2	1_2
7.6					6_1	10_1
9	5	10	85	5		
16	5	50	45	5		
17	5	50	45	5		
18	5	10	85	5	1_2	10_1

The online preparation was accomplished by placing empty 2 mL vials, each containing a 200 µL glass insert, on the tray. Four reagent bottles were placed on the autosampler with the following content: A) PBS buffer; B) 2 mg/mL protein sample; C) $MS(PEG)_8$ reagent in DMSO; D) deionized water for wash. Individual instrument methods were created for the preparation of each sample and standard according to the volumes shown in Table 1 using the user defined program function in the Thermo Scientific Dionex Chromeleon™ Chromatography Data System version 7.1, SR 1. A general user defined method for the addition of Tris buffer using a fifth reagent vial on the autosampler tray and other methods for no addition were also created.

Results and Discussion

Manually Prepared PEGylated IgG

After preparation of the samples was complete, they were stored frozen until a suitable analytical method was developed. The first part of this development process was to investigate SEC methods capable of reproducibly resolving both the free PEG and PEGylated proteins. Although several of the methods evaluated successfully resolved these species, the presence of non-volatile buffer salts made them unsuitable for PEG analysis with charged aerosol detection. The focus was then shifted to the 2D approach. The MW of the PEG reagent used was ~500 so this peak should elute near the front edge of the salt peak. The SEC column was chosen based on its ability to reproducibly elute the protein and buffer salts in less than 10 min using volatile mobile phase conditions (Figure 3).

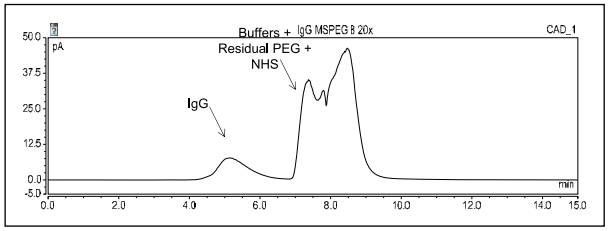

Figure 3. Analysis of PEGylated IgG sample using MAbPac SEC-1 column.

The 500 µL loop shown in Figure 2 was filled with the analytes of interest in 1.1 min due to the small internal diameter low flow rate column used in this method. The valves were then switched and the contents of the loop were transferred onto the C8 column. The success of the method depends on the elution properties of the PEG species and the organic content of both the SEC and initial conditions of the C8. When the organic content of the first dimension was similar to the elution strength of the compound of interest, transferring the contents of the 500 µL loop resulted in poor retention or peak broadening on the C8 column due to volume overloading. In this case, the organic strength of the transfer solvent was lower than the starting conditions of the C8 and was below the elution threshold of the PEG. Thus, the compound focused on the head of the C8 column while the buffer salts and other polar materials eluted early. Gradient conditions used for elution of the PEG were similar to those that would be used in a normal 1D reversed phase method (Figure 4).

At the end of the 30 reactions, possible unreacted and hydrolyzed PEGylation reagent remained in the sample solution. This is shown in Figure 4 with two peaks present even though the starting reagent material contained a single MW material. Other species can also form depending on the availability of free amine functionalities. The method permitted the quantification of all species formed, thereby enabling a better understanding of the degree of successful PEGylation reactions with the protein. The Corona detector requires mobile phase consisting of volatile buffers, and this requirement makes it easy to split the flow to a MS if further peak identification is desired. Multiple preparations of the IgG protein using different molar excesses of PEGylation reagent were also analyzed (Figure 5). The charged aerosol trace shows the movement of the protein to shorter retention times (higher MW) as the molar excess of the $MS(PEG)_8$ was increased. The peak response from residual PEG also increased when excess reagent was added. The inset chromatogram shows the peak for the protein using UV at 280 nm. This observed trend for the protein was confirmed by the charged aerosol detector.

