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Quick screening of intact antibody and antibody-drug conjugates with integrated microfluidic capillary electrophoresis and mass spectrometry

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Keywords

CE-MS, ZipChip, Q Exactive BioPharma mass spectrometer, BioPharma Finder software, Monoclonal antibody, Antibody-drug conjugates

Goal

Rapid screening of the heterogeneity of monoclonal antibodies and antibody-drug conjugates by an integrated microfluidic capillary electrophoresis (CE) and mass spectrometry (MS) workflow

Introduction

Monoclonal antibodies (mAb) and antibody-drug conjugates (ADC) constitute two of the most important biopharmaceuticals within the class of biotherapeutic drugs.[1] During drug development and manufacturing, undesired mutations and *in vitro* modifications may introduce sample heterogeneity, causing changes to the protein structure that may lead to the loss of drug efficacy.[2,3] Therefore, the availability of a quick screening method at the intact protein level to detect and assess any variability that might occur during drug development is attractive. Multiple analytical methods such as high-resolution liquid chromatography (HPLC), capillary electrophoresis (CE), and mass spectrometry (MS) have been used separately or together to characterize monoclonal antibodies.

Introduced here is a CE-MS solution achieved by coupling the ZipChip™ (908 Devices) system and the Thermo Scientific™ Q Exactive™ Orbitrap™ MS with the BioPharma option to rapidly assess the heterogeneity of mAb and

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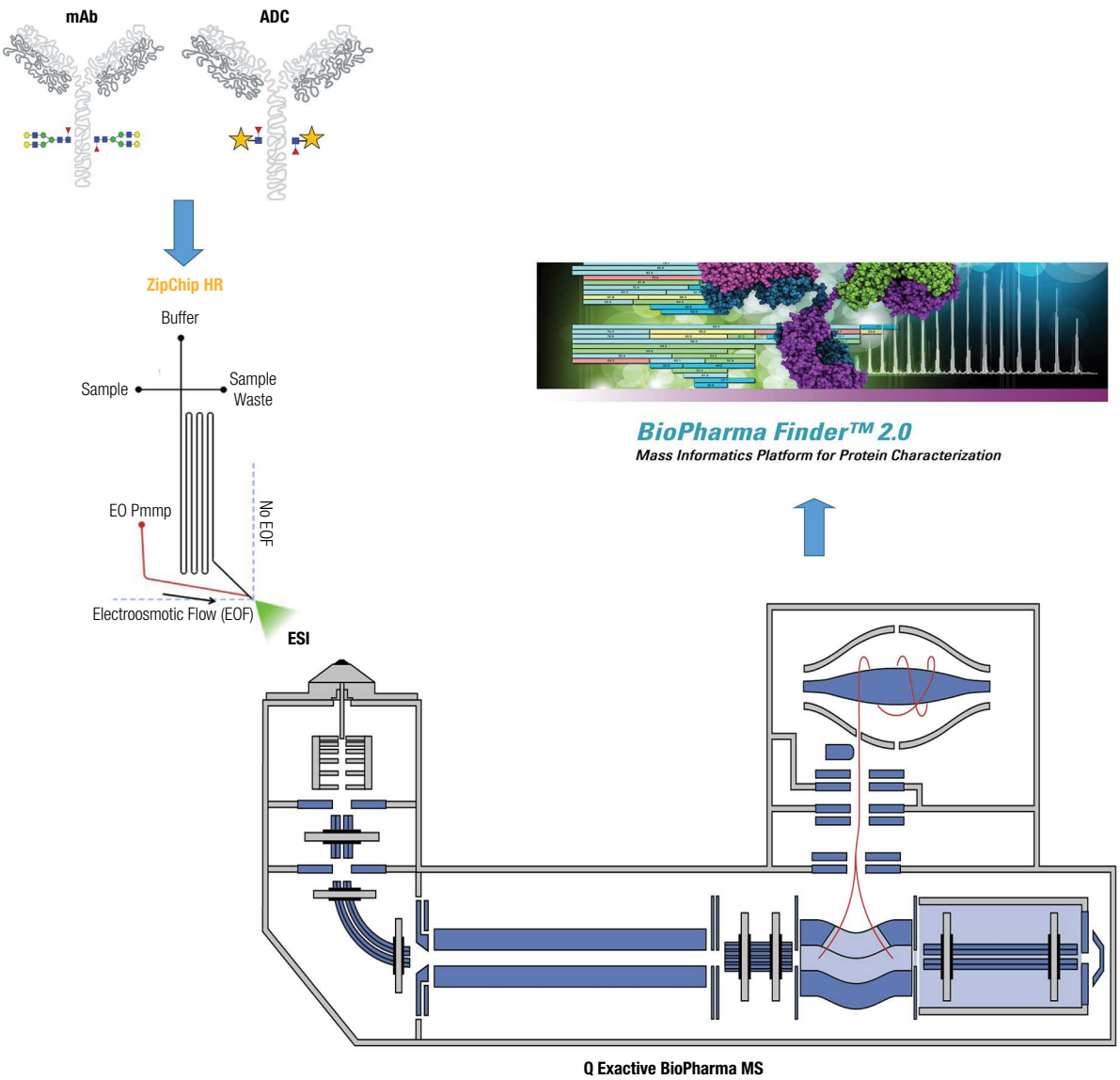
ADC as a complementary approach to existing analytical methods. CE has been previously described as a high-resolution separation method for intact proteins.[5–7] The implementation of charge-based separations as a microfluidic device, the ZipChip provides shorter analysis time due to its high electrical field strength.[8] The Q Exactive MS with BioPharma option is quickly becoming popular for biopharmaceutical characterization due to its high-resolution, accurate-mass (HRAM) performance for the detection of very large biomolecules with a mass range up to *m/z* 8000. This application describes the combination of these two techniques in one platform and demonstrates its ability for quick screening of biopharmaceutical drug heterogeneity.

