Analytical Methods to Qualify and Quantify PEG and PEGylated Biopharmaceuticals

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Overview

Purpose: The development and evaluation of a two-dimensional LC system with charged aerosol detection for the simultaneous characterization of protein PEGylation and unreacted PEGylation reagent.

Methods: An amine reactive PEGylation reagent was used with a traditional preparation method and then with the reaction carried out on the autosampler tray. The LC method uses a specialty size-exclusion column for the first dimension, which traps the PEG in a 500 µL loop. The loop's content is 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.

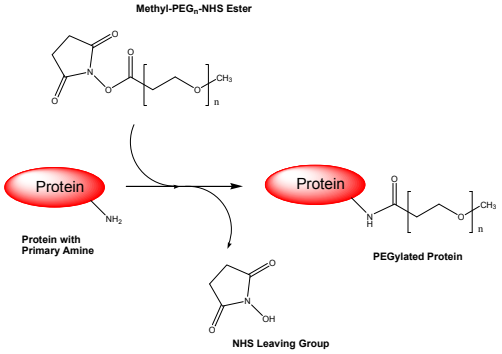
Results: In each case the protein peak shifted to earlier elution times with increasing quantities of PEGylation reagent indicating an increase in MW and successful PEGylation. The charged aerosol detector measured the residual reagent, both in its reactive form, as well as the spent hydroxylated form. This offers a straight forward approach for monitoring on-line reactions with subsequent quantification of the PEGylation reagent used. The dynamic range of the method and sensitivity of the technique were sufficient not only to monitor the high quantities of reagent but also test for residual PEG in the final product.

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 actual 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 chemistry (e.g. SEC) and then transferred and reanalyzed using a second chemistry (e.g. reversed-phase C8). Two-dimensional LC can be categorized as to whether or not the transfer is done on-line by the instrument and how this is actually accomplished. In this work, an on-line 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. On-line 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.



Methods

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 IgG study. Since the on-line reaction was spread over several hours, the second dilution used DMSO for this process. It should be noted that when performing on-line preparations with DMSO, this reagent has a freezing point of 19 ºC so tray cooling should be avoided.

Two proteins were evaluated in this analysis. The first protein, rabbit serum IgG (2 mg/mL), was prepared manually by hand pipetting the buffer, protein and PEGylation reagent (Table 1) into vials, waiting 30 minutes 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. Standards were also prepared on-line according to the volumes shown in Table 1.

Table 1	PEG 0x	PEG 5x	PEG 10x	PEG 20x	PEG 40x	PEG Std 1	PEG Std 2	PEG Std 3	PEG Std 4	PEG Std 5
Volume PBS	82.90	82.24	81.57	80.24	77.58	94.68	97.34	98.67	99.34	199.34
Volume Protein	17.10	17.10	17.10	17.10	17.10	0.00	0.00	0.00	0.00	0.00
Volume 10mM MS(PEG)8	0.00	0.67	1.33	2.66	5.32	5.32	2.66	1.33	0.67	0.67

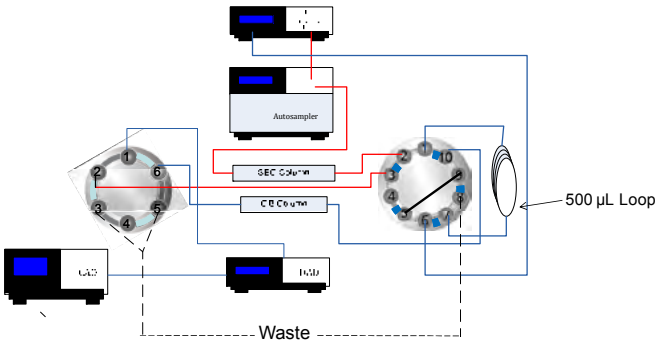
Liquid Chromatography

System: Thermo Scientific Dionex UltiMate 3000 2x Dual RSLC system equipped with: DPG-3600RS, WPS-3000TRS, TCC-3000RS with 10-port 2-position high pressure valve and 6-port 2-position high pressure valve, DAD-3000RS, Thermo Scientific Dionex Corona ultra RS Charged Aerosol Detector (CAD™).
Column: Thermo Scientific MAbPac SEC-1 5 µm, 300 Å, 4 × 300 mm
DAD settings: UV _1: 214 nm, UV_2 : 280 nm, 3D Field on (200–300 nm)
Corona™ ultra RS™: Filter setting 4, Nebulizer Temp: 15 ºC, Power Function 1.0
System configuration is shown in Figure 2
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 analysis. The mobile phases are (A) 100 mM ammonium acetate pH 4.7, (B) acetonitrile, and (C) deionized water. The timing of the gradient and loop fill is shown in Table 2.

