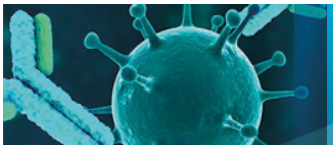

Sensitive Glycoform Profiling of β-Interferon-1a (Avonex) and recombinant human Erythropoietin by CESI-TOF-MS

Sensitive and high resolution glycoform profiling of intact rhEPO and Interferon (rhIFN-β) using CESI-MS with Neutral OptiMS Cartridge

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Introduction

130 protein-based therapeutics are licensed today. With the addition of anticipated new biotherapeutics, the market is forecasted to exceed $140 billion USD in 2017. These therapeutic drugs have changed and improved the quality of life of millions of people around the world in the past few decades. One of the most important quality attributes of a biopharmaceutical drug is its heterogeneity, which includes but is not limited to glycosylation patterns. Glycosylation may vary in degree and type, and is dependent on parameters such as cell line, host organism and cell culture conditions. During production, these variables can cause a broad spectrum of possible glycan species. Therefore the comprehensive analytical characterization of glycosylation heterogeneity is extremely important to ensure safety and efficacy of the biopharmaceutical drug.

Various analytical techniques, i.e., electrophoresis in either gel or capillary format as well as liquid chromatography, are widely used in the structural characterization of glycans. Glycan heterogeneity can be accessed by glycopeptide mapping, as well as released and labeled glycan analysis by capillary electrophoresis (CE) and or liquid chromatography (LC). Even though these are invaluable tools in the analysis of glycosylation, they all involve some level of sample processing by enzymatic digestion. Hence, it is very desirable, to access glycosylation patterns with minimal to no sample manipulation to avoid sample preparation artifacts in the analysis. This leads to intact protein analysis, which will require not only a high resolution separation technique (i.e. CE and or LC) but also a suitable detection system.

In the recent years, mass spectrometry has gained terrain as the detection mode of choice due to its soft ionization mode (ESI) for sample introduction and mass-selective detection with high resolution and high mass accuracy.

In this technical note, the integration of capillary electrophoresis (CE) and electrospray ionization (ESI) into one process (CESI) coupled to a time-of-flight mass spectrometer (TOF-MS), presents sensitive high resolution glycan profiling through reduced ion suppression at nanoliter per minute flow rates of two important biopharmaceutical molecules in intact mode: Recombinant human interferon-β-1a (rhIFN-β, Avonex) and recombinant human erythropoietin (rhEPO).[1]

The CESI-TOF-MS data revealed much improved results over traditional sheath liquid CE-MS by enabling the identification of 18 glycoform species, as well as deamidation, succinimide and oxidation products for rhIFN-β. The analysis of intact rhEPO using CESI-TOF-MS allowed for the identification of 74 glycoforms in 60 minutes. In addition, oxidation and acetylation products, adding up to more than 250 different isoforms being detected.

Materials and Methods

Chemicals. Acetic acid (99.8%) and ammonium hydroxide, recombinant human interferon-β-1a (rhIFN-β, Avonex 6,000,000 IU, lot no. 060016A) from Biogen Idec (Cambridge, MA) and recombinant human erythropoietin (rhEPO, NeoRecormon 30,000 IU, lot no. H0002H01) from Roche (Mannheim, Germany) were obtained as prefilled syringes. Insulin (from bovine pancreas), carbonic anhydrase II (from bovine erythrocytes), ribonuclease A (from bovine pancreas), and lysozyme (from chicken egg white) were from Sigma-Aldrich (Steinheim, Germany). Protein test mixtures were prepared by diluting protein stock solutions (1 mg/mL) to the appropriate concentration with deionized water.

CE System. CE experiments were carried out using a CESI 8000 High Performance Separation-ESI Module, Sciex (Brea, CA). Unless otherwise stated, the applied separation voltage was 30 kV with a supplemental forward pressure of 0.5 psi. The capillary

temperature was kept at 20°C. The capillary used in this study was the OptiMS Neutral Surface Cartridge (Sciex, Brea, CA). Prior to use, the capillary was rinsed with deionized water for 30 min at 50 psi and stored overnight filled with water. After use, the capillary was rinsed for 10 min with deionized water and air at 50 psi, respectively, and subsequently stored at +4°C. Before each run, the capillary was flushed with fresh BGE for 3 min at 50 psi. BGEs of acetic acid were prepared by diluting the appropriate amount glacial acetic acid to 30 mL with deionized water and adjusting the pH with 0.1 M ammonium hydroxide. The sample was injected for 10 s at 5 psi equaling 1% of the capillary volume.