Experimental

Materials

NIST mAb (reference material 8671)[9] is a recombinant, humanized IgG1 κ and its structure and post-translational modifications have been well characterized. The antibody sample is known to have a relative abundance of C-terminal lysine clipping, N-terminal pyroglutamination, glycosylation, as well as a lower abundance of oxidation, deamidation, and glycation modifications. An NIST mAb ADC analogue was made from the NIST mAb reference material using enzyme-based N-glycan labeling and SiteClick™[10] chemistry, resulting in covalent binding of two molecules of DIBO-Biotin per mAb.



Scheme 1. The workflow of intact mAb/ADC characterization. Characterization of mAb/ADC is accomplished by the separation of CE ZipChip, online analysis of Q Exactive BioPharma MS, and deconvolution/identification with BioPharma Finder 2.0 software.

Sample preparation

NIST mAb was stored in 12.5 mM of L-histidine and 12.5 mM of L-histidine HCl (pH 6.0). NIST mAb ADC was stored in 1× of Tris-buffered saline (TBS). Both samples were diluted with deionized water to 0.5 µg/µL and ready for analysis.

Capillary electrophoresis

A ZipChip HR chip (P/N 00950-01-00499) designed with a 22 cm separation channel and a built-in ESI emitter was used in the ZipChip system (P/N 00950-01-00492) (Scheme 1). A premixed background electrolyte solution containing 0.2% acetic acid and 10% isopropyl alcohol (ZipChip Intact Antibody Kit, P/N 00950-01-00502) was used. Samples were injected into the CE separation channel by pressure injection of 2 psi for 5 s, which corresponds to an injection volume of 0.65 nL. An electrical field strength of 770 V/cm with 2 psi pressure assistance was used during CE separation, resulting in an average analysis time of 3 min (Table 1).

Mass spectrometry

A Q Exactive Plus MS with BioPharma option (P/N 0726055) was used in this study in high mass range (HMR) mode for the analysis of intact mAb and ADC (Scheme 1). The HMR mode extends the detection capability of the Orbitrap™ mass spectrometry up to *m/z* 8000. In this work, a mass range between *m/z* 2500 and 6000 was sufficient to detect the mildly-denatured mAb at a MS scan rate of 12 scans/s. In-source CID (100 eV) was applied to assist desolvation (Table 1).

Data analysis

The raw data from the mAb and ADC measurements were analyzed with Thermo Scientific™ BioPharma Finder™ 2.0 software (P/N OPTON-30592). For the characterization of NIST mAb, a protein sequence of 1326 amino acids with 16 disulfide bonds was created in the protein sequence manager, which included a fixed modification for N-terminal pyroglutamination and variable modifications for glycosylation, C-terminal lysine clipping, and glycation. For the study of NIST mAb ADC, in which the glycosylation moiety had been removed with endoglycosidase S (Endo S) and replaced by DIBO-Biotin groups through azide activation (Scheme 2), the DIBO-Biotin was considered as the variable modification. The average mass of the NIST mAb and NIST mAb ADC were calculated with the sliding-window deconvolution method using the ReSpect™ algorithm and the identification automatically aligned with the structural information in protein sequence manager (Table 1).

