Analysis of Polyethylene Glycol (PEG) and a Mono and Di-PEGylated Therapeutic Protein Using HPLC and Q-TOF Mass Spectrometry

Application Note

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Abstract

PEGylated peptides and proteins generate significant challenges for the detailed structural characterization of biotherapeutics, mainly due to polyethylene glycol (PEG) heterogeneity. This application note describes a method for analyzing PEG and PEGylated protein using an Agilent HPLC coupled to an Agilent 6520 Accurate Mass Q-TOF LC/MS System. A charge stripping agent (triethylamine) was used as a post column addition to aid in obtaining a simpler mass spectrum and hence a better interpretation of the results. The results showed significant improvement in the mass spectrum quality for PEG and the PEGylated protein.

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Introduction

Liquid chromatography/mass spectrometry (LC/MS) technology is a powerful and sensitive technique for the characterization of proteins. LC/MS methods are used in both qualitative and quantitative analysis of protein pharmaceuticals for regulatory approval. PEGylation is one of the important covalent modifications used in the biopharmaceutical industry for improving the therapeutic value of proteins.[1] The PEGylation process involves the chemical attachment of polyethylene glycol (PEG) to the therapeutic protein. This modification is known to increase the half-life of the protein drug. The PEGylation reaction leads to product heterogeneity because PEG molecules are themselves heterogeneous. Therefore, it is very important to analyze the PEG reagents and PEGylated protein drugs before product release. In this study, the intact PEGylated therapeutic protein samples and the PEG reagents were analyzed using an Agilent 1260 Infinity LC coupled to an Agilent 6520 Q-TOF Mass Spectrometer. The PEG and PEGylated proteins were separated and eluted from the HPLC, while the charge stripping agent, triethylamine (TEA), was delivered using a syringe pump through a tee junction, as shown in Figure 1.[2,3] TEA was mixed with the PEGylated sample before it went into the mass spectrometer, where it acted as a charge reducing reagent. The addition of amine improved the mass spectrum and was useful for interpreting the results. The results showed that the PEGylated therapeutic protein under study, as well as PEG reagents, is heterogeneous. After PEGylation, the intact protein showed multiple peaks, with each peak differing by approximately 44 Da mass units which is the mass of ethylene glycol.

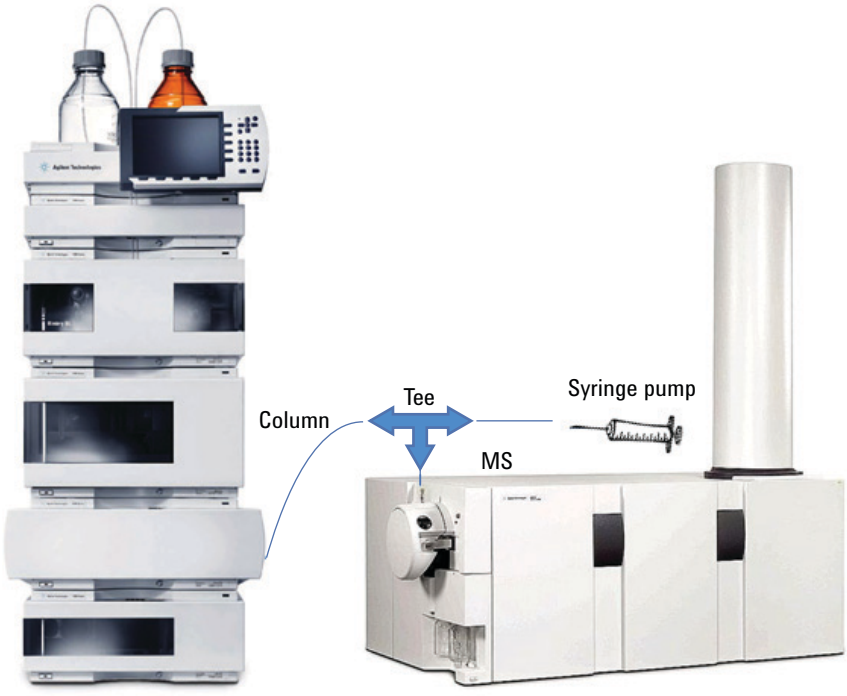

Figure 1. Instrument setup consisting of an Agilent 1260 Infinity LC System and an Agilent 6520 Accurate-Mass Q-TOF Mass Spectrometer. The syringe pump was used to deliver triethylamine solution through a T connector.

