ZipChip

Analysis of Reduced mAbs and Antibody-Drug Conjugates using the ZipChip Platform

INTRODUCTION

The detailed analysis of complex IgG type monoclonal antibodies (mAbs) is critical for a comprehensive understanding of a mAb population. Such analysis includes measurements of charge heterogeneity, localization of specific modifications, and in the case of antibody drug candidates, (ADC), measurement of the DAR. The biology of these molecules is complex. IgG type monoclonal antibodies (mAbs) are composed of two heavy polypeptide chains (~50kDa) and two light polypeptide chains (~25kDa) that are connected via a series of interchain disulfide bonds. Together these subunits make a tetramer of ~150kDa in mass. The complexity of this large protein is increased further due to post translational modifications that can occur during production, purification, and storage. These large proteins can also be modified with a small molecule drug load to create an antibody drug conjugate (ADC) that is even more complex, but also a potent drug therapy. Analyzing these complex proteins at the intact level provides valuable information about the general heterogeneity of the mAb population, but it is often useful to reduce the mAb or ADC to smaller subunits prior to analysis. There are several benefits to this approach: the complexity of the analyte is reduced, more specific localization of modifications can be determined, and the lower mass mAb subunits are easier to analyze via MS so higher mass accuracy and more confident identification of modifications can be achieved with a wider range of MS instrumentation. A common approach to this is to reduce the interchain disulfide bonds and analyze the individual heavy and light chains. In this work we describe a method to analyze reduced mAbs and ADCs using the ZipChip™Platform, which reduces or eliminates existing analytical limitation.

The ZipChip Platform is a front-end separation system for commercial mass spectrometers that directly couples high resolution separations to mass spectrometry analysis. This technology yields extremely fast and efficient separations of a wide range of molecules, from small polar metabolites to large intact proteins. ZipChip performs capillary zone electrophoresis (CZE) separations using a monolithic microfluidic device with on-line electrospray ionization (ESI) directly into a mass spectrometer (MS). Because the separation mechanism is based on charge and hydrodynamic radius, ZipChip often generates superior separations of intact proteins. The analysis of reduced antibodies and ADCs demonstrates the unique efficiency, speed, and simplicity of the ZipChip Platform. In this work, reduced mAbs and ADC mimics were analyzed using ZipChip. Two commercially available mAbs and ADC mimics of those molecules were processed using a simple reduction procedure and analyzed under denaturing conditions. The mAb and ADC subunits were resolved in the CZE separation with run times of 6.0 minutes or less. Variants of both the heavy and light chains were separated and identified using the mass information obtained from the mass spectrometer. For the ADC mimics, the average DAR was calculated from the conjugation levels detected on the two subunits.

MATERIALS AND METHODS

Samples and reagents. The three commercially available mAb standards used were the NIST mAb reference standard (National Institute of Standards and Technology, Ref. Material 8671), the SILuLite™ SigmaMAb Universal Monoclonal Antibody Standard and the SigmaMAb Antibody Drug Conjugate (ADC) Mimic (Sigma-Aldrich, St. Louis, MO). The NIST mAb ADC mimic was created using an NHS-dye antibody labeling kit according to manufacturer instructions (Pierce Biotechnology, Rockford, IL).

High purity ammonium bicarbonate (99%, ACROS Organics, New Jersey, USA), dithiothreitol (DTT) (Fisher Bioreagents, New Jersey, USA) and LC/MS

grade water (Fisher Scientific, New Jersey, USA) were used for reduction of the mAbs and ADC mimics. A 50 mM ammonium bicarbonate solution (pH~7.5) was prepared and a 100 mM DTT solution was then prepared using a portion of the ammonium bicarbonate buffer. Background electrolyte from the ZipChip Metabolites Kit #850-00033 (908 Devices Inc., Boston, MA) was prepared according to manufacturer instructions and used for analysis of the reduced mAbs and ADC mimics.

Reduction of mAbs and ADC mimics. The desired final sample concentration for this work was 0.5 mg/mL mAb reduced. To achieve this, 50 ug of the mAb or ADC mimic was diluted to 90 uL with the ammonium bicarbonate buffer. 10 uL of the DTT solution was then added for a final DTT concentration of 10 mM and final volume of 100 uL. The molecules were incubated at 37 C for ~30 minutes and then analyzed.

Analysis of reduced mAbs/ADC mimics. The ZipChip system was primed using the prepared metabolite background electrolyte. A ZipChip HR #810-00195 (908 Devices Inc., Boston, MA) chip was used for all analyses. A 1 nL injection of sample was performed on-chip and the separation was run at 500 V/cm. Pressure assist was set to activate at 0.5 minutes. Data was acquired using a Thermo Scientific™ Exactive Plus EMR™ mass spectrometer operated in Normal Mode with the following settings:

m/z range: 800-3000
Resolution: 17,500
In-source CID: 75
3 microscans
AGC target: 1E6
Max Ion Injection Time: 20 ms
Inlet capillary temperature: 200 C
S Lens RF: 80

Data processing. The data was processed using Thermo Scientific™ BioPharma Finder™ 1.0 SP1.

RESULTS & DISCUSSION

To evaluate ZipChip for characterizing reduced mAbs, two commercially available intact mAb standards were obtained: the SILu™Lite Sigma mAb and the NIST mAb reference material. The mAbs are recombinant human monoclonal antibodies expressed in mammalian cells and both products are geared toward use in method development and optimization of analytical techniques used to characterize mAbs.

