High resolution CESI-MS analysis of APTS-labeled N-glycans of biopharmaceutical interest

Bryan Fonslow[1], Boglarka Donczo[2], Marton Szigeti[2], Andras Guttman[1,2]
[1]SCIEX, Brea, CA and [2]Horvath Csaba Laboratory of Bioseparation Sciences, University of Debrecen, Hungary

ABSTRACT

Glycosylation plays an important role in cellular interactions, protein folding, and monoclonal antibody (mAb) stability. For characterization, N-linked glycosylation on mAbs is routinely analyzed by CE-LIF after endoglycosidase based glycan release and fluorophore labeling. CE separation provides structural resolution and migration time-based identification of glycans, but coupling with MS detection offers additional structural information. The integration of CE and ESI into a single dynamic process (CESI) provides the capability of performing CE separation and MS ionization with ultra-low flow rates, resulting in reduced ion suppression and improved sensitivity. CESI-MS has been optimized and evaluated for APTS-labeled mAb and formalin-fixed parafin-embedded glycan analysis.

INTRODUCTION

Since introduced as a biological tissue sample fixative by F. Blum in 1893, formalin fixation has been still an almost exclusively used method for tissue preservation. Fixation using this reagent makes clinically relevant samples stable that can be stored at ambient condition for decades in depositories. Combined with paraffin embedment, formalin fixed tissues become FFPE specimens (formalin fixed paraffin embedded) that are amenable for long-term storage and can be universally used in histopathological laboratories. The clinicopathological significance of formalin-fixed paraffin-embedded (FFPE) archival specimens has led many researchers to utilize and annotate these “unmasked treasures” beyond their main histopathological utilization even at the molecular level. Several clinico-molecular assays for oncological prognosis and treatment decision have been developed based on FFPE tissue analysis. Recently capillary electrophoresis has been introduced as a method to probe the N-glycosylation pattern of these precious samples.[1]

MATERIALS AND METHODS

Sample Preparation: A maltooligosaccharide ladder, IgG1 glycan standards, and released formalin-fixed paraffin-embedded (FFPE) glycans were labeled with APTS and cleaned-up. For deparaffinization, the FFPE samples were washed twice by xylene for 20 min and centrifuged at 5000 × g for 10 min. This was followed by ethanol wash, two times for 20 min and centrifugation at 5000 × g for 10 min. For protein extraction, all samples were first homogenized in a 1 mL Tapered Tissue Grinder (VWR, Budapest, Hungary) and solubilized with 100 µL of RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) [8] containing 10 µL of 50 mM DTT. The solubilization mixture was first incubated at 100° C for 20 min, then 65° C for 60 min. After the solubilization step, 10 µl of 50 mM iodoacetamide was added and incubated at 37° C for 30 min in dark. For buffer exchange, 10 kDa spin filters were used at 5000 × g for 10 min. To release the N-glycans, 49 µL of 20 mM $NaHCO_3$ buffer (pH 7.0) and 1 µL PNGase F Ultra enzyme (2.5 mU, Prozyme, Hayward, CA) were added to the sample on the filters and incubated for 1 h at 50° C. The released N-glycans were centrifuged though the spin filters at 5000 × g for 10 min and dried in a centrifugal vacuum evaporator. 6 µL of 20 mM APTS and 2 µL of 1 M $NaCNBH_3$ (in THF) was added to the dry pellet and incubated at 37° C overnight. The labeled samples were magnetic bead purified (CleanSeq, Beckman Coulter, Indianapolis, IN) and immediately used or stored at –20° C for later analysis.

CESI 8000 Plus Stand-Alone CE Mode Conditions: The separations were monitored by laser induced fluorescence (LIF) detection using a 488 nm solid state laser with a 520 nm emission filter. 50 cm effective (60 cm total) length with 50 µm id NCHO capillaries were used filled with NCHO gel buffer system (both from SCIEX) in reversed polarity mode (cathode at the injection side). The samples were injected by 1 psi pressure for 5 s.

CESI 8000 Plus MS Mode Conditions: CESI experiments were carried out with a SCIEX CESI 8000 Plus system equipped with a temperature controlled autosampler and a power supply with the ability to deliver up to 30 kV. CESI-MS analyses were performed using a SCIEX CESI 8000 Plus system. CESI-MS experiments also employed a TripleTOF® 6600 system. CESI-MS separations were achieved using an OptiMS bare fused-silica capillary cartridge by application of -30 kV and low pressures (2 for 7.5 min and 5 psi for 10 min). Ammonium acetate (7.5 mM, pH 4.5) with 10% isopropanol (IPA) was used as the background electrolyte (BGE). Samples were dissolved in 10% BGE to 10 µL and loaded into nanoVials (P/N 5043467) for ~15 nL pressure injections.

