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Simple Capillary Electrophoresis–Mass Spectrometry Method for Complex Glycan Analysis Using a Flow-Through Microvial Interface

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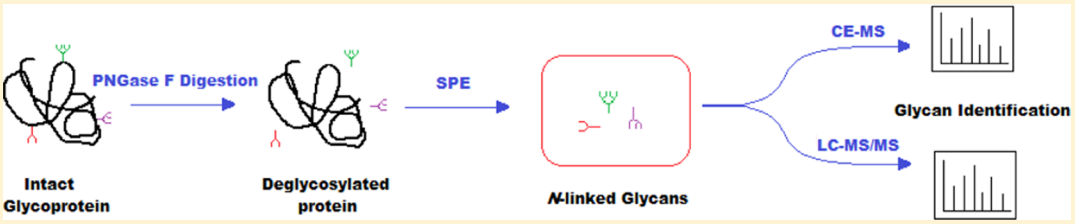
Supporting Information



ABSTRACT: A flow-through microvial is used to interface capillary electrophoresis and mass spectrometry (CE-MS) to develop a method for simultaneous profiling both neutral and sialylated glycans without derivatization or labeling. The CE separation was performed at near-zero electroosmotic flow in a capillary with neutral, hydrophilic coating, using 50 mM ammonium acetate in 20% methanol (pH 3.1) as the background electrolyte. The method was optimized with reversed CE polarity and negative ion ESI-MS. Enzymatically released *N*-glycans from human immunoglobulin G (IgG) were used as the test sample. The approach was also used to study the more complex *N*-glycans from recombinant human erythropoietin (rHuEPO) expressed in Chinese hamster ovary (CHO) cells. Glycoscreening of rHuEPO was performed using a triple quadrupole MS and an ultrahigh resolution TOF-MS. The high sensitivity and high mass accuracy of the TOF-MS revealed the presence of more than 70 glycans. Three mono- and di-sialylated tetra-antennary *N*-glycans and one mono-sialylated tri-antennary *N*-glycan of rHuEPO are reported for the first time. Further glycan heterogeneity was identified of the highly sialylated *N*-glycans of rHuEPO by extensive acetylation, Neu5Ac/Neu5Gc variation and the presence of *N*-acetyl-lactosamine repeats. For comparative purposes, porous graphitic carbon-based LC-MS/MS was also used to glycoprofile rHuEPO. This work demonstrates the potential of CE-MS to provide a comprehensive glycosylation profile with detailed features of the secondary glycan modifications. The CE-MS based method eliminates the need to label the *N*-glycans, as well as the requirement to desialylate before analysis, and could complement other established techniques for glycan characterization of therapeutic glycoproteins.

Characterizing glycosylation of proteins is still one of the most challenging tasks for bioanalytical chemists. The non-template control of the biosynthetic pathway of glycosylation is the basis for the great complexity of glycans.[1] Extensive microheterogeneity often occurs at the glycosylation site of proteins, leading to a large glycoform population. Because protein glycosylation plays a key role in a wide range of biological processes, aberrant protein glycosylation in mammalian systems is often associated with pathogenesis.[2]

Given the potential of recombinant glycoproteins as therapeutic drugs and diagnostic reagents, the analysis of protein glycosylation has attracted much interest in recent years.[3−5] Glycotyping, a comprehensive and detailed characterization of the various glycoforms present in a glycoprotein, has been used to detect subtle yet biologically relevant differences in glycan composition of different batches of glycoprotein pharmaceuticals.[6,7] Such differences can affect their biological activities and influence drug safety, efficacy, and stability.[8] The common practice to determine the glycan composition of recombinant or plasma-purified protein therapeutics involves the enzymatic release of the carbohydrate moiety with endoglycosidases (peptide N-glycosidase F or A), followed by the analysis and detection of either labeled or underivatized glycans.[3,9,10]

Various chromatographic methods have been used for the analysis of enzymatically released glycans. Hydrophilic-inter-

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action chromatography (HILIC),[4,11] porous graphitized carbon (PGC) LC-MS,[5,12] and reversed-phase liquid chromatography (RP-LC),[13,14] are routinely used for identifying glycan components in complex mixtures. However, since glycans are typically hydrophilic, they are not well retained by RP-LC, and common alternatives, such as HILIC and PGC, that better retain hydrophilic glycans, often result in longer retention times versus RP-LC. Additionally, these processes often require time-consuming derivatization processes with fluorophores that can introduce bias in the detection sensitivity of the glycans because of the purity of the labeling reagents or the derivatization steps.[15,16] Over the past few years, online coupling of capillary electrophoresis (CE) to *time-of-flight* mass spectrometry (TOF-MS) detection has emerged as an attractive and complementary technique for characterization of biomolecules.[17−20] Because CE-TOF-MS combines high separation efficiencies with high mass resolution and mass accuracy, it may be a powerful method for glycan analysis. CE-MS of carbohydrates has been previously done with coaxial sheath-liquid[21,22] and sheathless interfaces.[23] Although sheath-liquid CE-MS interfaces are relatively stable and robust, the required high volumetric flow of sheath-liquid contributes to considerable analyte dilution in comparison to sheathless CE-MS interfaces. The use of sheathless CE-MS interfaces often face difficulties in fabrication and operation. Unlabeled carbohydrate analysis using sheathless interfaces have been reported to be extremely slow (∼120 min) for routine use when CE in reverse polarity was performed.[23]