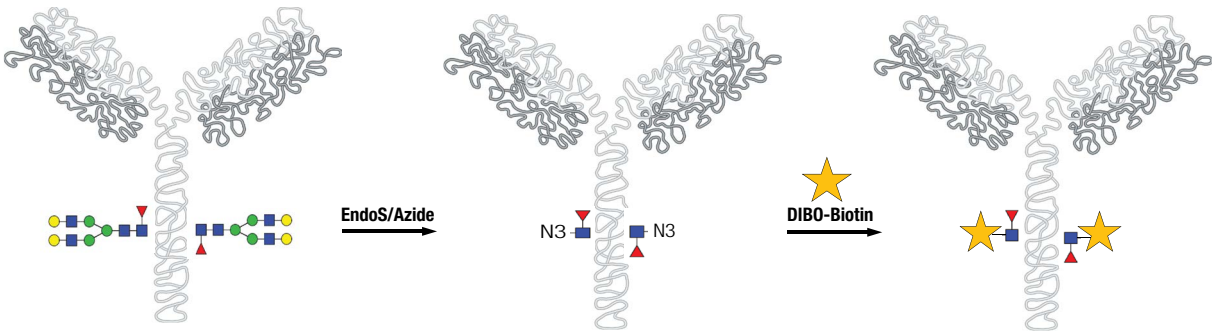
Table 1. Experimental conditions on the CE ZipChip, Q Exactive BioPharma MS, and BioPharma Finder 2.0 software.

CE ZipChip	
Chip:	HR chip
CE Electrical Field Strength:	770 V/cm
Pressure Assistance:	Enabled
Background Electrolyte Solution (BGE):	10% Isopropyl alcohol, 0.2% acetic acid
Sample Injection:	2 psi, 5 second
Q Exactive BioPharma MS	
Mode:	HMR mode
Scan Type:	Full MS
m/z Range:	2500 to 6000
Fragmentation:	In-source CID 100 eV
Resolution:	17,500
Sheath Gas Flow Rate:	2
Capillary Temp:	200 °C
S-lens RF Level:	100
BioPharma Finder 2.0 Software	
Disulfide Bond:	12 inter-chain; 4 intra-chain
Fixed Modification:	N-terminal pyroglutamination
Variable Modification:	C-terminal lysine clipping glycosylation
Source Spectra Method:	Sliding windows
Deconvolution Algorithm:	ReSpect™

Results and discussion

NIST mAb analysis

Recombinant therapeutic antibodies are large and complex molecules with a variety of modifications such as glycosylation, terminal clipping, oxidation, deamination, and glycation. These NIST mAb modifications have to be confidently identified through mass accuracy and the site of modification has to be localized through MS/MS analysis. Presented here is a powerful analysis method based on the ZipChip technology for fast separation of mAb samples in combination with HRAM Orbitrap MS for confident identification of the mAb and its variants. Near baseline separation of the lysine charge variants of intact NIST mAb sample was achieved within 3 minutes. (Figure 1A). Using the sliding deconvolution method, baseline-resolved mass spectra were obtained from the very low abundant 2-lysine variant whose relative abundance is only about 1.3% of the base peak. The charge distribution of the intact mAb mass spectrum remained within the mass range of *m/z* 4600–5800 (Figure 1A), which is close to the distribution of the mAb under native conditions.



Scheme 2. Synthesis of site-specific ADC from the NIST mAb with click chemistry.