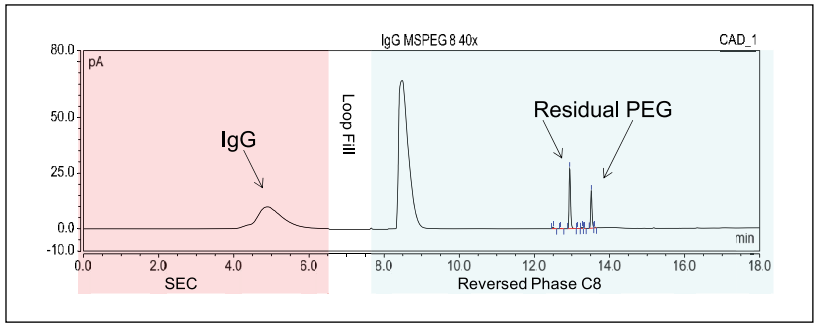

Figure 4. 2D analysis of PEGylated IgG sample with the SEC first dimension highlighted in red and the C8 second dimension highlighted in blue.

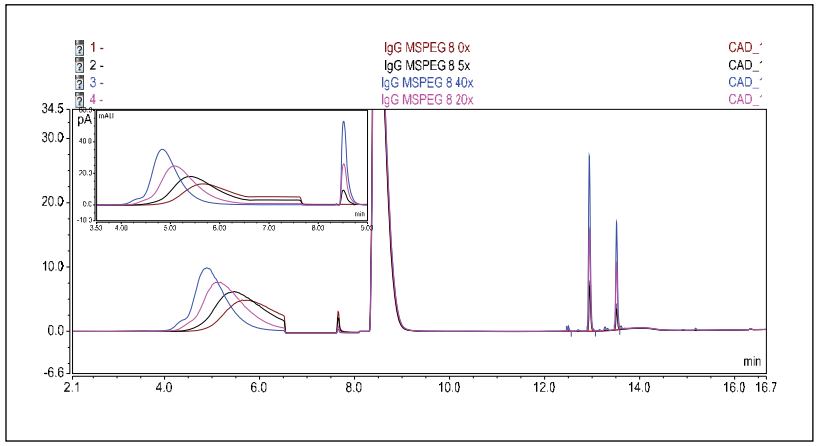

Figure 5. Overlay of four samples of IgG with varying amounts of $MS(PEG)_8$ analyzed by the 2D LC system with charged aerosol detection. Inset: UV at 280 nm.

Online Preparation of PEGylated BSA

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In this experiment a sequence list was prepared that alternated between the first vial, which contained protein plus PEGylation reagent, and a second vial, which also contained PEGylation reagent but the protein solution was replaced by an equal volume of PBS buffer. These vials were then sequentially analyzed at the initial time point (~2 min after the PEGylation reagent was added) and then again at ~40 min after the addition of Tris HCl buffer to quench further reactions. This sequence ensured identical reaction conditions and timing for all standards and sample vials. Additional sample/standard groups were prepared in a similar fashion using varying excess amounts of PEGylation reagent. The results for BSA indicated similar trends to those observed for IgG. The elution of the protein peak shifted on the SEC column as the molar excess of the reagent was increased due to the increasing size of the reacted product.

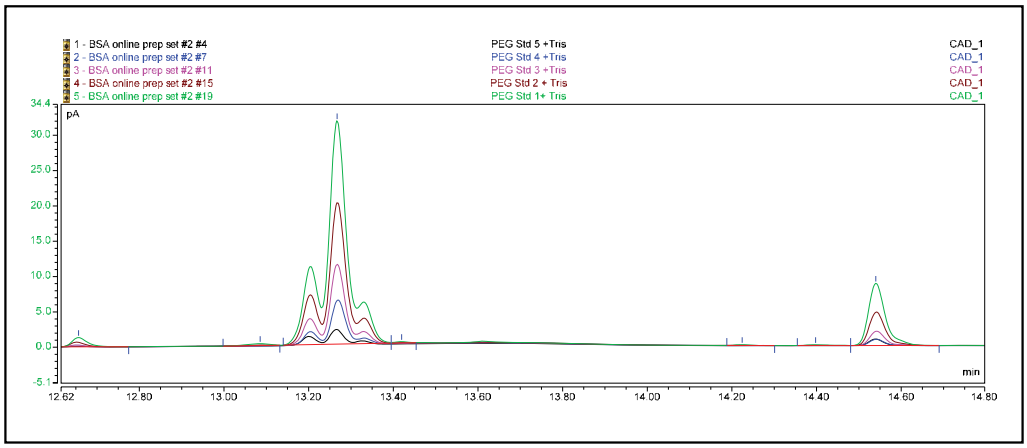

Figure 6. Overlay of chromatograms showing the reaction with BSA at 5 concentrations of PEGylation reagent illustrating both hydrolyzed and unreacted PEG reagent.