Mass Spectrometry. CESI−MS experiments were performed using a micrOTOF orthogonal-accelerated time-of-flight (TOF) mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a nanospray end plate and gas diverter to allow nanoESI. The mass accuracy was <5 ppm within a single day of measurements, and the resolution was about 20,000 in the range above *m/z* 1250, as was determined experimentally by infusion of the ESI tuning mix (Agilent Technologies, Waldbronn, Germany). Optimized spray conditions for low-flow infusion and CESI−MS experiments were as follows: dry gas temperature, 180°C; dry gas nitrogen flow, 1.2 L/min; nebulizer pressure, 0.0 bar. Electrospray in positive ionization mode was achieved using an ESI voltage of 0.9 kV.

Results

Assessing the analytical performance of the low-flow CESI−MS system for analysis of intact proteins

The neutral coated capillary is very attractive for the analysis of intact proteins because the coating acts as a barrier preventing the interaction with the surface. The neutral coating also minimizes the electroosmotic flow (EOF) to a minimum. The EOF is critical for a stable spray, thus a supplemental pressure of 0.5 psi corresponding to a 5 nL/min was applied along with the voltage during the separations. To evaluate the analytical performance of the neutral coated capillary, a mixture of lysozyme, ribonuclease A, carbonic anhydrase II, and insulin (5µg/mL each) was chosen. This mixture was analyzed using 50 mM acetic acid (pH 3.0) as the BGE.

Under these electrophoretic conditions, the peak shape of all four proteins were narrow, symmetrical and baseline resolved with theoretical plates between 80,000 and 130,000, indicating excellent separation conditions. The neutral coated capillary enabled the increase of separation resolution by a factor of 2 and a migration time window increase by 4 times compared to a positively coated capillary.[2] The quality of the spectra was good which allowed for the correct molecular mass assignment after deconvolution.

Glycoform Profiling of Intact Pharmaceutical Proteins:

Recombinant human interferon-β-1a (rhIFN-β)

rhIFN-β-1a (Avonex) is a disease modifying agent approved by the FDA for the treatment of relapsing forms of multiple sclerosis (MS) and to slow the physical disability suffered by those affected by MS[3] for over 20 years. This molecule is approximately

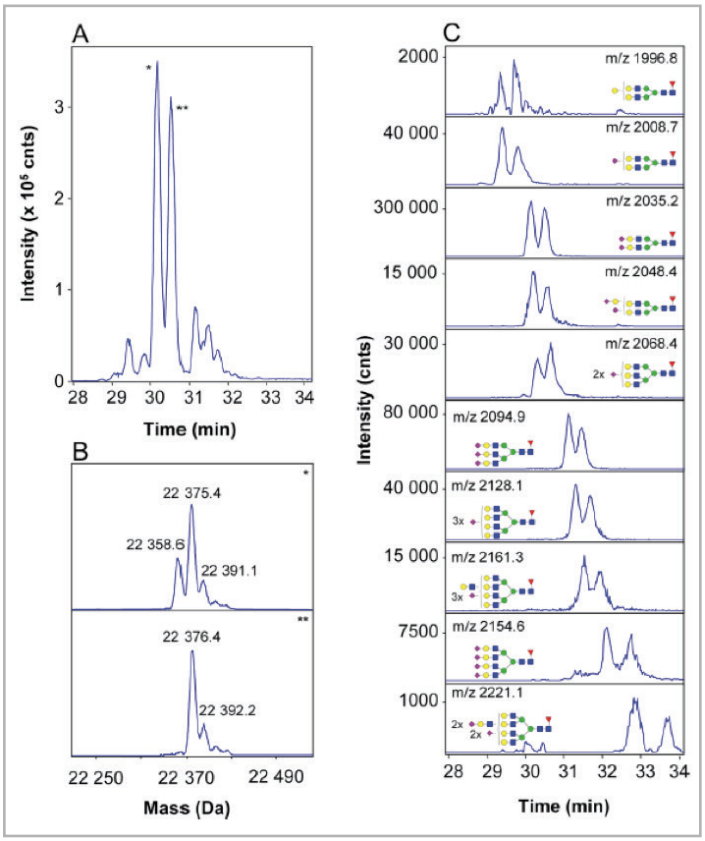

Figure 1. (A) BPE obtained with CESI-MS of rhIFN-β (45 µg/mL) employing a neutrally coated capillary. (B) Deconvoluted mass spectra obtained in the apexes of the peaks migrating at 30.1 min (*) and 30.5 min (**). (C) EIEs for 10 selected rhIFN-β glycoforms including their deamidated products at the indicated m/z value (±0.3 Da; 11+ charge state); assigned glycan structures are shown as insets. Symbols: green circle/yellow circle, hexose (mannose/galactose); red triangle, fucose; blue square, N-acetylhexosamine; purple diamond, sialic acid. Conditions: BGE, 50 mM acetic acid (pH 3.0); CE voltage, 15 kV; tip-to-end plate distance, 1.0 mm. Further conditions: see the Materials and Methods section. From Haselberg et al. Anal Chem, 2013,85,2289-2296, reproduced with permission.*