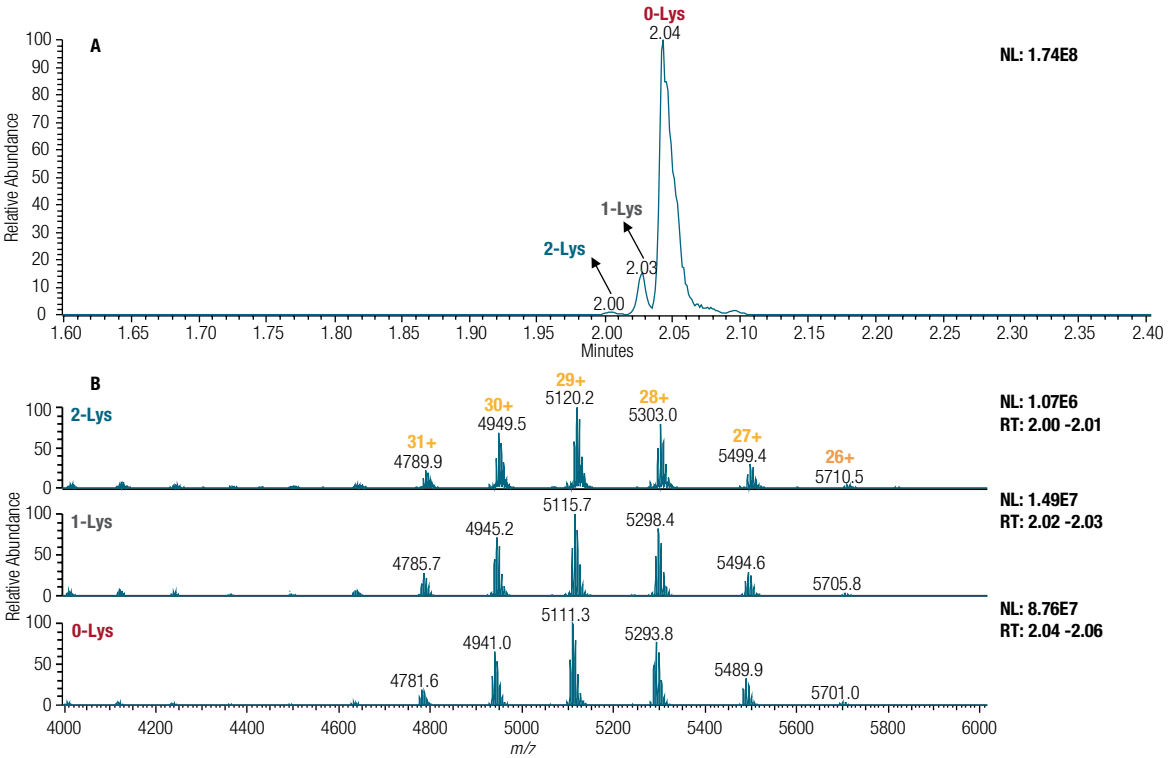


Figure 1. A) Electropherograms of NIST mAb in a 3 min run on a ZipChip CE device hyphenated to Q Exactive BioPharma MS. Three different lysine variants were conducted in a baseline separation B) Three mass spectra were averaged over the widths from the three peaks on the electropherograms from left to right.

The deconvoluted mass spectra of the 2-lysine, 1-lysine, and 0-lysine variants of the NIST mAb (Figure 2) show five major glycoforms. Each variant was separated by a mass difference of 162 Da indicative of the presence of hexoses. The same distribution of mAb glycoforms were observed for all three N-terminal lysine variants. Altogether, 17 variants were identified with mass error below 20 ppm at the intact protein level by BioPharma Finder 2.0 software (Table 2), as determined from the analysis of three injection replicates. Sum intensity of all NIST mAb variants peaks was determined and relative quantification of the 17 variants was assessed to span over three orders of magnitude in dynamic range (Table 2). The mAb variant containing two additional N-terminal lysines and the glycoforms G2F/G2F was detected at 0.19% signal intensity (row 17 in Table 2). Relative quantification for 17 variants was enabled through the combination of high resolution CE separation together with the high mass accuracy and long-term mass stability provided by the Orbitrap mass analyzer.

