Poster Note xxxxx

Monitoring Peptide PEGylation by HPLC with Charged Aerosol Detection

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ABSTRACT

Purpose: The goal of this work was to develop a fast and accurate method to monitor PEGylation reactions by using HPLC with complementary UV/Vis and charged aerosol detection.

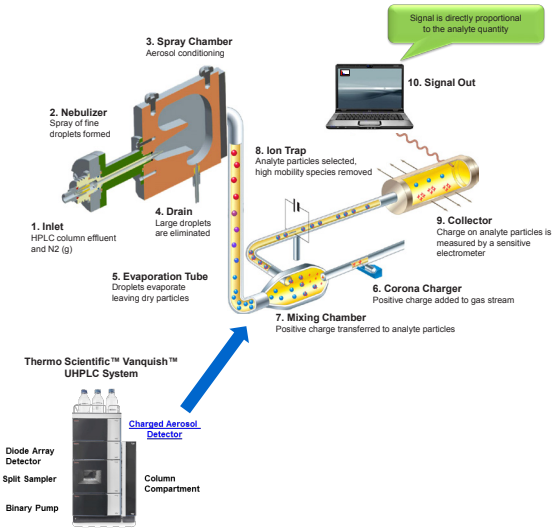
It is an increasingly common practice to modify biotherapeutic peptides, proteins and small molecules by attaching chemical groups designed to enhance properties such as bioavailability, potency or stability. One example of this is the PEGylation reaction that attaches hydrophilic polyethylene glycol (PEG) moieties tailored to increase the drug's aqueous solubility and circulating half life. Chemists developing extended-life biotherapeutics need to measure free PEG, partially PEGylated intermediates, and final PEGylated peptide/protein in order to optimize reaction conditions, assess the quality of final product, and study drug product stability.

Methods: Peptides and proteins were modified by reaction with MS(PEG)n reagents. These are methyl-terminated, polyethylene glycol compounds with defined PEG chain lengths of discrete molecular weight (n equals 4 to 24 PEG units). They are activated as NHS esters for covalent PEGylation of primary amines on proteins through the formation of stable, irreversible amide bonds.

A high resolution separation on the MAbPac RP resolves PEGylation reagents, Tris-quenched PEG, unreacted insulin, and several PEGylated insulins that differ in the number and sites of attached PEG. The UV/Vis detector provides information on the quantity and molar ratio of insulin and PEGylated insulin, but can't measure PEGylation reagents or hydrolyzed PEG. Charged aerosol detection (Figure 1) easily quantifies the PEG related species that are missed by UV, and provides information on the number of PEGs attached to each peptide.

Results: The method was used to characterize the reaction products from MS(PEG)8 PEGylation of insulin and human immunoglobulin G (IgG). Quantitative performance including precision, detection limits and dynamic range is presented. Figures of merit include sensitivity at the low-nanogram on-column level, dynamic range over two orders of magnitude, and peak area precision averaging less than two percent RSD.

Figure 1. Charged aerosol detector and principle of operation.



MATERIALS AND METHODS

Liquid Chromatography

Thermo Scientific™ Vanquish™ UHPLC system with:

- Thermo Scientific™ Vanquish Charged Aerosol Detector H:
  - Evaporation Temperature: 50 °C
  - Power function: 1.00
  - Data collection rate: 2 Hz
  - Signal Filter: 3.6 sec

Data Analysis

Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System (CDS) 7.2

Reagents

Reagent-grade or Optima™ LCMS-grade

Reagents

Reagent-grade or Optima™ LCMS-grade

Separation

Column:	Thermo Scientific™ MAbPac™ RP 4 µm, 3 × 100 mm
Column Temp:	60 °C, Still Air mode
Flow Rate:	0.6 mL/min
Injection Vol.:	1 µL
Mobile Phase A:	0.1% trifluoroacetic acid (TFA) in deionized water
Mobile Phase B:	0.1% TFA in acetonitrile
Gradient: Time, %B:	–5, 10; 0, 10; 10, 40; 12, 90; 15, 90

Sample Preparation

Insulin calibration standards (Alfa Aesar 67626) were prepared by dilution from a stock of 1 mg/mL in deionized water with 10% acetonitrile and 0.1% TFA. Insulin for PEGylation reactions was dissolved in 50 mM borate buffer pH 8.5 at a concentration of 4 mg/mL. Human IgG (Sigma I4506) was prepared by dissolving approximately 10 mg in HPLC-grade water to yield a 1 mg/mL solution.