a 23 kDa glycoprotein with the glycosylation site at the Asn80. As described in the introduction, it is expected that variation in glycosylation patterns of rhIFN-β-1a may affect the biological activity, requiring great scrutiny from quality control of produced batches.[4]

The analysis by CESI-MS of rhIFN-β revealed a unique separation profile consisting of multiple peaks nearly baseline resolved (Figure 1A). The deconvoluted mass spectra were constructed across the peak profile at 12s intervals. The mass with highest intensity (22,375.4 Da) corresponds to the molecular mass of rhIFN-β with a fucosylated disialylated biantennary glycan structure comprising four hexose and five N-acetylhexosamine units. This glycoform has been described as the most abundant present in rhIFN-β[3]. Another peak at 22,358.4 Da was observed and assigned to a succinimide intermediate which is formed during deamidation. The deamidated form with a mass of 22,376.4 Da migrates at 30.5 mi. It is worth noting that the deamidated variant carries a mass increase of only 1 Da, however, it also increases the overall negative charge resulting in an increase in mobility which is why the deamidated form is observed at 30.5 min, 0.4 min after the parent peak. Also observed were minor peaks of +16 Da relative to the main peak which after deconvolution corresponds to a mass of 22,391.1 Da, assigned to an oxidation product. Other interferon standard samples were investigated under these same conditions and no oxidation was found, indicating that the oxidation is likely to be present in the sample and not an artifact of ESI.

From deconvoluted spectra 18 different glycoform masses were extracted and are listed in Table 1. Deamidation, oxidation and succinimide products of these glycoforms were also observed, giving an overall estimate of 80 different isoforms of rhINF-β detected in one run. Fig. 1C shows 10 of the glycoforms observed with corresponding deamidated forms. The resolution of glycoforms was mainly due to the differences in sialylation and also to lesser extent in number of HexHexNAc residues. It is worth noting that previous results from direct infusion ESI-MS,[1] only 6 glycoforms were observed. Additionally with sheath-liquid CE-MS only 10 glycoforms were observed using a sample 10 times more concentrated.[5] From the EIEs peak areas for all glycoforms could be derived (Table 1). Based on these peak areas and the injected amount, glycoform concentrations were estimated to be between 0.5 and 1000 nM, demonstrating the excellent sensitivity and dynamic range of the system.

This work clearly demonstrated that a much improved glycan coverage of intact rhINF-β can be achieved with the combination of high resolution glycan separation only obtained with a neutral coated surface and high sensitivity of the nano-flow regime offered by CESI-MS and high resolution mass spectrometry.

Recombinant Human Erythropoietin (rhEPO)

EPO is a hormone essential to red blood cell production. rhEPO is an approximately 30 kDa glycoprotein where up to 40% of its molecular weight is due to glycosylation.[6] EPO contains three N-glycosylation sites on Asn24, Asn38 and Asn83, and one O-glycosylation site on Ser126. EPO is well known for its high heterogeneity making the glycoform profiling very challenging.

Figure 2A shows the base peak electropherogram of rhEPO obtained using CESI-MS with a neutral coated capillary. The separation profile spans over a 20 min window with a high signal-to-noise ratio. The deconvoluted spectrum in fig 2B corresponds to the peak at 38 min with a main mass of 29,597 Da. Minor peaks corresponding to oxidation and acetylation products were also observed.

The deconvoluted spectra were obtained every 12s across the entire peak profile and the masses of the most intense protein peaks can be found in Table 2. Overall, 74 distinct glycoforms were detected varying in sialic acid (SiA) and hexose N-acetyl-hexosamine (HexHexNAc) residues. Almost all glycans were detected with corresponding oxidized and acetylated variants, which combined resulted in more than 250 isoforms detected.[7]

Taking into account the glycans observed, the contour map from fig. 2C reveals the powerful glycan separation obtained. Take the glycoforms with charge state of +14, as an example (clear highlighted area). In this figure, it is clear that the difference of one sialic acid, results in a shift of 2 min in migration time (Fig. 2C2). Now, if one considers the group of sialoforms, the neutral HexHexNAc residue, contributes to a different of 0.5 min, even though these species are not charged (Fig. 2C3).

