# Sensitive Glycoform Profiling of Interferon-beta-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 the Neutral OptiMS cartridge***

Rob Haselberg,[1] Gerhardus J. de Jong,[2] and Govert W. Somsen[1]

*1 Division of BioAnalytical Chemistry, VU University, The Netherlands*

*2 BioMolecular Analysis, Utrecht University, The Netherlands*

## Introduction

Today, 130 protein-based therapeutics are licensed. 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 the safety and efficacy of a 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) 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 or LC), but also a suitable detection system.

In 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 2 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 min. In addition, oxidation and acetylation products were identified, 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 (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 Plus High Performance Separation-ESI Module from 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 cartridge with neutral surface (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 background electrolyte (BGE) for 3 min at 50 psi. BGEs of acetic acid were prepared by diluting the appropriate amount of glacial acetic acid to 30 mL with deionized water and adjusting the pH with 0.1 M ammonium hydroxide (50 mM acetic acid, pH 3.0). 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-acceleration time-of-flight (TOF) mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a nanospray endplate and gas diverter to allow nano electrospray ionization (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.

## Important:

- A separation current above 5 µA might cause permanent damage to the separation capillary.
- Generally, please do not apply >2000V to generate electrospray as it may result in capillary damage.

## 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 keeps 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 µL/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 4 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 increased the migration time window 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)*

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

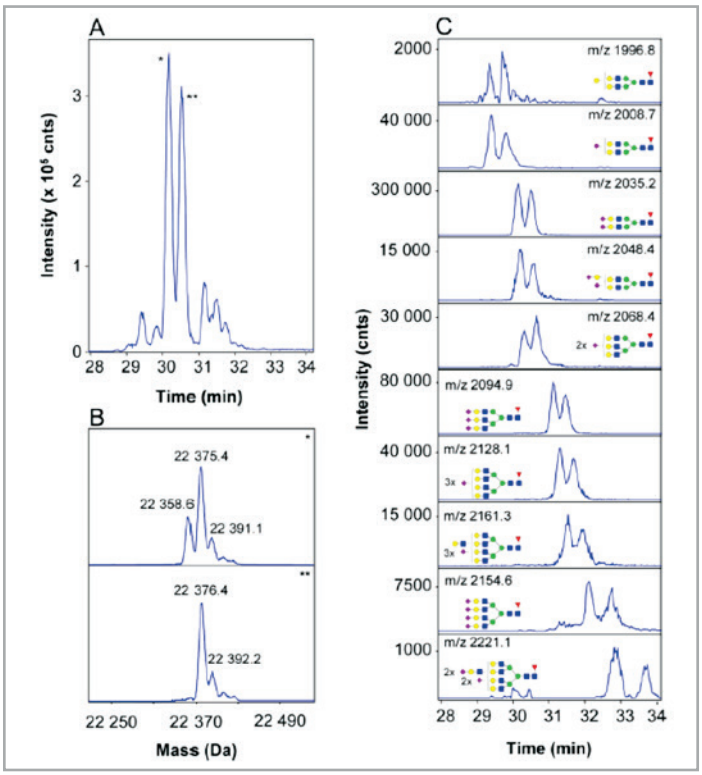



**Figure 1.** (A) BPE obtained with CESI-MS of rhIFN-β (45 µg/mL) employing a neutrally coated capillary. (B) Deconvoluted mass spectra obtained in the apexes of the peaks migrating at 30.1 min (*) and 30.5 min (**). (C) EIEs for 10 selected rhIFN-β glycoforms, including their deamidated products at 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-endplate 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.*

approximately 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 12 s 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 4 hexose and 5 N-acetylhexosamine units. This glycoform has been described as the most abundant present in rhIFN-β3. 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 while the deamidated variant carries a mass increase of only 1 Da, 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 a single run. Figure 1C shows 10 of the glycoforms observed with corresponding deamidated forms. The resolution of glycoforms was mainly due to the differences in sialylation and to a lesser extent in number of HexHexNAc residues. It is worth noting that in 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 extracted-ion electropherograms (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 the high sensitivity of the nanoflow 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 3 N-glycosylation sites on Asn24, Asn38 and Asn83, and 1 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 (BPE) 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 Figure 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 12 s 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 when combined, resulted in more than 250 isoforms detected.[7]

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

Table 2 displays the glycoforms detected and their relative intensity, assuming equal detection response. The relative intensity spans more than 3 orders of magnitude between the most abundant $SiA_{13}Hex2_2HexNAc_{13}Fuc_3$ to the least abundant $SiA_{14}Hex3_2HexNAc_{29}Fuc_3$. 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.