Table 2	Pump Left (C8)				Valve Position	
Time	% 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 on-line preparation was accomplished by placing empty 2 mL vials on the tray, each containing a 200 µL glass insert. 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 7.1 SR 1 Chromatography Data System. A general user defined method for the addition of Tris buffer using a fifth reagent vial on the autosampler tray and methods for no addition were also created.

FIGURE 2. Schematic of 2D LC system configuration.

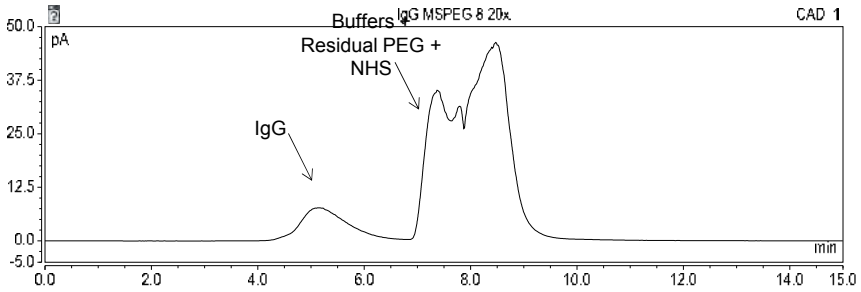


Results

Manually Prepared PEGylated IgG

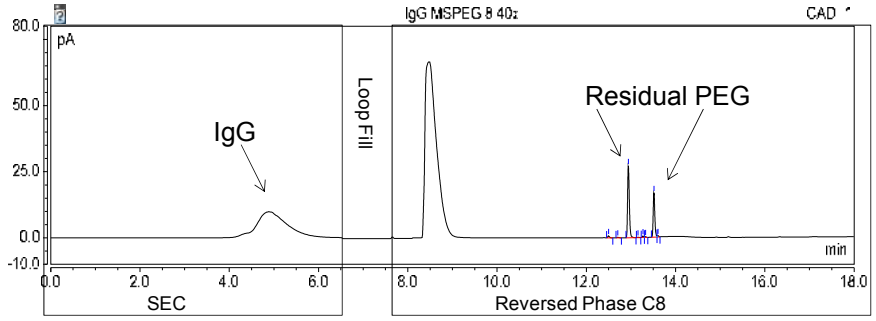
After preparation of the samples was complete, they were stored frozen until a suitable analytical method was developed. The first part of this development process was to investigate SEC methods capable of reproducibly resolving both the free PEG and PEGylated proteins. Although several of the methods 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 minutes using volatile mobile phase conditions (Figure 3).

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



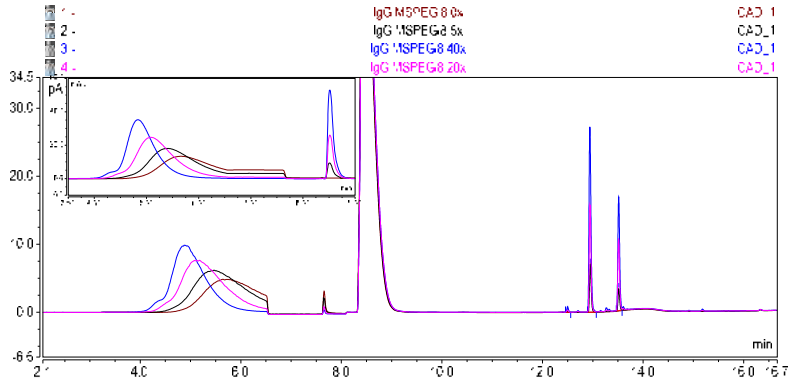
The 500 µL loop shown in Figure 2 was filled with the analytes of interest in 1.1 minutes due to the small internal diameter low flow rate column used in this method. The valves were then switched and the contents of the loop were transferred onto the C8 column. The success of the method depends on the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

FIGURE 4. 2D analysis of PEGylated IgG sample with the SEC 1st dimension highlighted in red and the C8 2nd dimension highlighted in blue.