We have previously described a novel flow-through microvial interface in which a chemical modifier solution mixes with the CE effluent and delivers the analyte from the capillary terminus to the source.[24] The major advantages of this interface are that (i) the modifier solution is delivered at low flow rates, typically 100−500 nL/min to minimize analyte dilution, and (ii) the stainless steel electrospray emitter holds the separation capillary inside, making the operation very robust. The beveled shape of the electrospray emitter stabilizes the ESI spray in a wide range of flow rates.[25] Successful applications of this low flow sheath-liquid interface have been reported for the analysis of fluorescently labeled carbohydrates,[26] separation of isomeric *O*-acetylated *N*-glycans in fish serum,[27] monitoring prostate cancer biomarkers,[28] sensitive metabolic profiling studies,[29] and for the coupling of capillary isoelectric focusing (cIEF) to MS.[30] In this article, we describe a relatively simple approach including enzymatic digestion, SPE purification, CE separation, and MS detection of underivatized neutral and acidic *N*-glycans from immunoglobulin G (IgG) and recombinant human erythropoietin (rHuEPO). Unlabeled glycans were analyzed to preserve their native structural characteristics and to avoid time-consuming derivatization procedures. Glycosylation of rHuEPO was also studied using online liquid chromatography−mass spectrometry (LC-MS) to evaluate its ability, in comparison with CE-MS, to detect underivatized glycans along with their secondary modifications.

EXPERIMENTAL SECTION

Materials. See Supporting Information.

Glycoprotein Deglycosylation with PNGase F. *N*-glycans from purified rHuEPO (see Supporting Information) and IgG from human serum were released by enzymatic digestion with PNGase F. rHuEPO (50 μg) or 100 μg of human IgG were adjusted to a final volume of 50 μL with 10 mM Tris-HCl at pH 8.0. Samples denaturation was achieved by adding 2.5 μL of denaturation solution, containing 0.1% sodium dodecyl sulfate and 50 mM β-mercaptoethanol, and heating at 100 °C for 5 min. Samples were then treated with 2.5 μL of detergent solution containing 0.75% NP-40, followed by 2 μL of PNGase F (1000 mU). The solutions were incubated overnight at 37 °C.

Electrophoretic Procedure. CE-MS analyses of SPE-purified *N*-glycans (see Supporting Information) were performed using hydrophilic hydroxypropyl cellulose-coated capillaries (HPC-capillaries) prepared in-house according to the method of Shen and Smith.[31] Capillaries were of 70 cm length and 50 μm id. Separations were carried out in reverse-polarity mode at −25 kV with an overimposed pressure of 10 mbar (0.145 psi) to reduce analysis time for neutral glycans. Background electrolytes (BGE) were composed of 30−100 mM ammonium acetate (pH 3.0−4.0) with 20% methanol. The samples were injected at 1.0 psi for 10 s, corresponding to a volume of 19 nL.

CE-ESI-MS System. CE-ESI-MS analyses of *N*-glycans were carried out with a PA800 plus capillary electrophoresis system (Beckman Coulter, Brea, CA) coupled to an API 4000 triple-quadrupole mass spectrometer (AB SCIEX, Concord, Canada). A modified capillary cartridge that permits external detection was used for the analysis. The CE-MS interface with a flow-through microvial was developed in our laboratory and has been described previously.[24,26−30,32] The electrospray voltage was set at −3.5 kV. Data acquisition and system control were performed using the Analyst 1.4.2. software (AB SCIEX, Framingham, MA, U.S.A.). The modifier solution was delivered by a syringe pump (Harvard Apparatus, Holliston, MA, U.S.A.) at a flow rate of 0.3 μL/min and was composed of 10 mM ammonium acetate (pH 3.1) containing 75% of 2-propanol and methanol (2:1 ratio). The same interface setup and solutions were used for CE-ESI-MS with an HP ^{3D}CE (Agilent Technologies, Waldbronn, Germany) coupled to a maXis ultrahigh resolution TOF mass spectrometer (Bruker Daltonik, Bremen, Germany). The mass spectrometer was operated in negative ionization mode and acquired data in full scan mode with a mass range from m/z 500−3000 at a spectra rate of 1 Hz. Optimization of the transfer parameters and MS calibration were performed with TuneMix (Agilent Technologies) in order to obtain the best sensitivity at satisfactory resolution ($R \approx 20\,000$ at m/z 1334). Instrument control and data analysis were performed using ESI Compass 1.3 application software from Bruker (Bruker Daltonik, Bremen, Germany). The conditions for modifier solution and its delivery rate are the same as the ones when the API 4000 MS was used.