PEGylation Reactions

PEGylation was performed per the MS(PEG)8 (Thermo Scientific™ Pierce™ 22509) instructions. Insulin was reacted with 200 mM MS(PEG)8 at 1, 3, 6 and 15 molar equivalents in either 50 mM borate buffer or 50:50 (v/v) borate buffer: DMSO. IgG was reacted with MS(PEG)8 at 5, 10, 20 and 40 molar equivalents. Following the labeling reaction, the reaction mixtures were quenched with 1 M Tris-HCl. The mixtures were divided and one portion was desalted by ion-exchange. Both portions were analyzed by UHPLC-UV-CAD.

Insulin has two polypeptide chains, each with an amino-terminal end. In addition, the B chain has a reactive lysine, thus there are three potential sites for modification by the amine-reactive MS(PEG)8 (Figures 2 and 3).

Figure 2. Insulin showing three sites available for reaction with MS(PEG)8[2].

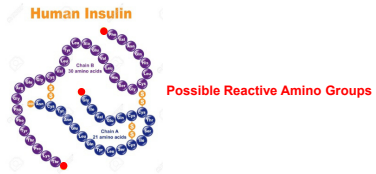
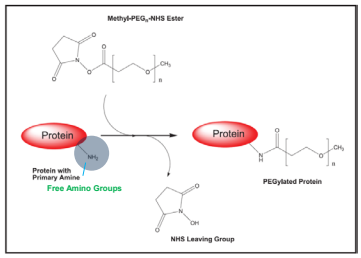


Figure 3. Reaction of MS(PEG)8 with the primary amine of a peptide or protein.



RESULTS

Monitoring Peptide PEGylation

Under the conditions used for the labeling reaction insulin has three free amino groups available for reacting with the MS(PEG)8 reagent, so there are seven possible PEGylated insulin products: one tri-PEGylated, three di-PEGylated, and three mono-PEGylated. Reversed phase UHPLC efficiently separated insulin from all of these PEGylated products. Also well-separated is the quenched reaction byproduct, Tris-MS(PEG)8, observed in the top UHPLC-CAD trace in Figure 4. This is useful because it allows assessment of mass balance throughout the reaction. UV detection alone is unable to provide this information.[3,4] Other reagents and byproducts elute under these conditions near the column void peak.

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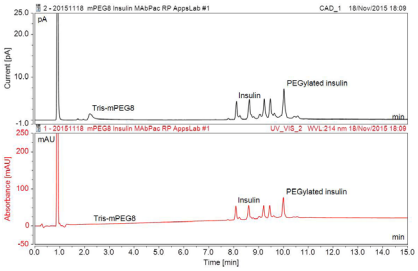
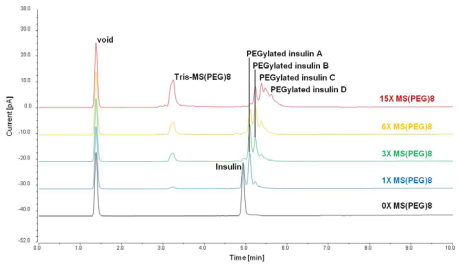
Figure 4. Insulin PEGylation reaction analyzed by UHPLC-UV-CAD on the MAbPac RP column.



Reaction Monitoring

The UHPLC-UV-CAD method was used to monitor a typical experiment used to optimize a PEGylation reaction. By regulating the molar ratio of NHS-PEGylation reagent to target molecule, the extent of labeling can be controlled. Here, insulin was reacted with 3 to 15-fold molar excesses of MS(PEG)8. As can be seen in Figure 5, the use of Increasing excesses of MS(PEG)8 resulted in formation of more and later eluting (i.e., more highly PEGylated) species. Also seen is an increase in Tris-quenched MS(PEG)8, corresponding to the higher starting concentration of PEGylation reagent.