Table 2 displays the glycoforms detected and their relative intensity, assuming equal detection response. The relative intensity spams over more than 3 orders of magnitude between the most abundant $SiA_{13}Hex_{22}HexNAc_{13}Fuc_3$ to the least abundant $SiA_{14}Hex_{32}HexNAc_{29}Fuc_3$. Applying the relative intensities of the glycoforms detected to the injected sample amount, a glycosylation concentration can be estimated ranging from 0.35 to 630 nM, once again illustrating the superior sensitivity of CESI-MS. No non-glycosylated species were found during these analyses.

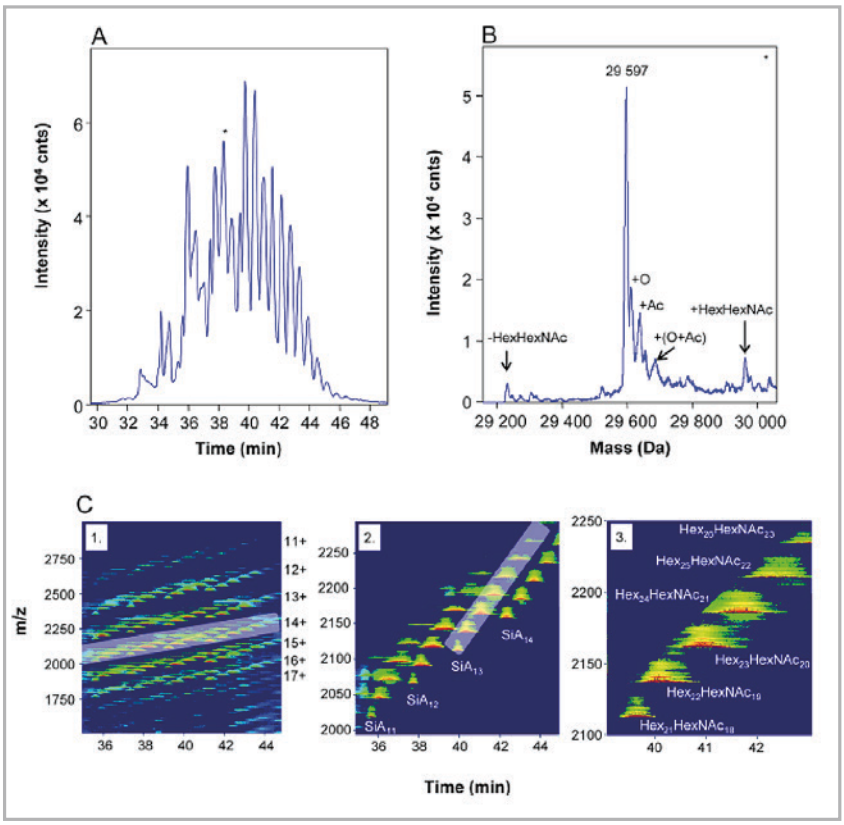

Figure 2. CESI-MS of rhEPO (200 µg/mL) employing a neutrally coated capillary. (A) BPE; (B) deconvoluted mass spectrum obtained in the apex of the peak migrating at 38.0 min; (C1) contour plot with zooms of (C2) the 14+ charge state of the glycoforms and (C3) the SiA13 sialoforms of the 14+ glycoforms. Conditions: CE voltage, 30 kV; BGE, 2 M acetic acid (pH 2.1); Further conditions: see Figure 1. From Haselberg et al. Anal Chem, 2013,85,2289-2296, reproduced with permission.*

Time (min)	M_w (Da)	Glycan
28.4	21 793	
28.5	21 955	
29.3	22 084	
29.3	22 246	
29.6	22 449	
30.1	22 375	
30.2	22 537	
30.4	22 740	2x
30.5	23 105	2x
30.7	22 666	
30.7	23 470	2x
31.1	23 031	
31.3	23 396	3x
31.5	23 761	3x
31.9	24 126	2x
32.1	23 687	
32.4	24 052	3x
32.8	24 417	2x 2x

Table 1. Symbols: green circle/yellow circle, hexose (mannose/galactose); red triangle, fucose; blue square, N-acetylhexosamine; purple diamond, sialic acid. From Haselberg et al. Anal Chem, 2013,85,2289-2296, reproduced with permission.*