RESULTS AND DISCUSSION

Analysis of *N*-Glycans from Human IgG. Complex bi-antennary, core-fucosylated and partially galactosylated, and sialylated oligosaccharides are typically found at the single *N*-glycosylation site of human IgG, located in the constant region of the heavy chain.[33] The variable presence of bisecting GlcNAc and α-2,3- or α-2,6-linked sialic acids on terminal galactose residues contribute to the glycan heterogeneity.[34] Despite human IgGs are characterized by relatively simple glycosylation profiles, changes in their glycosylation are associated with immune responses and with the activity of recombinantly expressed monoclonal antibodies (mAbs) for therapeutic purposes.[34,35]

Regarding the clinical role of mAbs, it is crucial an in-depth characterization of their glycosylation for activity, safety and quality control during development and production.[36] Because

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of the utmost importance of glycosylation in human IgG for its biological and clinical functions, here we present a simple and efficient method for analyzing its *N*-glycosylation profile by CE-MS. The method will be further used to explore the *N*-glycosylation of rHuEPO.

Based on our experience in analyzing underivatized *N*-glycans from fish serum,[27] we used neutral coated capillaries and acidic background electrolytes (BGEs) to optimize the CE-MS conditions for underivatized *N*-glycans from human IgG (Supporting Information). Baseline separation between neutral and sialylated *N*-glycans was achieved (Figure 1) and glycans

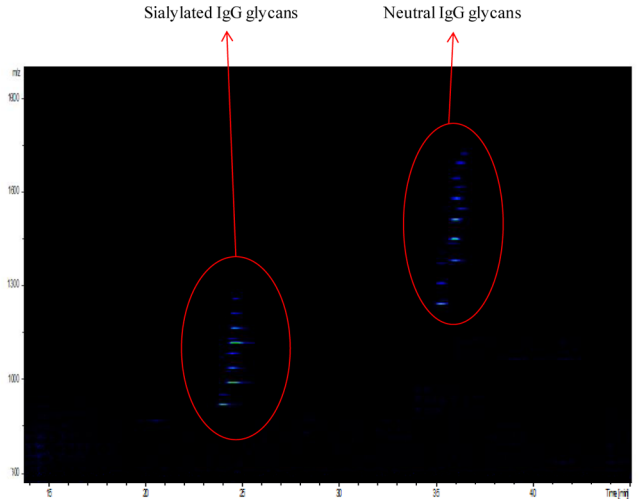

Figure 1. Density map for the CE-ESI-MS separation of neutral and acidic *N*-glycans from human IgG. Conditions: Neutral (HPC)-coated capillary 70 cm × 50 μm; BGE, 50 mM ammonium acetate/20% methanol (pH 3.1); separation voltage, −25 kV + 10 mbar; negative ion ESI-MS; modifier solution, 10 mM ammonium acetate (pH 3.1) containing 75% of 2-propanol: methanol (2:1). The intensity of the color reflects the glycan abundance with light green being less abundant that dark blue.

were detected as $[M - H]^-$ and $[M - 2H]^{2-}$ ion species. Sialylated glycans migrated faster than neutral glycans because of their electrophoretic mobility enhanced by the acidic BGE in reversed polarity mode.[23] Among sialylated species, separation occurred based on the number of sialic acid residues and the hydrodynamic volume of the glycans. The overimposed pressure of 10 mbar (0.145 psi) and the interaction of neutral glycans with acetate ions allowed the glycans to migrate toward the anode. Although baseline separation in the time dimension was not achieved for all the glycan species, the orthogonal baseline MS resolution allows direct analysis of underivatized neutral and acidic glycans in a single run, providing a detailed glycoprofile of the complex glycoprotein. The presented methodology also eliminates the need to label the glycans, as well as the need to remove sialic acid residues prior to their analysis as it has been commonly reported for acidic glycans of different sources.[37,38]

As outlined in Table S.I. (Supporting Information), 22 glycans of IgG were identified. Putative glycan monosaccharide compositions were assigned based on accurate precursor mass data and previous reports on *N*-glycosylation analysis of human IgG.[39−41] The number of glycans observed in IgG contrasts with the large heterogeneity found in other glycoproteins of the immune system.[42] For instance, a single GPI-anchored glycoprotein, CD59, has been reported to contain over 120 glycoforms.[42]