As shown in Table 1, PEGylation reagent standards were prepared at five concentrations to fully encompass the PEG concentrations added to the protein samples. An overlay of chromatograms obtained at the 40 min time point after Tris HCl buffer addition for the five PEG standards is shown in Figure 6. Two major peaks were observed. The first group of peaks at around 13.3 min was related to hydrolyzed PEGylation reagent and the second peak at 14.5 min was unreacted PEGylation reagent. Response curves were generated from the five PEG standards for the initial time point and post Tris HCl buffer addition (t=40 min) time points. These response curves were then used to calculate the molar excess of unreacted PEGylation products at their respective reaction times and concentration levels. The levels of excess of PEGylation reagent in both hydrolyzed and unreacted form at each time point were summed to provide the total level of residual PEG remaining in the protein sample. Since the actual molar excess of reagent added was known, the residual amount of PEG was simply subtracted from this value to provide the molar excess value for the amount of reagent used in PEGylation of protein at four different levels and two time points. These values are plotted in Figure 7.

There is good correlation between the amount of PEGylation reagent added and the amount of protein PEGylation. Figure 7 shows that at the initial time point the amount of PEGylation reagent used has a near linear relationship with the PEG attached to the protein. After 40 min, the slope of the line begins to increase with increasing amounts of reagent added. At the 40 molar excess point, the slope begins to flatten out indicating that additional reagent will have little impact on the overall extent of PEGylation. This online setup allows for optimal amounts of PEGylation reagent to be determined without the need for extensive sample preparation by a chemist.

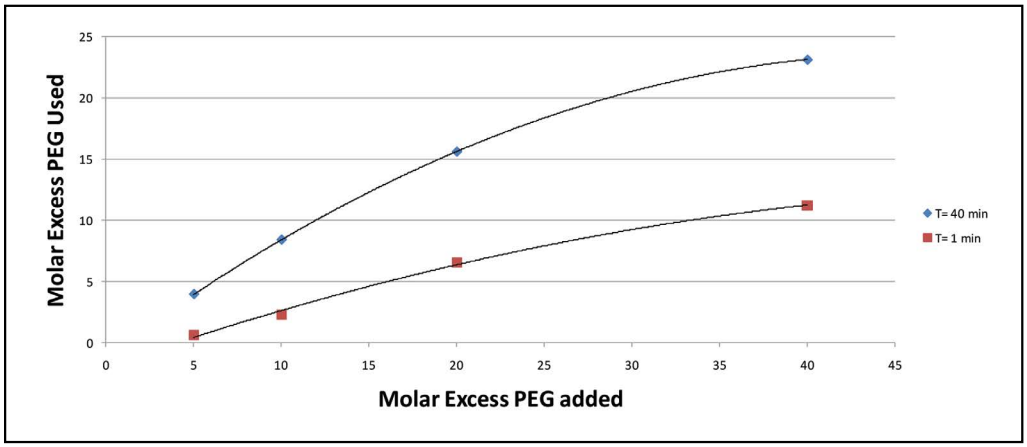

Figure 7. Correlation of PEGylated product and residual PEGylation reagents.

Conclusion

- The MS(PEG)$_n$ series of PEGylation reagents can selectively react at a protein's free amino sites to improve solubility and bioactivity.
- The UltiMate 3000 x2 Dual RSLC system with a dual gradient pump enables simple configuration for an online 2D LC system.
- The Corona ultra RS detector can be used in combination with a 2D LC configuration to qualify and quantify both the PEGylated product and the residual PEGylation reagents and byproducts.
- The use of user defined program methods with Chromeleon software permits sample preparation and reaction quenching to be performed on the autosampler tray in a highly controlled fashion.
- Online sample monitoring of the reaction process allows for consistent degree of protein PEGylation.

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