MS/MS Conditions: A SCIEX TripleTOF® 6600 system with a NanoSpray® III source and CESI adapter were used. Negative ESI was performed at -1750 V scanning from 250 - 2000 m/z with 250 ms (MS).

Data Analysis: High resolution MS and MS/MS spectra were analyzed using SCIEX PeakView®. Glucose unit (GU) values were calculated by the GUcal software (http://www.gucal.hu) and preliminary assignment of the glycan structures of interest were aided by the Glycobase ver 3.0 from NIBRT (Dublin, Ireland).

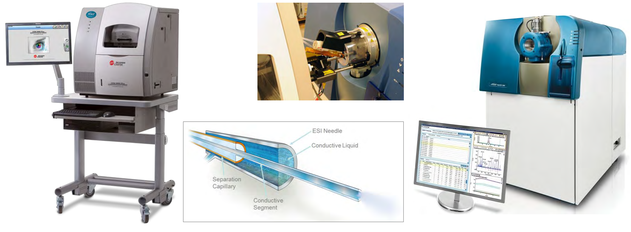

Figure 1. CESI 8000 Plus High Performance Separation-ESI Module coupled to a TripleTOF® 6600 System. The core of the CESI-MS technology is an etched porous capillary tip that allows for electrospray ionization without dilution of the BGE and sample by a make-up liquid. Instead a conductive liquid is used to apply the ESI voltage through the porous tip.

RESULTS

The transition from current CE-LIF APTS-glycan analysis methods to CESI-MS requires the use of MS-compatible buffers. Separations of an APTS-labeled carbohydrate ladder and mAb glycan standards were analyzed to evaluate the general capabilities of CESI-MS for the analysis of APTS-labeled released glycans from proteins of interest. Separation and detection of the carbohydrate ladder and mAb glycan standards illustrated efficient separations (~6 sec peak widths) and sensitive detection in negative ESI mode.

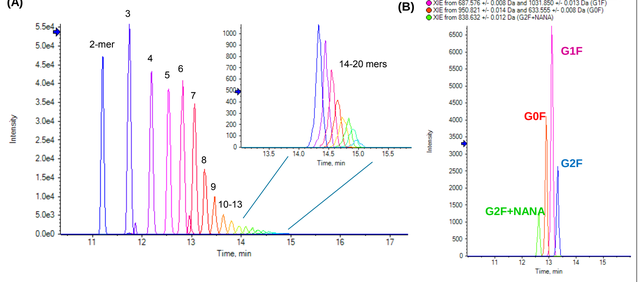

Figure 2. CE-LIF analysis of (A) an APTS-labeled carbohydrate ladder and (B) a fucosylated series of mAb glycan standards.

Separations of mAb glycan standards using a MS-compatible ammonium acetate buffer (pH 4.5) were also compared to the industry-accepted CE-LIF analyses using the stand-alone CE functionality of the CESI 8000 Plus instrument. Very similar separation profiles were observed, indicating ease of method transfer and correlation.

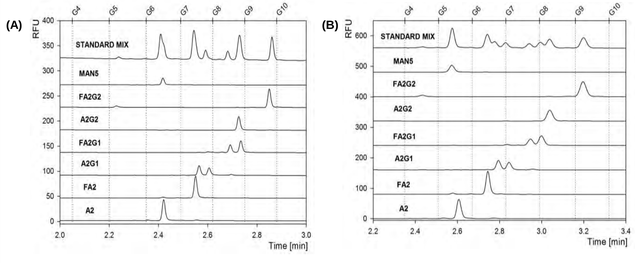

Figure 3. CE-LIF analysis of mAb glycan standards **(A)** with non-MS compatible BGE and **(B)** with MS-compatible 7.5 mM ammonium acetate, pH 4.5 with 10% isopropanol.

The CESI-MS method was evaluated using real samples with additional sample preparation. Prior to CESI-MS experiments, the released glycan samples were first characterized with industry-accepted CE-LIF analyses using the stand-alone CE functionality of the CESI 8000 Plus instrument. FFPE glycans from different mouse tissues were analyzed and compared. Glycan sequencing was performed with a combination of glycolytic enzymes to determine the carbohydrate content of each glycan.