CE-MS Analysis of *N*-Glycans from rHuEPO. Previous CE-MS studies on *N*-glycosylation of rHuEPO were performed using bare-fused silica capillaries in basic background electrolyte (BGE) conditions.[10,43] To enhance mobility differences of glycoforms and to avoid compromising spray stability or signal intensity that may occur in basic BGE, our CE-MS method employs neutral hydrophilic (HPC)-coated capillaries under acidic BGE conditions. *N*-glycan population from rHuEPO is mainly composed of core-fucosylated tetra-antennary glycans containing variable numbers of sialic acids. Tri-antennary *N*-glycans containing up to 3 sialic acid residues and di-antennary *N*-glycans containing up to 2 sialic acid residues are also present but with lower abundance than tetra-antennary structures.[6,8,44] Because *N*-glycans from rHuEPO are highly sialylated, they remain negatively charged over a broad pH range and can be easily CE separated and detected in negative ion ESI-MS without derivatization or glycan labeling. Analyzing underivatized glycans is desirable not only to simplify the sample preparation but also to preserve their native structural characteristics. The most common derivatization strategy for CE analysis of *N*-glycans is reductive amination,[45] in which the combination of acidic conditions and high temperatures present a considerable risk for loss of sialic acids, which could contribute to an increased heterogeneity.[46] Removal of the excess of labeling reagent can also be problematic and labor-intensive because of the similar properties of the labeling and the derivatized glycans. Therefore, the analysis of underivatized glycans is preferred to reduce the risk of bias because of sample handling and the potential loss of sialic acids.

Figure 2 shows the base peak electropherogram (BPE) of the 17 most abundant *N*-glycans from rHuEPO detected with a

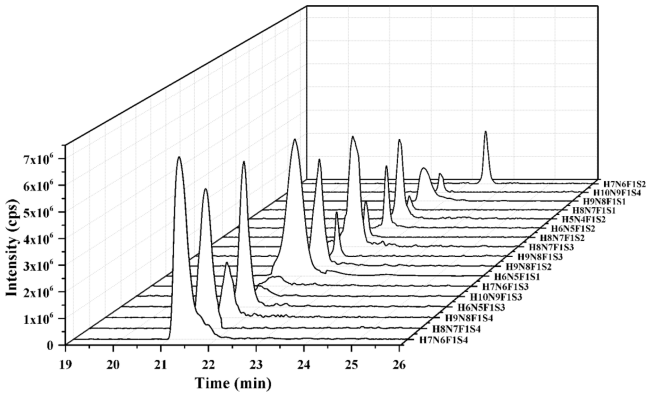

Figure 2. EIE for the CE-ESI-MS separation of *N*-glycans from rHuEPO. Only the 17 most abundant *N*-glycans are displayed. The glycans were detected under the same CE-MS conditions as in Figure 1. The monosaccharide composition of the glycans is indicated in the graph. Nomenclature is as follows: H = mannose or galactose (Hex), N = N-acetylglucosamine (HexNAc), F = fucose, and S = sialic acid (Neu5Ac). Data was acquired using the triple quadrupole MS.

triple quadrupole MS. Separation of *N*-glycans is achieved based on different electrophoretic mobilities influenced by the number of sialic acids residues, *N*-acetyl-lactosamine (LacNAc) repeat units, and the hydrodynamic volume of the glycans. Glycans with an increased number of sialic acids display a stronger mobility toward the anode and appear earlier in the electropherogram. As expected, heavier glycans containing the

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Table 1. Identification and Annotation of the 17 Most Abundant *N*-Glycan Monosaccharide Compositions of rHuEPO Shown in Figure 2[a]

	composition						observed m/z		
glycan	H	N	F	S	theor. mass (Da)	obsd mass (Da)	$[M-2H]^{2-}$	$[M-3H]^{3-}$	$[M-4H]^{4-}$
H7N6F1S4	7	6	1	4	3683.33	3683.21		1226.7	919.7
H8N7F1S4	8	7	1	4	4048.67	4048.62		1348.5	1011.1
H9N8F1S4	9	8	1	4	4415.07	4415.10		1470.6	1102.7
H6N5F1S3	6	5	1	3	3026.74	3026.76	1512.3	1007.8	
H10N9F1S3	10	9	1	3	4490.10	4489.95		1495.6	
H7N6F1S3	7	6	1	3	3393.10	3392.97	1695.4	1129.9	
H6N5F1S1	6	5	1	1	2444.22	2444.23	1221.0		
H9N8F1S2	9	8	1	2	3833.51	3833.47		1276.8	
H9N8F1S3	9	8	1	3	4124.80	4124.69		1373.8	
H8N7F1S3	8	7	1	3	3758.20	3757.90		1251.6	
H8N7F1S2	8	7	1	2	3467.49	3467.60		1154.8	
H6N5F1S2	6	5	1	2	2736.50	2736.46	1367.1		
H5N4F1S2	5	4	1	2	2371.17	2371.30	1184.6		
H8N7F1S1	8	7	1	1	3171.85	3171.79		1056.2	
H9N8F1S1	9	8	1	1	3539.24	3539.40	1768.6	1178.7	
H10N9F1S4	10	9	1	4	4781.42	4781.37		1592.7	
H7N6F1S2	7	6	1	2	3101.84	3101.77	1549.8	1032.8	

[a]Glycans were observed as $[M-2H]^{2-}$, $[M-3H]^{3-}$, or $[M-4H]^{4-}$ ion species. Data was obtained using the triple quadrupole MS. Nomenclature of the glycan monosaccharide composition as indicated in Figure 2.

