Analysis of PEG and a PEGylated Therapeutic Protein Using an Agilent HPLC-Chip Coupled to a Q-TOF Mass Spectrometer

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 shows the development of a method to analyze PEG and PEGylated protein using an Agilent 1260 Infinity HPLC-Chip coupled to an Agilent 6520 Accurate-Mass Q-TOF LC/MS System. A charge stripping agent (triethylamine) was used as a post-electrospray addition to aid in obtaining a simpler mass spectrum and hence allow for a better interpretation of the results. The results showed improvement in the mass spectrum quality for PEG and the PEGylated protein.

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Introduction

PEGylation is one of the important covalent modifications used in biopharmaceutical industry for improving the therapeutic value of proteins.[3] PEGylation involves the chemical attachment of PEG to the therapeutic protein. This modification is known to increase the half life of the protein drug. However, the PEGylation reaction leads to product heterogeneity because PEG molecules themselves are heterogeneous. Therefore, it is very important to analyze PEG reagents and PEGylated protein drugs before product release. Liquid chromatography/mass spectrometry (LC/MS) technology is a powerful and sensitive technique for the characterization of such complex and heterogeneous products. In this study, the intact PEGylated therapeutic protein samples and PEG reagents were analyzed using a C8 packed microfluidic HPLC-Chip coupled to Q-TOF/MS system. The PEG and PEGylated proteins were separated and eluted from the HPLC-chip, and sprayed through the HPLC-chip nano electrospray tip. A modified version of the ESI-MS based charge stripping approach[1,2] was employed in this study. The charge stripping agent, triethylamine (TEA), was delivered through a calibrant delivery system (CDS) present in the instrument which is generally used to add reference masses (Figure 1). The addition of amine improved the mass spectrum by reducing the charges on the PEGylated protein to yield an interpretable spectrum. The results showed that the PEGylated therapeutic protein under study, as well as PEG reagents, are heterogeneous. After PEGylation, the intact protein showed multiple peaks with each peak differing by approximately 44 Da mass units, which is the mass of the ethylene glycol.

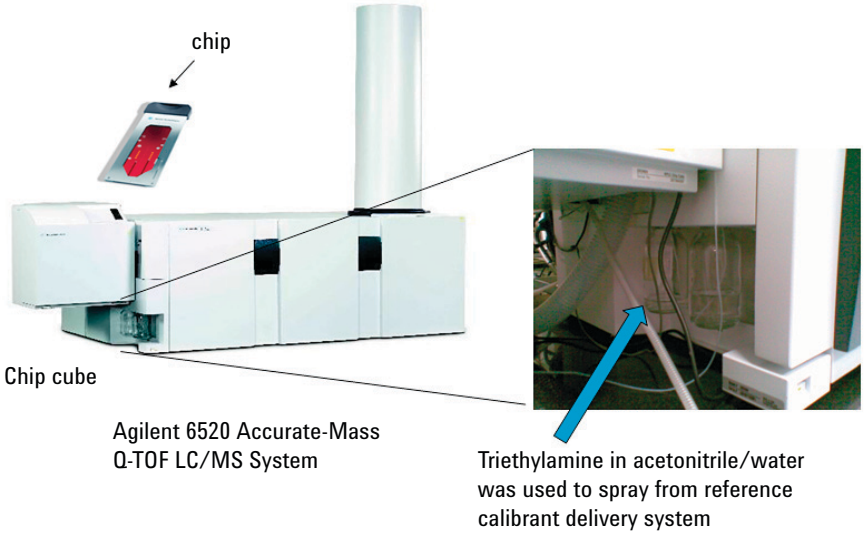

Figure 1. Microfluidic based HPLC-Chip system coupled to an Agilent 6520 Accurate-Mass Q-TOF LC/MS System. The expanded view shows the calibrant delivery system which was used to spray triethylamine solution.

Experimental

Materials and methods

A therapeutic protein sample was obtained from GangaGen Biotechnologies Pvt. Ltd. Para-nitrophenylcarbonate (mPEG-PNP, 10 kDa) was obtained from Creative PEG works. Triethyl amine and formic acid were purchased from Sigma-Aldrich. LC/MS grade water and acetonile were used. The therapeutic protein 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 onto a cation exchange column. The bound mono-PEGylated protein was eluted using a linear NaCl gradient.

For this study, the Agilent HPLC-Chip/MS Interface (p/n G4240A) was coupled to the 6520 Accurate-Mass Q-TOF LC/MS System. The charge reducing reagent was delivered using the calibrant deliver system (CDS) on the instrument as shown in Figure 1. The LC and MS parameters used for the experiment are shown in Table 1.