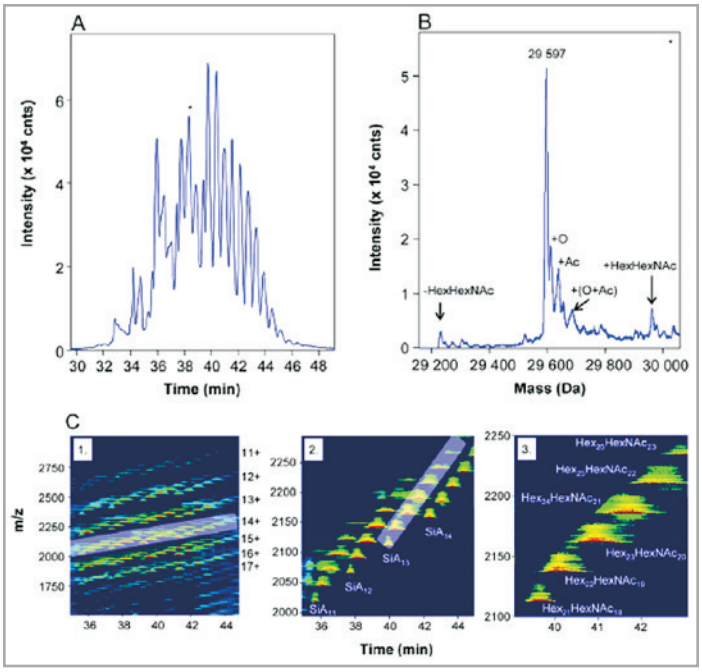
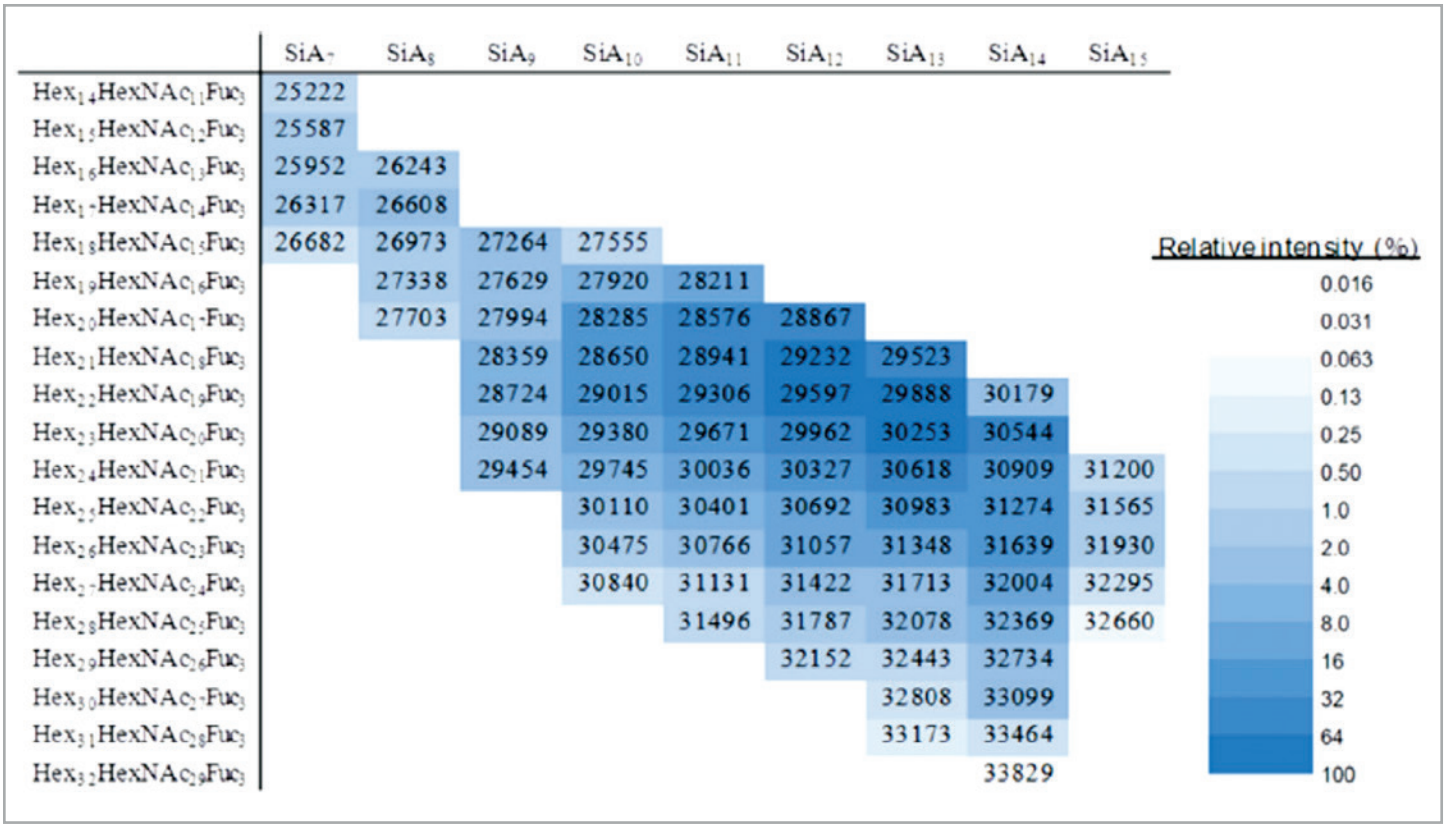