same number of negative charges have a reduced mobility and are detected later in the electropherogram. Smaller species containing variable amounts of sialic acids migrate faster than larger species according to the LacNAc extension of the chain. Although the detected glycans are not fully time-resolved, sialylated glycans displaying the same charge but containing variable number of hexoses (Hex) and/or LacNAc units (161.05 or 364.1 Da, respectively) can be profiled and distinguished in the MS, showing the excellent separation power of the technique to resolve structurally similar *N*-glycans. Some abundant glycans, such as tetra-antennary glycans with 3−4 sialic acid residues are detected as broad peaks because of the co-migration of their acetylated and/or Neu5Ac/Neu5Gc variations and because of the diffusion process that can occur under negligible EOF conditions. Band broadening properties in CE-MS have been recently discussed in a publication from our research group.[32]

Putative monosaccharide compositions, observed masses and m/z values for glycans shown in Figure 2 are summarized in Table 1. The precursor mass data obtained from the triple quadrupole MS were used to search for matching compounds in the Consortium of Functional Glycomics (CFG) for *N*-linked glycans. In agreement with other reports, all released glycans of rHuEPO contain a fucosylated core with variable degrees of sialylation.[47−49] Using the developed method, we detected the same population of glycans from rHuEPO expressed by CHO cells previously reported.[10,47,49−51] We also identified additional species that were not reported before: two tetra-antennary glycans (mono- and di-sialylated) and one mono-sialylated tri-antennary glycan, with the monosaccharide compositions H8N7F1S1, H9N8F1S2, and H6N5F1S1, respectively. Glycans with acetylation and/or Neu5Ac/Neu5Gc variations with putative compositions H8N7F1S1 and H9N8F1S2 were also observed (Table S.II, Supporting Information). Assignment of glycan monosaccharide composition could be based on the observed precursor mass alone because of the restricted combinations of monosaccharide residues of *N*-glycans.[52] For each glycan monosaccharide composition there are several possible isomeric structures and different types of glycosidic linkages that cannot be identified by single stage CE-MS analysis. Differences in electrophoretic mobilities, resulting in differential migration time for glycans, can be useful to predict the structure of certain glycans. For instance, the monosaccharide composition of glycan H8N7F1S3 (Table 1) matches both with the structure of a tri-antennary glycan with two LacNAc units and with the structure of a tetra-antennary glycan with one LacNAc unit. The longer migration time of glycan H8N7F1S3 in comparison to that of glycan H7N6F1S3, a tetra-antennary species,[10] suggests that glycan H8N7F1S3 may be a tetra-antennary species with one LacNAc repeat. The increased volume of glycan H8N7F1S3 in comparison to glycan H7N6F1S3, reduces its electrophoretic mobility and appears later in the electropherogram.

To accurately determine the mass of the glycans, our flow-through microvial interface was connected to a maXis ultra high-resolution TOF MS, which offers a wider mass range, higher resolution and higher mass accuracy than the triple quadrupole MS. In addition to the glycans detected before, a mono-sialylated tetra-antennary glycan with three LacNAc units (monosaccharide composition H10N9F1S1) that has not been previously reported by CE-MS was detected with the TOF MS. In total, more than 70 *N*-glycans were observed of which several could be identified, as shown in Supporting Information Table S.II. A complete list of the glycans observed for BRP rHuEPO is shown in Supporting Information Table S.II, including monosaccharide composition, theoretical mass, observed mass, and m/z values. Glycans were identified based on their true isotopic pattern provided by the ESI Compass 1.3 application software from the maXis TOF MS in combination with the information provided by the Consortium of Functional Glycomics (CFG) for *N*-linked glycans. The high mass accuracy, in the low ppm range (<1 ppm) and the resolving power, of nearly 20 000 at m/z 1334 (Agilent ESI low concentration tuning mix) of the TOF MS was also useful to