At the end of the 30 reactions, possible unreacted and hydrolyzed PEGylation reagent remain in the sample solution. This is shown in Figure 4 with two peaks being 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 CAD 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 CAD trace shows the movement of the protein to shorter retention times (higher MW) as the molar excess of the $MS(PEG)_8$ is 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 6. 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.



On-line Preparation of PEGylated BSA

In this experiment a sequence list was prepared that alternated between the first vial containing protein-plus PEGylation reagent and a second vial also containing 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 ensures 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 (above). The elution of the protein peak shifted on the SEC column as the molar excess of the reagent was increased due to increasing size of the reacted product.

Table 1 shows the fifth PEGylation standard preparation which had a PEGylation reagent concentration, half that of the lowest protein preparation. This allowed for PEG standards to fully encompass the PEG in protein samples. An overlay of chromatograms obtained at the 40 min time point after Tris addition for the five PEG standards is shown in Figure 6. Two major peaks were observed. The first group of peaks was related to hydrolyzed material and the second peak at 14.5 min was unreacted PEG reagent. Response curves were then generated from the five PEG standards for the initial time point and post Tris 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.

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

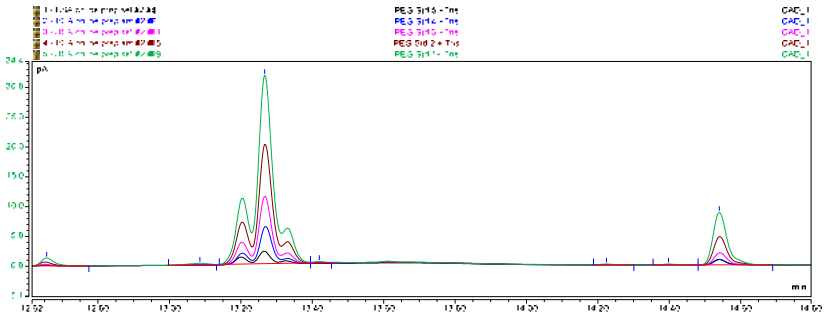
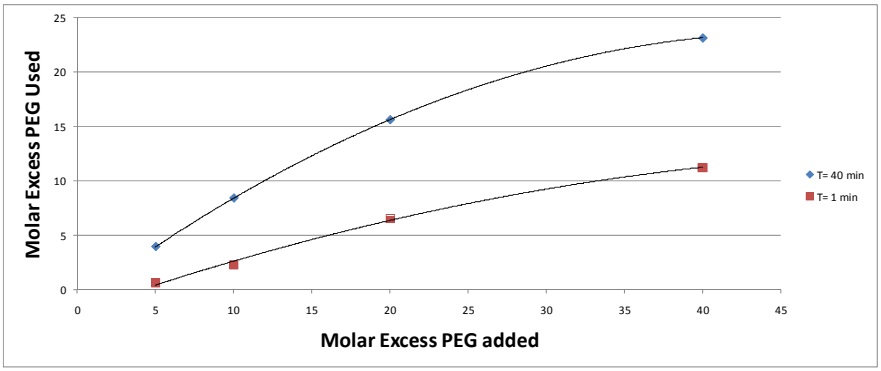


FIGURE 7. Correlation of PEGylated product and residual PEGylation reagents.



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 on-line setup allows for optimal amounts of PEGylation reagent to be determined without the need for extensive sample preparation by a chemist.

Conclusion

- The use of MS(PEG)$_n$ series of PEGylation reagents can selectively label a protein at their 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 on-line 2D LC system.
- The Corona ultra RS detector can be used in combination with the 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.
- On-line sample monitoring of the reaction process allows for consistent degree of protein PEGylation.

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