**Figure 2.** CESI-MS of rhEPO (200 µg/mL) employing a neutrally coated capillary. (A) BPE; (B) deconvoluted mass spectrum obtained in the apex of the peak migrating at 38.0 min; (C1) contour plot with zooms of (C2) the 14+ charge state of the glycoforms and (C3) the SiA13 sialoforms of the 14+ glycoforms. Conditions: CE voltage, 30 kV; BGE, 2 M acetic acid (pH 2.1). For 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.*



| | $SiA_7$ | $SiA_8$ | $SiA_9$ | $SiA_{10}$ | $SiA_{11}$ | $SiA_{12}$ | $SiA_{13}$ | $SiA_{14}$ | $SiA_{15}$ |
|---|---|---|---|---|---|---|---|---|---|
| $Hex_{14}HexNAc_{11}Fuc_3$ | 25222 | | | | | | | | |
| $Hex_{15}HexNAc_{12}Fuc_3$ | 25587 | | | | | | | | |
| $Hex_{16}HexNAc_{13}Fuc_3$ | 25952 | 26243 | | | | | | | |
| $Hex_{17}HexNAc_{14}Fuc_3$ | 26317 | 26608 | | | | | | | |
| $Hex_{18}HexNAc_{15}Fuc_3$ | 26682 | 26973 | 27264 | 27555 | | | | | |
| $Hex_{19}HexNAc_{16}Fuc_3$ | | 27338 | 27629 | 27920 | 28211 | | | | |
| $Hex_{20}HexNAc_{17}Fuc_3$ | | 27703 | 27994 | 28285 | 28576 | 28867 | | | |
| $Hex_{21}HexNAc_{18}Fuc_3$ | | | 28359 | 28650 | 28941 | 29232 | 29523 | | |
| $Hex_{22}HexNAc_{19}Fuc_3$ | | | 28724 | 29015 | 29306 | 29597 | 29888 | 30179 | |
| $Hex_{23}HexNAc_{20}Fuc_3$ | | | 29089 | 29380 | 29671 | 29962 | 30253 | 30544 | |
| $Hex_{24}HexNAc_{21}Fuc_3$ | | | 29454 | 29745 | 30036 | 30327 | 30618 | 30909 | 31200 |
| $Hex_{25}HexNAc_{22}Fuc_3$ | | | | 30110 | 30401 | 30692 | 30983 | 31274 | 31565 |
| $Hex_{26}HexNAc_{23}Fuc_3$ | | | | 30475 | 30766 | 31057 | 31348 | 31639 | 31930 |
| $Hex_{27}HexNAc_{24}Fuc_3$ | | | | 30840 | 31131 | 31422 | 31713 | 32004 | 32295 |
| $Hex_{28}HexNAc_{25}Fuc_3$ | | | | | 31496 | 31787 | 32078 | 32369 | 32660 |
| $Hex_{29}HexNAc_{26}Fuc_3$ | | | | | | 32152 | 32443 | 32734 | |
| $Hex_{30}HexNAc_{27}Fuc_3$ | | | | | | | 32808 | 33099 | |
| $Hex_{31}HexNAc_{28}Fuc_3$ | | | | | | | 33173 | 33464 | |
| $Hex_{32}HexNAc_{29}Fuc_3$ | | | | | | | | 33829 | |



**Table 1.** 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 2 intact proteins of therapeutic interest was demonstrated thanks to the combination of 3 powerful analytical tools. The high-resolution separation was accomplished thanks to a neutral coated capillary combined with the high sensitivity provided by the nanoflow regime of CESI-MS and high-resolution mass spectrometry.

## References

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