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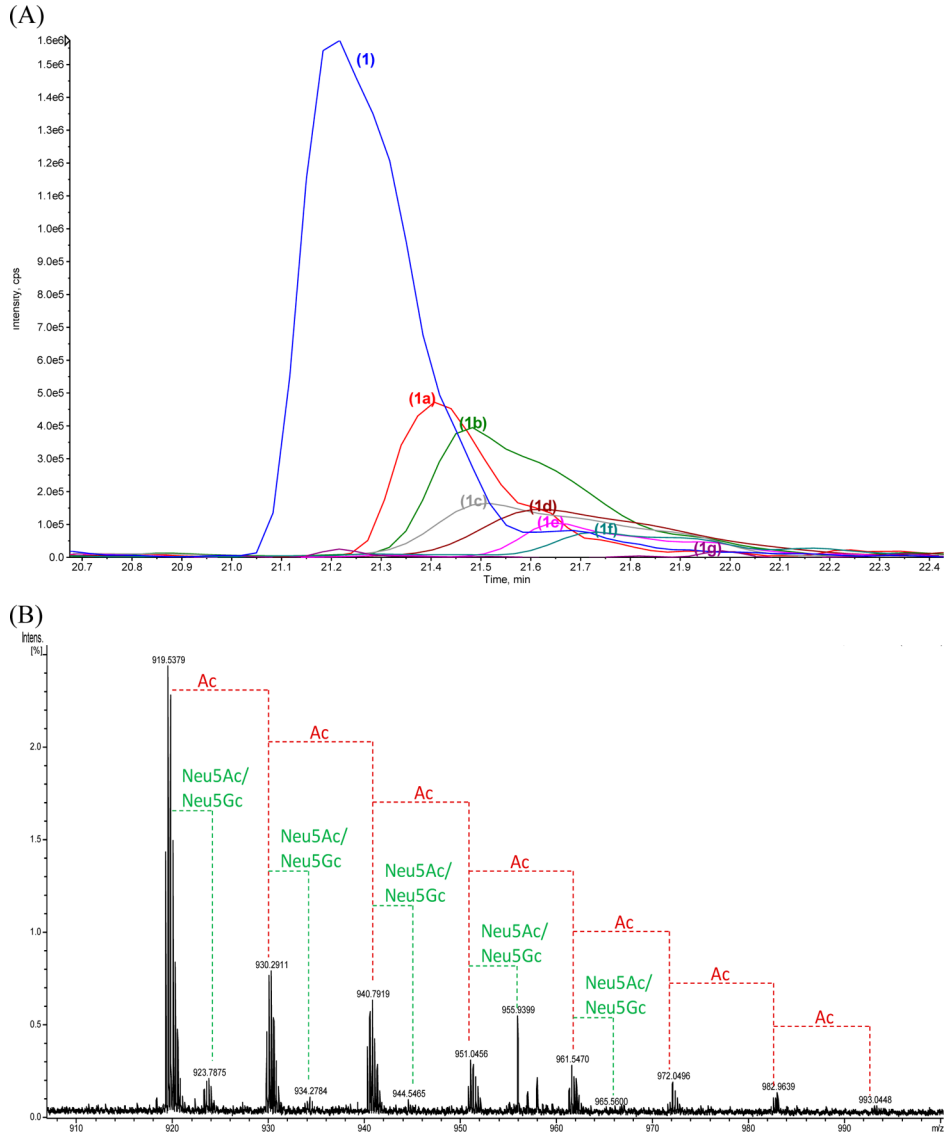

Figure 3. (A) EIE obtained for the CE-MS separation of the tetra-antennary tetra-sialic glycan H7N6F1S4 (peak 1) and its acetylated species containing from 1 to 7 *O*-acetyl groups (peaks 1a to 1g, respectively). (B) Average MS spectrum obtained for panel A where it can be observed the *m*/*z* values of the *O*-acetylated and Neu5Ac/Neu5Gc variations species of H7N6F1S4. The glycans were detected under the same CE-MS conditions as in Figure 1

confirm glycan modifications previously observed with the triple quadrupole MS.

Detection of Glycan Modifications in rHuEPO. In agreement with other reports, our CE-MS method revealed the presence of three types of secondary glycan modifications in enzymatically released *N*-glycans from rHuEPO.[4,8,10,43,44] These modifications were mainly observed in highly sialylated glycans and include acetylation of sialic acids, N-acetylneuraminic (Neu5Ac) / N-glycolylneuraminic acid (Neu5Gc) variation, and elongation of the glycan chains because of the presence of LacNAc repeats. Up to three modifications occurred per glycan.

Acetylation of *N*-glycans was detected due to mass shifts of 42 Da from the naked glycan masses. The presence of a set of peaks differing in 10.5 *m*/*z* (Th) for glycans observed as $[M - 4H]^{4-}$ ion species or 14 *m*/*z* (Th) for glycans observed as $[M - 3H]^{3-}$ ion species, accounts for the acetylation of glycans. For instance, the presence of a set of seven peaks additional to the most abundant ion at *m*/*z* 919.53 for the tetra-sialylated glycan H7N6F1S4, indicates the occurrence of 7 acetylated forms that were resolved in the mass dimension (Figure 3A and 3B). Acetylated species were predominantly detected as $[M - 4H]^{4-}$ ion species, while $[M - 3H]^{3-}$ ions species were less abundant. Higher acetylated forms of glycan H7N6F1S4 have a reduced electrophoretic mobility compared to less acetylated species and are detected later in the electropherogram. This is illustrated in the extracted ion electropherogram (EIE) (Figure 3A), where the migration order of the glycans are in agreement with the number of acetyl groups. Our CE-MS method succeeded in detecting mobility differences due to only 42 Da, corresponding to acetylation, in high molecular mass analytes (2300−4800 Da, approximately) with an optimal MS resolution. Acetylation of other glycans such as the tri-sialylated H7N6F1S3 and the tetra-sialylated with one LacNAc repeat H8N7F1S4, containing 4 and 6 acetylated forms, respectively, were also observed and are discussed in the Supporting Information. All acetylated *N*-glycans observed in rHuEPO are included in Supporting Information Table S.II.