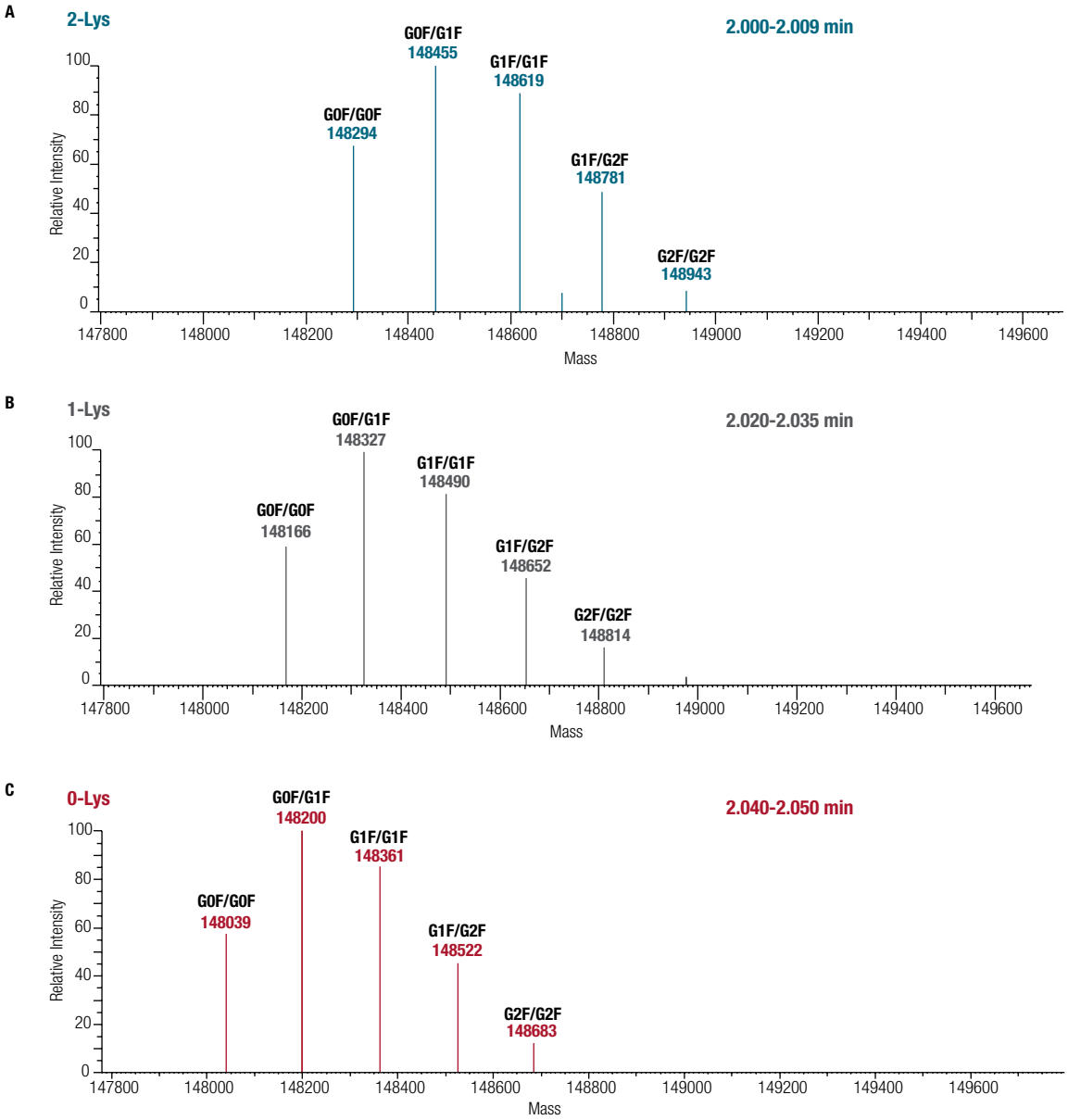


Figure 2. Three deconvoluted mass spectra from different migration time intervals, which correspond to different lysine variants. A) NIST mAb with two lysine adducts including five glycoforms was detected B) NIST mAb with one lysine adduct including five glycoforms was detected C) NIST mAb without lysine adduct including five glycoforms was detected.

Table 2. Seventeen identified variants of NIST mAb from three replicates of run by using BioPharma Finder 2.0 software for data analysis.