Table 1. LC/MS conditions.

HPLC-Chip conditions	
HPLC-Chip	5 µm, Agilent ZORBAX 300SB-C8 (300 Å), 40 nL enrichment column and a 75 mm × 43 mm analytical column
Injection volume	1 µL (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
Sample Loading	With Agilent 1260 Infinity Capillary Pump at 3 % B.
Gradient	At 0 minutes → 3 % B At 30 minutes → 50 % B At 32 minutes → 95 % B At 34 minutes → 95 % B At 34.1 minutes → 3 % B
Stop time	36 minutes
Flush volume	4 µL
Flow rate	3 µL/min from an Agilent 1260 Infinity Capillary Pump (p/n G1382A) to the enrichment column and 600 nL/min from the Agilent 1260 Infinity Nanoflow Pump (p/n G2225) to the analytical column.
Q-TOF MS conditions	
Ion mode	Positive ion mode, HPLC-Chip
Drying gas temperature	350 °C
Drying gas flow	5 L/min (nitrogen)
Capillary voltage	1,900 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 high resolution (3,200 *m/z*), 4 GHz, MS only mode, mass range 300–2,200 *m/z*
Data analysis	The data obtained from LC/MS were analyzed using Agilent MassHunter Qualitative Analysis software and Agilent MassHunter BioConfirm software

Results and Discussion

LC/MS analysis of PEG with a post-electrospray addition of TEA

Figure 2 shows the mass spectra of the main peak of 10 kDa mPEG-PNP obtained using LC/MS with post-electrospray addition of TEA at different concentrations. Without the TEA post-electrospray addition, PEG was highly charged and all charge state ions were narrowly distributed. It is very difficult to obtain the PEG molecular weight distribution from these data by deconvolution (Figure 3A). Inspection of the spectrum shows a poorly resolved deconvoluted spectrum without well-defined peaks. Increasing the concentration of the post-electrospray TEA, lead to a well-distributed charge state of mPEG-PNP. The decreased charge states of PEG resulted in distinct charge state groups in a wide *m/z* range.

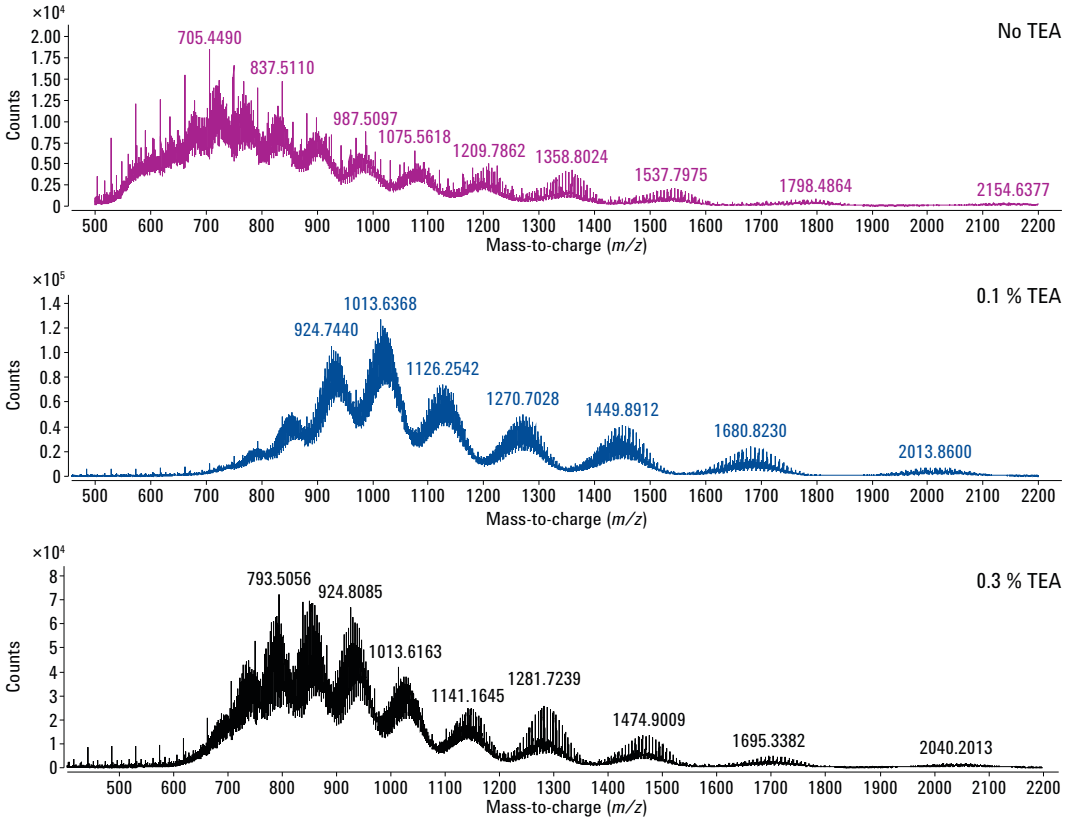

Figure 2. Mass spectrum of 10 kDa mPEG-PNP at different concentration of TEA.