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CE-MS analysis of sialylated glycans shows that a higher degree of acetylation is present in glycans containing a higher degree of sialylation. This indicates that the acetylation occurs at the sialic acid level due to the occurrence of Neu5,9Ac$_2$, as it has been reported before in rHuEPO.[49] Based on the number of sialic acid residues and *O*-acetyl groups observed, it can be predicted that not only mono-*O*-acetylated sialic acid residues are present but also multiple *O*-acetylated forms of sialic acids occurs. For instance, the presence of 5, 6, and 7 *O*-acetyl substituents in tetra- and tri-sialylated glycans implies multiple *O*-acetylations occurring on a single sialic acid residue. This phenomenon has also been reported during the glycoprofile of rHuEPO,[4,10,43] Dynepo,[53] a novel recombinant human EPO, darbepoetin,[48] an hyperglycosylated glycoform and other commercial EPOs produced in CHO cell lines.[8] The degree of acetylation of sialic acid deserves special attention because it is correlated with the circulatory half-life of the glycoproteins in the human serum and its in vivo biological activities.[53,54] Also, variability in the glycosylation pattern can potentially affect the biological, physicochemical and immunological properties of rHuEPO.[55]

rHuEPOs engineered in Chinese hamster ovary (CHO) cells have a distinct set of glycans relative to recombinant EPOs engineered in human fibrosarcoma cell lines and baby hamster kidney (BHK) cells, because of the slightly different glycosylation machineries including the sugar-transferring enzymes.[56,57] Contrary to human cells, CHO cells do not express sialyl-α-2,6-transferase, α-1,3/4-fucosyltransferase and bisecting N-acetylglucosamine transferase.[47] However, these cells contain the enzyme responsible for N-glycolylneuraminic acid synthesis (CMP-N-acetylneuraminic acid hydroxylase [CMAH]) that is not present in humans due to an internal frame shift mutation in the CMAH human gene.[58,59] For that reason, rHuEPO expressed in CHO cells has been reported to contain between 1 and 1.5% of Neu5Gc residues relative to the total sialic acid content, while EPO products engineered by gene-activation in human cells contain no Neu5Gc.[8,57,60]

Our CE-MS method detected the presence of Neu5Gc, instead of Neu5Ac, in some *N*-glycans from rHuEPO. In some cases, this sialic acid variation occurred simultaneously with the acetylation of glycans previously discussed (Figure 3B). The presence of a set of peaks differing in 4 m/z (Th) for glycans observed as $[M - 4H]^{4-}$ ion species, correspond to mass shift of 16 Da from the unmodified glycan masses, and accounts for the Neu5Ac/Neu5Gc variation. For instance, the tetra-sialylated glycan H7N6F1S4 and four of its acetylated forms, containing one to four acetyl groups, displayed lower intense peaks at 4 m/z (Th) higher than the corresponding acetylated glycan. This type of modification was also observed in the tetra-sialylated glycan H8N7F1S4 containing a LacNAc repeat, the tri-sialylated glycan H7N6F1S3, and their corresponding acetylated forms (Supporting Information). Other glycans where the Neu5Ac/Neu5Gc variation occurred are shown in Supporting Information Table S.II, along with their corresponding putative monosaccharide composition. This phenomenon has not been extensively reported and there are only a few studies in the literature dealing with the presence of Neu5Gc in rHuEPO.[8,10,44,53,60] Even though Neu5Gc is a common sialic acid in most mammals, it has been demonstrated to be absent in healthy humans and only small amounts have been found in some tumors and meconium.[61]

The CE-MS method succeeded in detecting this heterogeneity of acidic monosaccharides without removing the sialic acid from the glycan chain by acid hydrolysis as it has been commonly reported.[8,53,62] As reviewed lately, the LC-MS detection of sialic acids and its heterogeneous subtypes is challenging.[63] Mass spectrometric results indicate that the variation occurred at the glycan level as shown in Figure 3B and Supporting Information (Figures S1.B and S1.D).

The third type of glycan modification corresponds to the elongation of the glycan chain due to the presence of LacNAc repeat units. For instance, tetra-antennary glycans, the most abundant oligosaccharides present in rHuEPO, were observed to contain up to three LacNAc extensions. Glycans with monosaccharide compositions H8N7F1S4, H8N7F1S2; H9N8F1S4, H9N8F1S3; H10N9F1S3 and H10N9F1S4 were identified to carry one, two and three units of LacNAc, respectively which are in agreement with other reports in the literature.[10,47,49,51] These extended oligosaccharides represent the most important difference between circulating human EPO and rHuEPO, and they have been reported as a critical structural feature of rHuEPO that should be considered for its biological significance.[64] Recently, Bones et al.[4] have reported an increased number of LacNAc extensions containing up to five units in BRP rHuEPO. In that study, successful detection of tetra-antennary glycans with extensive LacNAc repeats was based on a combination of weak anion exchange chromatography (AEC) and HILIC that allowed for the first time the detection of up to five LacNAc repeats.[4]