Experimental

Materials and methods

The therapeutic protein sample was obtained from GangaGen Biotechnologies Pvt. Ltd. Para-nitrophenylcarbonate (mPEG-PNP, 10 kDa) was obtained from Creative PEG works. Triethyl (TEA) and formic acid was purchased from Sigma-Aldrich. LC/MS grade water and acetonile were used. The therapeutic protein (P128) was PEGylated at a molar ratio of 1:5 using 10 kDa para-nitrophenylcarbonate (mPEG-PNP). Unreacted PEG was removed by applying the reaction mixture to a cation exchange column. The bound mono and di-PEGylated protein was eluted using a linear NaCl gradient.

For this study, the 1260 Infinity LC System was coupled with the 6520 Accurate-Mass Q-TOF Mass Spectrometer. Post column addition of amine solution (1 % TEA in 50:50 acetonitrile/water), using a syringe pump continuously with steady flow rate of 0–10 µL/min during the LC/MS runs, through a tee junction as shown in Figure 1. Table 1 shows the LC and MS parameters used for the run. The data obtained from LC/MS were analyzed using Agilent MassHunter Qualitative Analysis software and Agilent MassHunter BioConfirm software.

Table 1. LC/MS conditions.

LC conditions	
Column	mRP Hi-recovery protein column (p/n 5188-5231), 4.6 mm × 50 mm
Injection volume	1 µL for PEG, 2 µL for di-PEGylated protein, 5 µL for mono-PEGylated protein (Needle with wash, flush port active for 5 seconds)
Sample thermostat	5 °C
Mobile phase A	0.1 % formic acid in water
Mobile phase B	90 % acetonitrile in water with 0.1 % formic acid
Gradient	At 0 minutes → 3 % B At 8 minutes → 75 % B At 15 minutes → 100 % B At 16 minutes → 100 % B At 17 minutes → 3 % B
Stop time	25 minutes
Column temperature	60 °C
Flow rate	0.2 mL/min from an Agilent 1260 Infinity Binary Pump (p/n G1312B)
Q-TOF MS conditions	
Ion mode	Positive ion mode, ESI (Profile)
Drying gas temperature	350 °C
Drying gas flow	6 L/min (nitrogen)
Nebulizer	40 psi
Capillary voltage	3,800 V
Fragmentor voltage	300 V for the PEGylated protein and 250 V for the PEG alone.
Skimmer voltage	65 V
Oct RF Vpp	750 V
Acquisition parameters MS mode	Data were acquired on minimum storage (20,000 *m/z*), 1 GHz, MS only mode, mass range 300–10,000 *m/z*

Results and Discussion

LC/MS analysis of PEG with a post column addition of TEA

Figure 2 shows the mass spectra of the main peak of 10 kDa mPEG-PNP obtained by LC/MS with a post column addition of TEA at different concentrations. When no TEA was added, the PEG was highly charged and all charge state ions were narrowly distributed. It is difficult to obtain the PEG molecular weight distribution from these data by deconvolution (Figure 3A). The deconvoluted spectrum shows no well-defined deconvoluted peaks. Increasing concentration of post-column addition of TEA leads to well distributed charge-states of mPEG-PNP (Figure 2). The charge states of PEG had decreased resulting distinct charge state groups in a wide *m/z* range. Figure 3B shows the deconvoluted mass spectra for 10 kDa mPEG-PNP at 10 µL/min TEA infused. The ethylene glycol unit (44 Da) is clearly detected.