Figure 5. Insulin PEGylation reactions monitored by UHPLC-UV-CAD on the MAbPac RP column.



Method Performance

Method precision, dynamic range and detection limit were evaluated for PEGylation of insulin. Calibration data are shown in Figure 6, (Hubaux-Vos lines in blue), and method performance summarized in Table 1.

Figure 6. CAD calibration data for monitoring insulin PEGylation by UHPLC-UV-CAD on the MAbPac RP column.

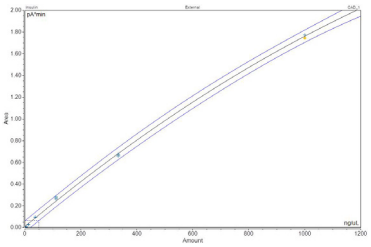


Table 1. CAD method performance for monitoring insulin PEGylation by UHPLC-UV-CAD on the MAbPac RP column.

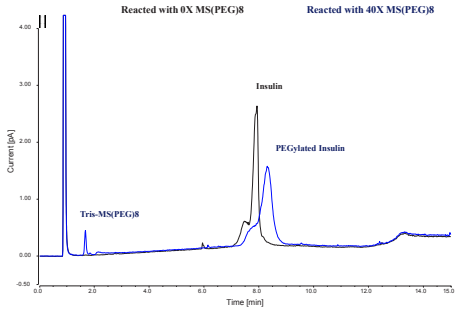
Component	Precision[1]		Calibration[2]			
	Ret. Time (%RSD)	Peak Area (%RSD)	Range (ng/µL)	R^2	RSD (%)	Detection Limit ng/µL
Tris-MS(PEG)8	0.22	0.66				
Insulin	0.06	1.33	1.4 – 1000	0.9932	3.75	49.3
PEG-insulin 1	0.01	2.00				
PEG-insulin 3	0.05	1.96				

1. for n = 3 replicates of a reaction mixture with 3X molar excess of MS(PEG)8
2. Quadratic with Offset, n = 3, Hubaux-Vos method

Monitoring Protein PEGylation

The HPLC method was modified slightly to monitor PEGylation of IgG. The column temperature was 80 °C, the flowrate 0.8 mL/min, the mobile phase contained 0.2% TFA, and the gradient ran from 5 to 60 %B in 15 min. Figure 7 shows a typical chromatogram. Although the native IgG (black trace) and the PEGylated product (blue trace) were not baseline-resolved, probably because the MS(PEG)8 was so small compared to the 150 kDa IgG, the separation of the two was sufficient to assess optimization of the MS(PEG)8:protein ratio, and Tris-MS(PEG)8 eluting at 1.8 min was very well resolved.

Figure 7. IgG PEGylation reaction analyzed by UHPLC-UV-CAD on the MAbPac RP column.



Future Steps

This preliminary work shows that UHPLC with charged aerosol detection is well suited to monitor the reagents, intermediates and products of protein and peptide PEGylation reactions.

Future work will include:

- Applying this UHPLC-CAD method to monitor the stability and forced degradation behavior of PEGylated peptide and proteins.
- Improving the separation of IgG and PEGylated IgG.
- The development of optimized workflows for PEGylation reaction and PEGylated product stability monitoring that are simple and robust enough for routine quality control purposes.
- Further investigations into applying UHPLC-CAD to monitor the reagents, intermediates and products of other protein conjugation reactions such as XTENylation, HESylation, PASylation or conjugation with glycopolymers.[5]

CONCLUSIONS

- PEGylation reagents, byproducts and products can be quantitatively measured by a simple direct method that minimizes sample manipulation and cleanup.
- The starting peptides or proteins and final PEGylated products are separated with high resolution by reversed phase UHPLC using a simple, mass spectrometry compatible mobile phase.
- Complimentary detection is used to obtain maximum information; UV provides information on the peptide backbone and UV-active amino acids, and CAD provides information on the PEGylation reagents, byproducts, and the PEGylated products.
- The UHPLC-UV-CAD method can be applied to optimize reaction conditions, assess the quality of final product, and study drug product stability. The uniform response of charged aerosol detection also provides simple, accurate and precise estimates of relative concentration even in the absence of pure primary standards.

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