Protein Name	Modification	Average Mass(Da)	Theoretical Mass (Da)	Mass Error (ppm)	Sum Intensity	Relative Abundance
NIST mAb	1xG0FG1F	148199.66	148199.21	3.1	8.54E+09	100.00
NIST mAb	1xG1FG1F	148361.30	148361.35	0.3	7.30E+09	85.52
NIST mAb	1xG1FG2F	148523.57	148523.49	0.6	3.33E+09	38.98
NIST mAb	1xG0FG0F	148037.72	148037.07	4.4	3.18E+09	37.29
NIST mAb	1xG2FG2F	148685.06	148685.63	3.8	1.23E+09	14.35
NIST mAb_Hexose	1xG2FG2F	148845.21	148847.77	17.2	1.61E+08	1.89
NIST mAb minus 1GlcNac	1xG0FG0F	147831.30	147833.88	17.4	9.78E+07	1.15
NIST mAb plus 1K	1xG0FG1F	148327.72	148327.41	2.1	1.67E+09	19.57
NIST mAb plus 1K	1xG1FG1F	148489.69	148489.55	1.0	1.44E+09	16.81
NIST mAb plus 1K	1xG0FG0F	148166.13	148165.27	5.8	1.07E+09	12.56
NIST mAb plus 1K	1xG1FG2F	148651.44	148651.69	1.7	6.99E+08	8.18
NIST mAb plus 1K	1xG2FG2F	148811.79	148813.83	13.7	2.67E+08	3.13
NIST mAb plus 2K	1xG0FG1F	148456.98	148455.61	9.3	1.13E+08	1.32
NIST mAb plus 2K	1xG1FG1F	148618.90	148617.75	7.7	1.09E+08	1.28
NIST mAb plus 2K	1xG0FG0F	148294.86	148293.47	9.4	8.65E+07	1.01
NIST mAb plus 2K	1xG1FG2F	148780.66	148779.89	5.2	5.81E+07	0.68
NIST mAb plus 2K	1xG2FG2F	148942.67	148942.03	4.3	1.61E+07	0.19

NIST mAb ADC analysis

Recombinant therapeutic antibodies have been used to treat a variety of diseases. This successful therapeutic approach has been extended to a new class of biopharmaceutical drugs: antibody-drug conjugates, where the highly potent drug payload is covalently linked to the antibody to specifically target and affect the site of disease. To date, only two ADCs are marketed while more than 60 ADC molecules are currently undergoing clinical evaluation.[11] Historically, the introduction of a drug molecule to the mAb through statistical conjugation brings a heterogeneous drug load distribution to the ADC, which could result in altered therapeutic effects with various pharmacokinetic implications and potentially a narrower therapeutic window. Site-specific conjugation methods with a more homogenuous drug distribution are now considered a standard method in ADC development.[12,13] The site-specific ADC analogue analyzed here was synthesized from the NIST mAb with a known payload of two at the site of glycan modifications to provide a reference for other ADC molecules.

The same experimental setting used for the NIST mAb analysis was applied to the NIST mAb ADC analysis. Similar to the NIST mAb analysis, near-baseline separation of the lysine variants of the ADC was achieved within 3 minutes (Figure 3A). The mass spectrum is less complex due to the removal of the complex glycosylation modification and replacement with a simple biotin moiety (Figure 3B). Five major ADC variants were identified by BioPharma Finder software (Table 3). We observed high abundance of 0-lysine, 1-lysine, and 2-lysine NIST mAb ADC variants each carrying a payload of two. Variants with zero or one payload were not observed. The drug-to-antibody ratio (DAR) was calculated as 2.0 in this site-specific NIST mAb ADC. Two additional peaks with the molecular weight of ADC + 162 Da and ADC + 324 Da were detected and identified as ADC+1 hexose and ADC+2 hexose molecules. These Hexose adducts were also observed in the NIST mAb on the glycoforms of G2F/G2F (Figure 4). They have also been reported as the glycation adducts from the NIST mAb[9] and were not removed during the the PNGase F digestion.