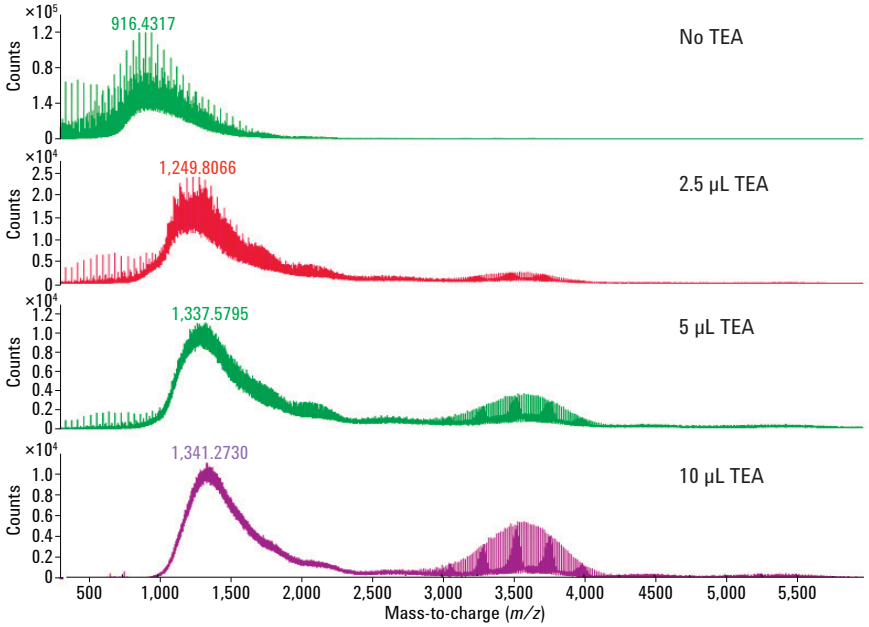

Figure 2. Mass spectrum of 10 kDa mPEG-PNP at different concentration of TEA.

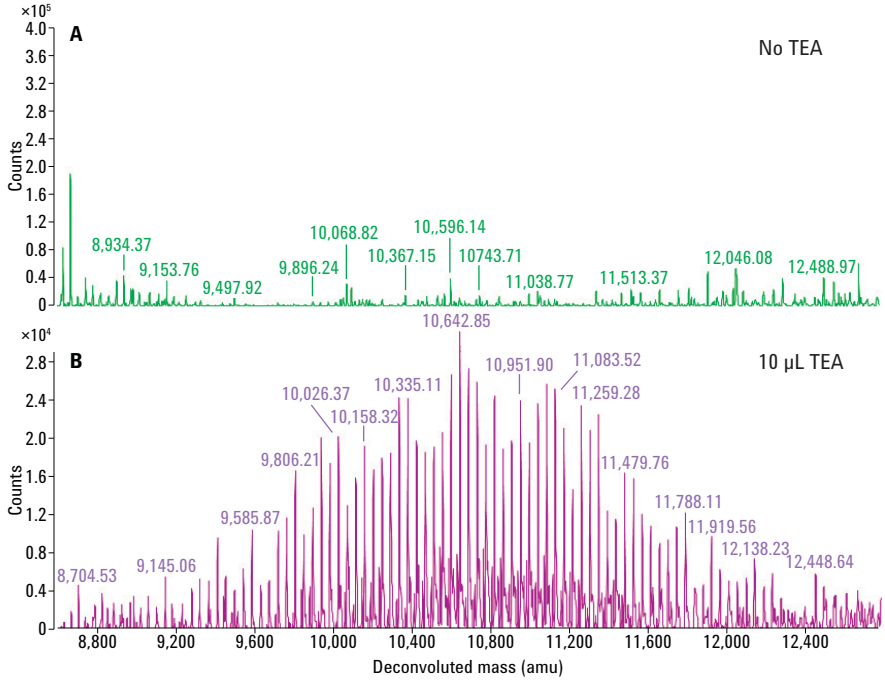

Figure 3. Deconvoluted mass spectrum of 10 kDa mPEG-PNP with no TEA added (A). Deconvoluted mass spectrum of 10 kDa mPEG-PNP, with 10 µL TEA added (B).