PEG masses with different ethylene glycol (44 Da) could also be easily seen under these conditions. Figure 3B shows the deconvoluted mass spectra for 10 kDa mPEG-PNP at 0.3 % TEA concentration. An expanded view (Figure 3C) of the spectra clearly shows well resolved 44 Da oxyethylene unit of PEG.

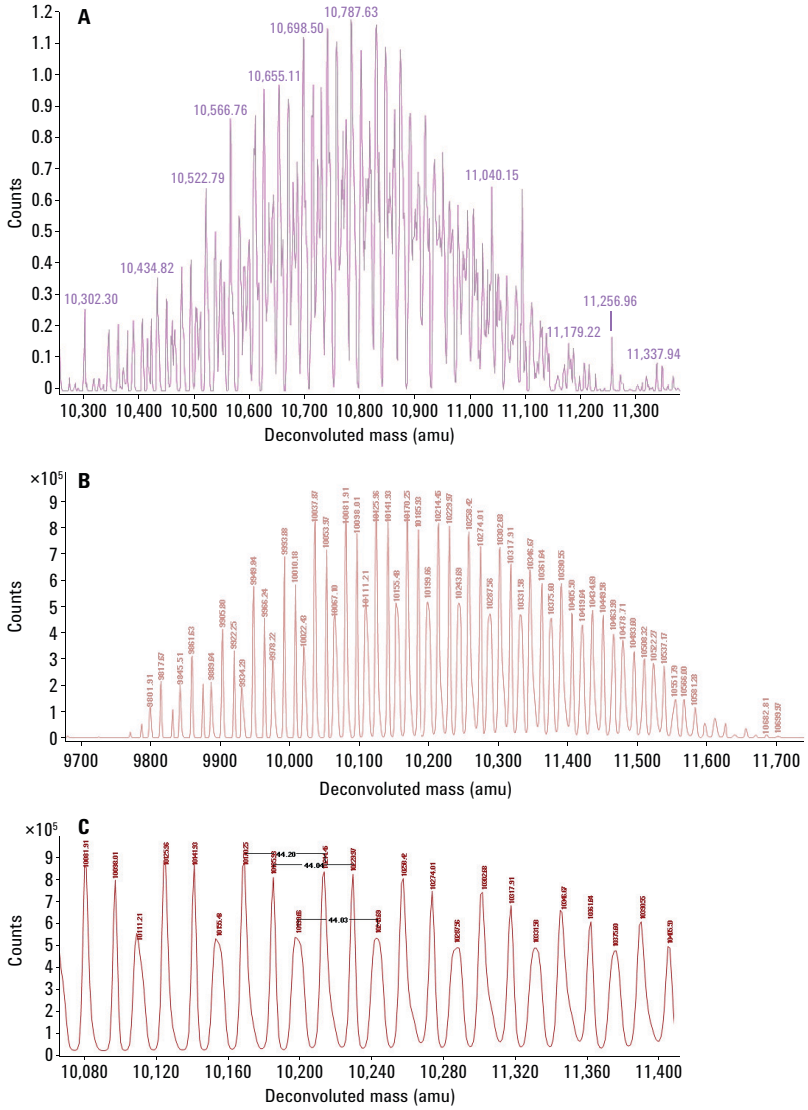

Figure 3. Deconvoluted mass spectrum of 10 kDa mPEG-PNP without TEA (A), deconvoluted mass spectrum of 10 kDa mPEG-PNP with 0.3 % TEA (B), and expanded view of deconvoluted mass spectrum of 10 kDa mPEG-PNP with 0.3 % TEA (C).

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

Figure 4 shows mass spectra of a PEGylated therapeutic protein obtained by LC/MS with a post-electrospray addition of 0.3 % TEA. The intact molecular weight of the native protein after deconvolution is approximately 26,489 as shown in the inset of Figure 4A. Also seen is a small oxidized native protein at around 26,505. After this protein was modified by 10 kDa PEG, a shift in mass from 26 kDa to 36 KDa was observed as depicted in Figure 4B. Heterogeneity in PEG masses with different ethylene glycol units (44 Da) could be easily seen in the expanded view of the deconvoluted spectrum. The small peak is the oxidated protein as noted earlier with native protein. The LC/MS analysis also confirmed the monopegylated status of the protein as no other species was observed.