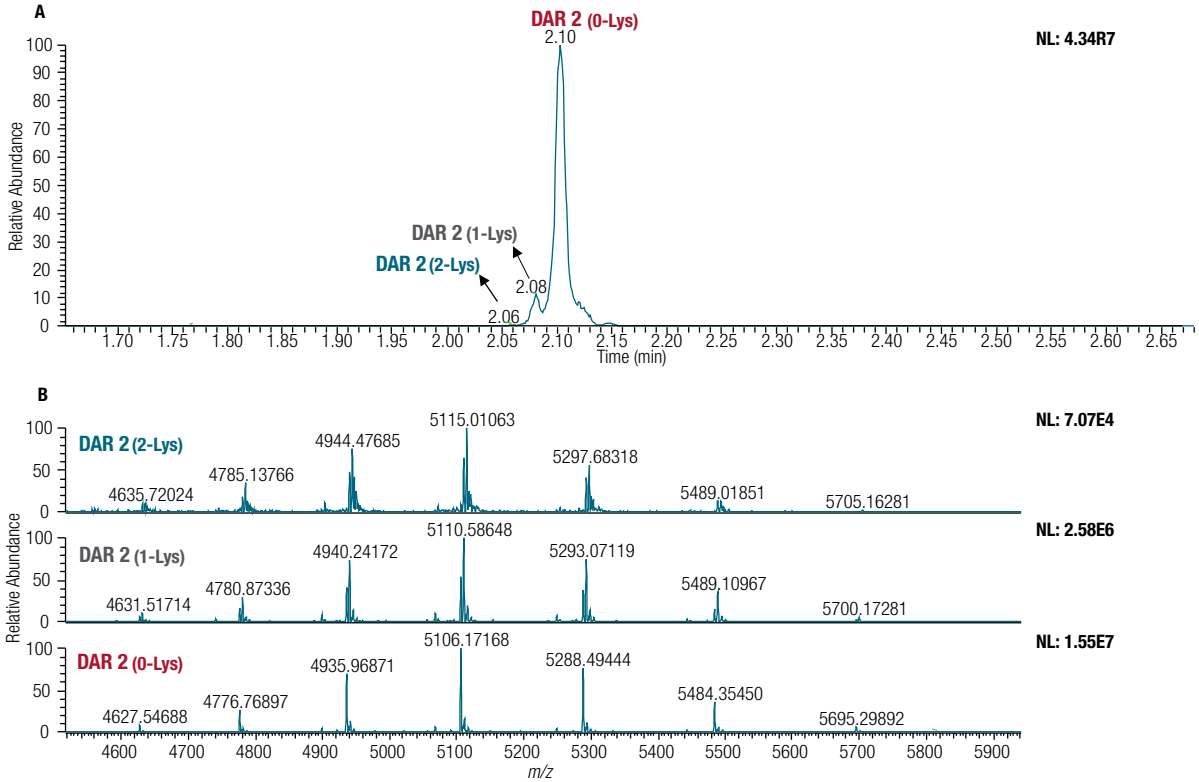


Figure 3. A) Electropherograms of NIST mAb ADC in a 3 min run on a CE ZipChip hyphenated to Q Exactive BioPharma MS. Three different forms of lysine variants were separated B) three mass spectra were averaged from the three peaks on the electropherograms over their widths from left to right.

Table 3. Five identified variants of NIST mAb ADC using the BioPharma Finder software.

Protein Name	Modification	Average Mass(Da)	Theoretical Mass (Da)	Mass Error (ppm)	Relative Abundance
NIST mAb_Endo_Azide	1xtwo_DIBO_Biotin	148049.09	148049.67	3.9	100.00
NIST mAb_Endo_Azide	1xtwo_DIBO_Biotin_1 Hexose	148213.40	148211.81	10.8	14.13
NIST mAb_Endo_Azide	1xtwo_DIBO_Biotin_2 Hexose	148373.95	148373.95	0.0	2.10
NIST mAb plus 1K_Endo_Azide	1xtwo_DIBO_Biotin	148177.60	148177.87	1.8	8.68
NIST mAb plus 2K_Endo_Azide	1xtwo_DIBO_Biotin	148306.59	148306.07	3.5	0.12