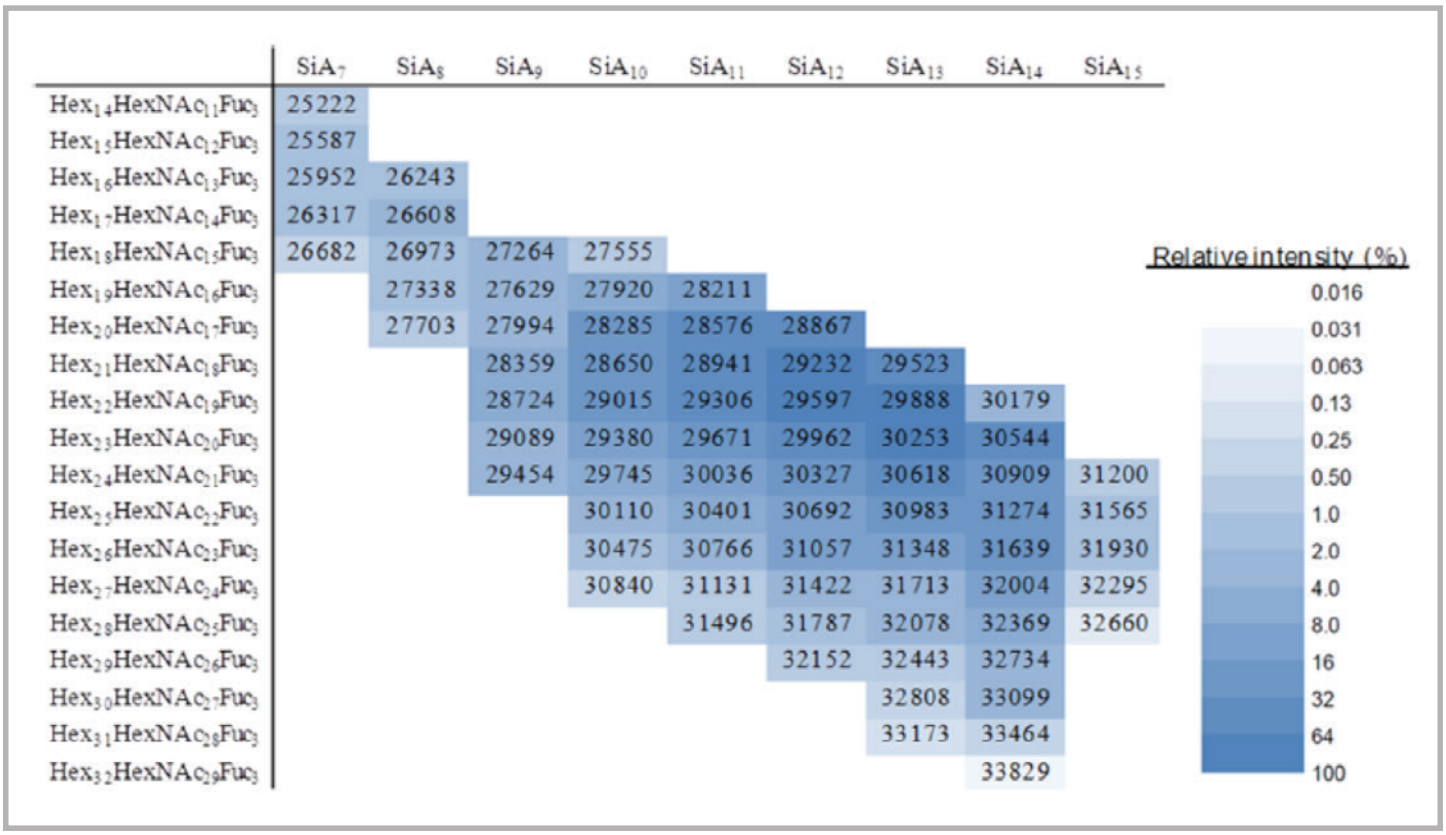

Table 2. Glycan Composition and Molecular Mass of the rhEPO Glycoforms Observed with CESI-MS Employing a Neutrally-Coated Capillary. The relative intensity of the different glycoforms is indicated by the color intensity (log scale). From Haselberg et al. Anal Chem, 2013,85, 2289-2296, reproduced with permission.*

Conclusions

In this Technical Note, the glycosylation profiling of two intact proteins of therapeutic interest was demonstrated thanks to the combination of 3 powerful analytical tools, the high resolution separation accomplished thanks to a neutral coated capillary combined with the high sensitivity provided by the nano-flow regime of CESI-MS, and high resolution mass spectrometry.

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