LC/MS analysis of a mono-PEGylated protein with a post column addition of TEA

Figure 4 shows the deconvoluted mass spectra of a mono-PEGylated and a di-PEGylated therapeutic protein obtained by LC/MS with a post column addition of 10 µL/min TEA. The intact molecular weight of the native protein after deconvolution was approximately 26,489, which is shown in the inset of Figure 4A. Figure 4A shows a shift in mass from 26 kDa to 36 KDa after this protein was modified by the addition of a 10 kDa PEG molecule. Heterogeneity in PEG masses with different ethylene glycol units (44 Da) is easily seen in the deconvoluted spectrum.

The LC/MS analysis also confirmed the mono-PEGylated status of the protein as no other species was observed except for a small fraction of unmodified native protein (data not shown). Figure 4B shows the deconvoluted mass spectrum of the di-PEGylated form of P128 with a 10 kDa PEG modification. The mass of the di-PEGylated protein is approximately 46 kDa confirming that two 10 kDa PEG molecules are attached to the 26 kDa protein. Here, heterogeneity in PEG masses with different ethylene glycol units (44 Da) is easily seen in the deconvoluted spectrum.

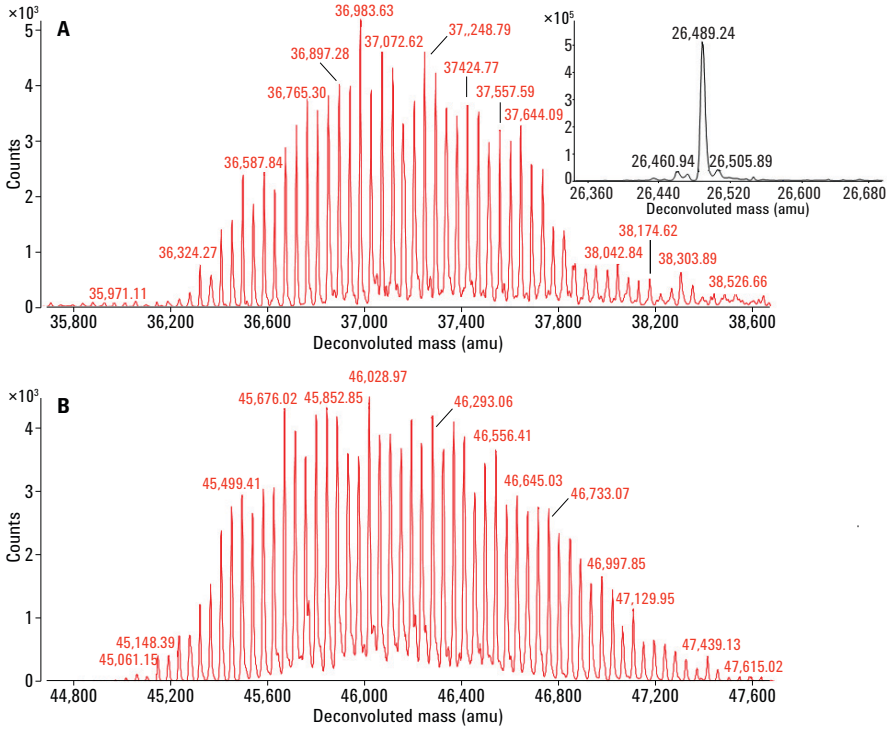

Figure 4. Deconvoluted mass spectrum of 10 kDa mono-PEGylated therapeutic protein, with 10 µL/min TEA addition (A); inset shows the deconvoluted mass spectrum of unmodified protein. Deconvoluted mass spectrum of 10 kDa di-PEGylated therapeutic protein, with 10 µL/min TEA addition (B).

Conclusions

An LC/MS method with post column addition was developed on an Agilent 6520 Q-TOF LC/MS System to analyze PEG and PEGylated proteins. Post column addition of an amine resulted in an improvement in mass spectrum quality and was useful in deconvolution of the complex charge states of PEG and PEGylated proteins. The average mass and the mass distribution of the PEGylated protein was easily determined. The results showed that the PEGylated therapeutic proteins under study, as well as PEG reagents, are heterogeneous. The developed method is useful for studying the PEGylation process during the formulation stages of therapeutic drug development.

References

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