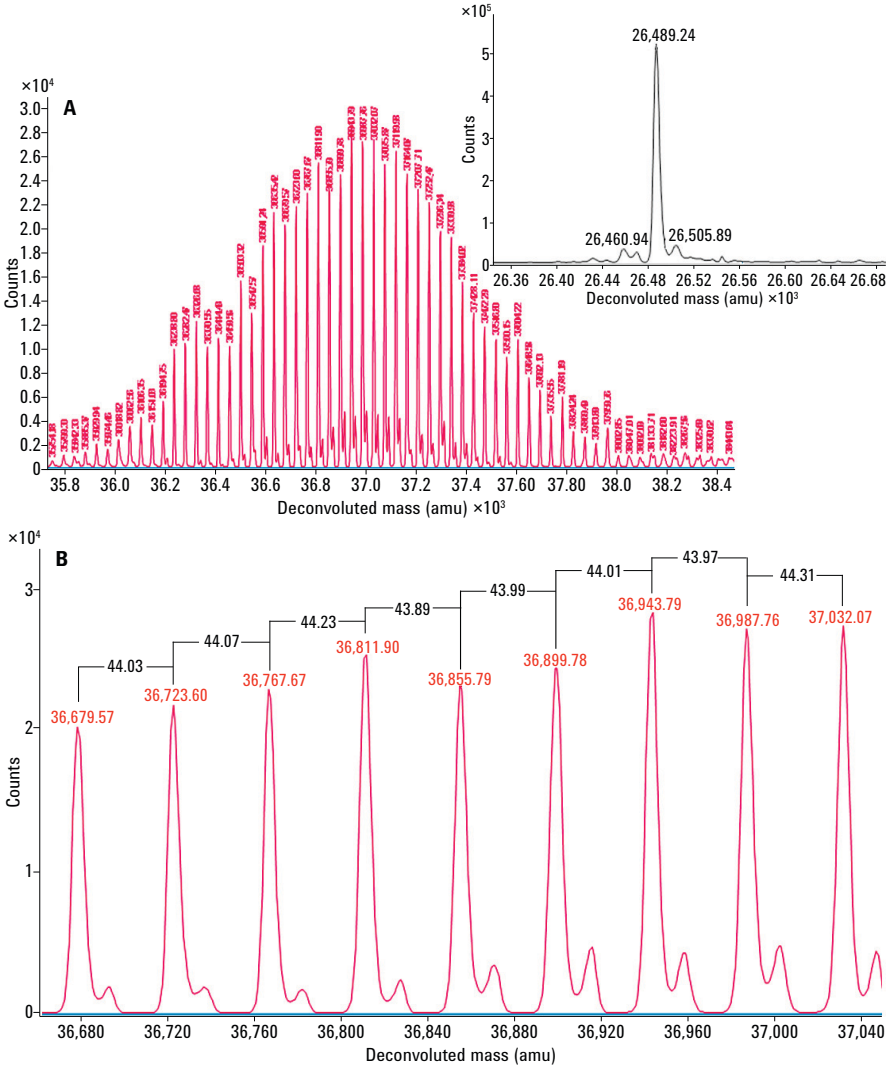

Figure 4. Deconvoluted mass spectrum of 10 kDa PEG modified therapeutic protein with 0.3 % TEA (A); the inset shows the deconvoluted mass spectrum of the unmodified protein. Expanded view of a deconvoluted mass spectrum of a 10 kDa PEG modified therapeutic protein, with 0.3 % TEA, showing a 44 Da mass difference for each ethylene glycol unit (B).

Conclusions

An LC/MS method was developed to analyze PEG and PEGylated proteins. Post-electrospray addition of amine caused an improvement in mass spectrum quality and was useful in deconvolution of the complex charge states of the PEGylated proteins. The results showed that the PEGylated therapeutic protein under study, as well as the PEG reagents, are heterogeneous. The average mass and the mass distribution of the PEGylated protein can easily be determined.

HPLC-Chip offers excellent sensitivity for intact protein analysis. An HPLC-Chip coupled to a high resolution high mass accuracy Q-TOF mass spectrometer with post-electrospray addition of amine is a unique configuration for PEG and PEGylated proteins. This method allows optimization of PEGylation reaction conditions using limited amounts of valuable therapeutic proteins in the early research and development stage of the development process.

References

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