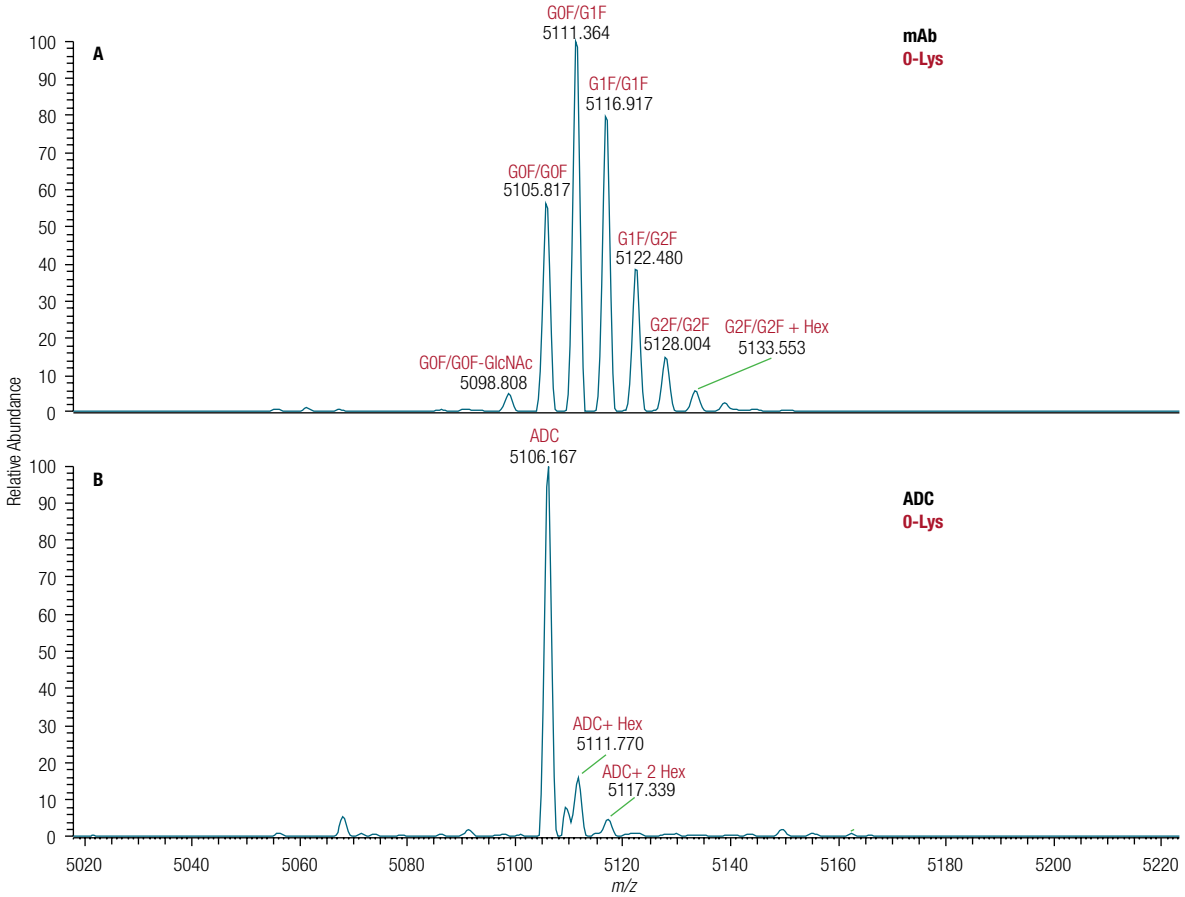


Figure 4. Identification of peaks from raw mass spectra acquired by CE-MS from A) NIST mAb and B) NIST mAb ADC.

Conclusion

In this study, fast analysis of the heterogeneity of the intact NIST mAb and ADC samples was achieved within 3 min using a CE-MS workflow. Identification of highly differently abundant sample components was accomplished by automatic peak identification through sliding-window deconvolution. Samples were directly injected on the chip, eliminating the need for desalting or additional sample preparation before analysis. The minimal sample preparation and online desalting capability make this approach even more amenable to quick screening and characterization of mAbs and ADCs during various phases of drug development and production. Compared to infusion-based MS methods, the separation of charge variants by capillary electrophoresis reduces sample complexity for MS detection and allows a more complete sample profiling. In this study, 17 NIST mAb variants with signal intensities spanning over four orders of magnitude were identified. The high sensitivity and dynamic range of the Q Exactive Orbitrap mass spectrometer were able to detect and relatively quantify variants with abundance as low as 0.19% of the base peak intensity. One of the additional benefits of this platform is its low sample consumption. Only about 0.2 ng of mAb or ADC sample was used for the successful analysis, which makes it particularly well suited during early stage drug development where sample might be limited.

For mAb characterization, near isobaric masses in heterogeneous mAb preparations pose a substantial analytical challenge. In this study, the ZipChip coupled to a Q Exactive MS was able to identify and quantify all known variants with a high dynamic range in a few minutes, making the workflow the method of choice for quick screening of the heterogeneous mAb and ADC samples during biopharmaceutical drug development.

Abbreviations:

ADC: antibody-drug conjugate
CE: capillary electrophoresis
cIEF: capillary isoelectric focusing
ESI: electrospray ionization
HILIC: hydrophilic interaction liquid chromatography
HMR mode: high mass range mode
HRAM: high-resolution accurate-mass
IgG: immunoglobulin G
mAb: monoclonal antibody
MS: mass spectrometer

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