LC-MS/MS Analysis of *N*-Glycans from rHuEPO. Underivatized, nonreduced *N*-glycans of rHuEPO were also analyzed using online porous graphitized carbon (PGC) LC-MS/MS to compare its ability to glycoprofile complicated glycan mixtures with that of CE-MS. Table S.III in the Supporting Information shows a list of the glycans detected with PGC LC-MS/MS. A total of 27 glycan monosaccharide compositions including a few acetylated species and three neutral glycans were detected. Glycan heterogeneity such as Neu5Ac/Neu5Gc variations and extensive acetylation of tri- and tetra-sialylated glycans H7N6F1S3, H7N6F1S4, and H8N7F1S4, observed with our CE-MS method, were not detected with PGC LC-MS. The tetra-sialylated glycan containing one LacNAc unit H8N7F1S4 was not detected by PGC LC-MS either.

Successful LC-MS/MS analysis of glycans using PGC often requires oligosaccharide reduction in the presence of mild reducing agents or derivatization with hydrophobic compounds prior to MS detection.[5,65,66] However, those additional steps can eliminate subtle modifications including *O*-acetylation present in glycans (unpublished observation). Therefore, the analysis of underivatized species is the preferred method of choice to preserve the structural characteristics of these complex analytes. Although both chromatographic and electrophoretic separation techniques are suitable for the profiling of glycans, CE is advantageous because of its demonstrated capability to analyze underivatized glycans and offers an alternative and complementary analytical tool to chromatographic based techniques. Our CE methodology has the advantage of keeping the detailed structural information about the glycans, which results in the detection of more species, not only because of the type of MS employed, but also because of the unique characteristics of the technique. Moreover, CE-MS requires short analysis time for the study of intact *N*-glycans, in comparison to LC-MS, which requires longer times for separation and column re-equilibration. Taken

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together, this makes CE a well-suited analytical approach for extensive glycan characterization.

CONCLUSIONS

In this study, we demonstrated the excellent potential of CE-MS for glycan analysis using a flow-through microvial interface. With our CE-MS method, we were able to characterize heterogeneous mixtures of enzymatically released *N*-glycans from human IgG and rHuEPO. Glycans released from IgG were used to optimize the conditions for CE-MS analysis of complex *N*-glycan mixtures. In total, 22 *N*-glycans, including neutral and sialylated species of IgG were identified. The use of hydrophilic HPC-coated capillaries that provide almost zero EOF was necessary to allow successful separation of the *N*-glycan mixtures. Underivatized glycans were used, because the labeling procedure often affects the detection of glycan modifications by inducing, for example, de-*O*-acetylation or complete destruction of chemically unstable substituted sialic acids. Also, it was not necessary to remove sialic acid residues. Sialylated and neutral *N*-glycans from IgG were simultaneously glycoprofiled to provide a general overview of the complex glycan mixture, although it was not possible to achieve high resolution for both neutral and sialylated species.

Application of the optimum CE-MS conditions for the analysis of rHuEPO glycosylation, revealed the presence of more than 70 *N*-glycans, including *O*-acetylation, Neu5Ac/Neu5Gc heterogeneity and extension of the glycan chains due to LacNAc repeats. The CE-MS method made it possible to unravel the complexity of the carbohydrates and showed detailed information about their minor glycan modifications. This revealed the ability of the method to glycoprofile glycans based on the degree of sialylation and the patterns of their substituents. The ultrahigh resolution TOF MS confirmed the presence of modified glycan structures, and putative glycan monosaccharide compositions were determined by using the high resolution and high mass accuracy of the TOF MS. Although, the method has several advantages for the glycosylation characterization of rHuEPO, in terms of the simple instrumentation, small sample consumption, relatively short analysis time and the feasibility of coupling CE with different types of MS instruments, its major limitation is probably the limited concentration sensitivity. However, in the context of therapeutic glycoprotein characterization, highly concentrated pharmaceuticals are commonly available. Therefore, the presented CE-MS method can be regarded as an orthogonal and complementary approach for glycoprofiling protein pharmaceuticals.

In addition, PGC LC-MS/MS confirmed the presence of sialylated glycan structures containing variable degrees of acetylation. However, other glycan modifications were not detected with PGC LC-MS/MS which indicates the potential advantages of CE-MS over LC-MS for unraveling the structural complexity of glycans released from glycoproteins.

Quality control of recombinant therapeutic proteins requires determining the glycosylation profile and the occurrence of secondary glycan modifications that may shed light on additional biological properties that affect both pharmacodynamics and pharmacokinetics. In combination with the high quality of MS data, an improved insight into the glycan heterogeneity of rHuEPO was possible. Our CE-MS analysis can be considered as a promising alternative to assess the glycosylation profile of therapeutic drugs and to evaluate the quality of biosimilar products.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

The authors declare no competing financial interest.

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