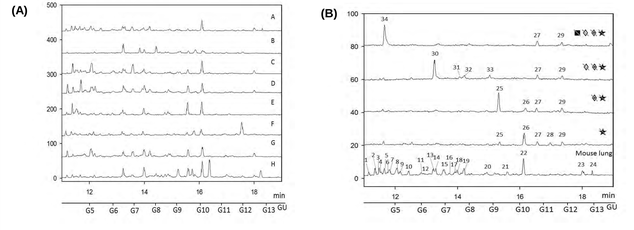

Figure 4. (A) CE-LIF profiles of the released and APTS labeled N-glycans from different mouse tissue samples. A: lung B: brain C: hearth D: spleen E: liver F: kidney G: tumor tissue H: intestine. (B) Exoglycosidase array based sequencing of mouse lung N-glycans. Traces: sialidase (★), sialidase + fucosidase (◆★), sialidase + fucosidase + galactosidase (◊◆★) and sialidase + fucosidase + galactosidase + N-acetylglucosamine (■◊◆★).

Analysis of FFPE mouse lung and brain glycans were then performed by CESI-MS. Similar numbers of glycan peaks were extracted from the MS data for each sample. Accurate molecular masses were calculated from m/z’s and charge states to find potential carbohydrate compositions for each ATPS-glycan electrophoretic peak.

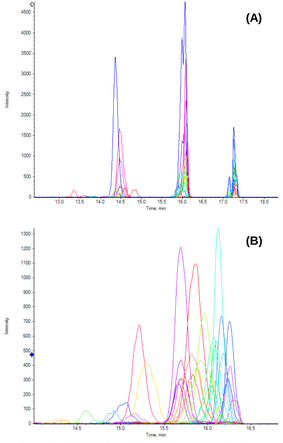

Figure 5. CESI-MS profiles of the released and APTS labeled N-glycans from mouse (A) lung and (B) brain tissue samples.

Measured m/z	Monoisotopic mass (APTS-glycan)	Monoisotopic mass (Glycan)	Potential glycan composition	Tissue
1004.340	2010.696	1556.275	H7N2	Lung
1045.869	2093.754	1639.333	H10; H5N4	Lung
1069.371	2140.758	1686.337	H4N5	Lung
1076.381	2154.778	1700.357		Lung, Brain
1081.884	2165.784	1711.363	H4N3A1F1	Brain
1085.372	2172.760	1718.339	H8N2	Lung
1104.404	2210.824	1756.403	H7N3	Brain
1118.390	2238.796	1784.375	H5N4F1	Lung
1126.386	2254.788	1800.367	H11; H6N4	Lung, Brain
1129.411	2260.838	1806.417		Brain
1147.401	2296.818	1842.397	H5N5	Brain
1150.402	2302.820	1848.399		Lung, Brain
1158.396	2318.808	1864.387	H3N6F1	Lung
1177.760	2357.536	1903.115		Brain
1183.929	2369.874	1915.453	H4N3A1F1	Lung, Brain
1191.929	2385.874	1931.453	H5N4A1	Lung
1199.414	2400.844	1946.423	H6N4F1	Lung, Brain
1203.938	2409.892	1955.471		Brain
1223.919	2449.854	1995.433	H5N5F1	Lung, Brain
1226.449	2454.914	2000.493	H6N5	Brain
1231.428	2464.872	2010.451	H3N6A1	Lung
1256.455	2514.926	2060.505	H4N4A2	Brain
1284.966	2571.948	2117.527	H4N5A1F1	Brain
1329.485	2660.986	2206.565	H11N2; H6N6	Brain
1357.995	2718.006	2263.585		Brain
1365.995	2734.006	2279.585	H5N5A1F1	Brain
1386.506	2775.028	2320.607	H8N5	Brain
1439.023	2880.062	2425.641	H5N5A2; H5N5A1F2	Brain
1459.535	2921.086	2466.665		Brain
1463.011	2928.038	2473.617		Lung
1511.538	3025.092	2570.671	H5N5A2F1; H7N7	Brain
1540.563	3083.142	2628.721		Brain
1694.622	3391.260	2936.839	H6N6A2F1	Brain

Table 1. List of APTS-glycan masses measured between FFPE mouse lung and brain tissue samples. Potential glycan compositions were assigned based on accurate molecular weights for the majority of measured masses.

CONCLUSIONS

The combined CESI-MS and stand-alone CE functionality of the CESI 8000 Plus system provides powerful methods to characterize and quantify released glycans from biologics and FFPE samples. Both analyses deliver high resolution separations, structural characterization, and relative quantitation. When coupled with the TripleTOF® 6600 system, the high sensitivity CESI-MS analyses are achieved from small sample amounts (~10 ng) and volumes (1 – 5 µL) using the nanoVial. Further analyses by MS/MS would facilitate confirmation of glycan structures and correlation to CE-LIF sequencing results.

REFERENCES

1. Donczo, B., Szigeti, M. Ostoros, G., Gacs, A., Tovari, J., Guttman, A. N-Glycosylation analysis of formalin fixed paraffin embedded samples by capillary electrophoresis, *Electrophoresis*. **2015**.

TRADEMARKS/LICENSING

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