The Sigma mAb is an IgG 1 mAb with a lambda light chain. The intact mAb has a mass of ~144 kDa with

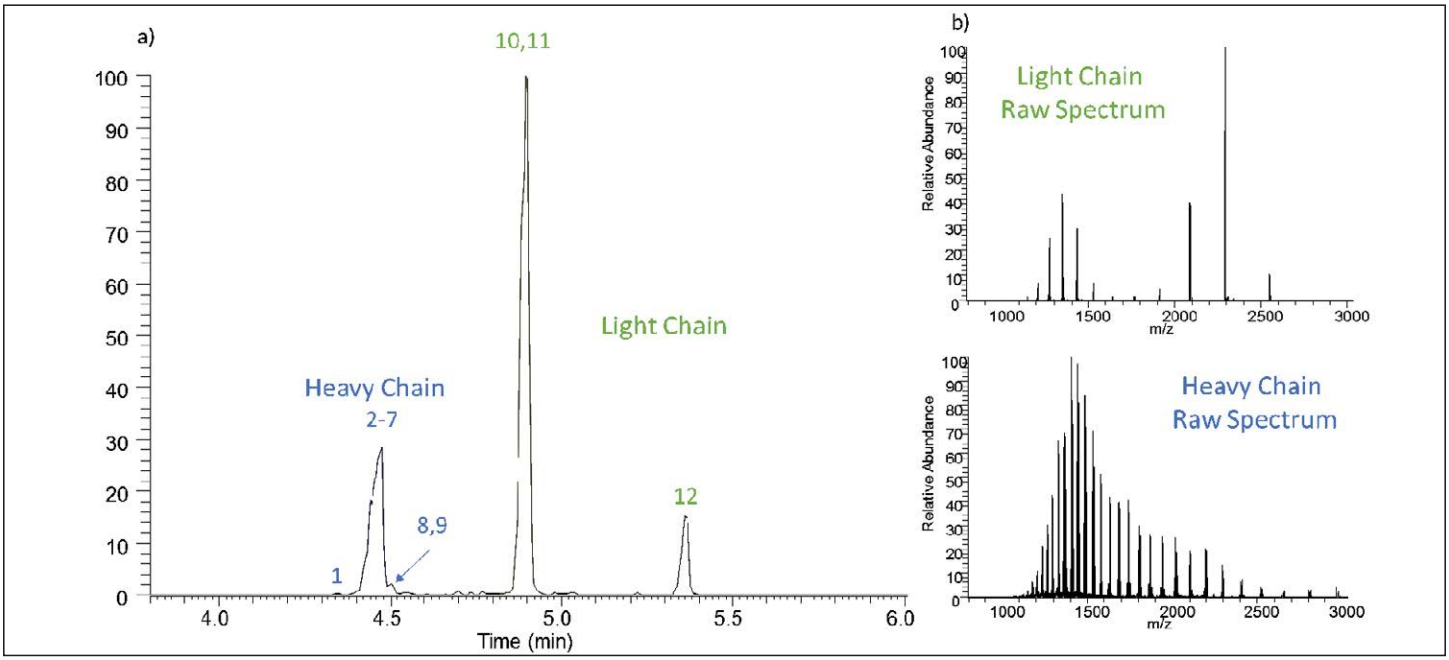

Figure 1: a) ZipChip separation of the reduced Sigma mAb. The heavy and light chain species are well resolved in the electrophoretic separation. The heavy chain separates primarily by differences in glycosylation whereas the two resolved light chain species differ in the number of intrachain disulfide bonds. b) Representative raw mass spectra for the heavy and light chains. The spectra were generated by averaging the scans across the width of the peaks indicated by the shaded rectangles.

glycans typical of expression in CHO cells. Analysis of the reduced Sigma mAb resulted in the separation provided in Figure 1a. The heavy chain and light chain species were well resolved within a 6.0 minute run time. A mass spectrum was generated for each of the peaks in the separation by averaging the individual mass scans across the width of the peak. Representative mass spectra for the heavy and light chains are provided in Figure 1b. Deconvolution of the raw mass spectra was performed using Thermo Scientific™ BioPharma Finder™ 1.0 SP1 and variants of the heavy and light chains were automatically identified based on mass and confirmed by migration time. The identified variants are listed in Table 1. For all variants identified, the mass accuracy was greater than 20 ppm.

Closer inspection of the data reveals that variants of the subunits were also resolved in the ZipChip separation. The heavy chain separated primarily based on differences in the glycan structure on the conserved region glycosylation site. Aglycosylated heavy chain (peak 1) was the first heavy chain variant detected, indicating that this species has the highest electrophoretic mobility. Heavy chain variants bearing neutral glycan structures (peaks 2-7) have a slower migration time. Since these glycan structures have little impact on the net charge of the molecules, it can be inferred that the addition of the glycans primarily affects the electrophoretic mobility by altering the hydrodynamic radius of the molecules in solution. Glycans bearing sialic acids decrease the electrophoretic mobility further due to their negative charge (peaks 8,9). These glycans not only alter the hydrodynamic radius, but also the net charge. As compared to the heavy chain, the light chain is more homogeneous. Deconvolution of the mass spectra revealed glycation on the light

Peak	Average Mass	Fractional Abundance (%)	Matched Delta Mass (ppm)	Matched Sequence	Modifications	Intra-chain Disulfides
1	48949.49	0.36	4.66	Sigma mAb HC		4
2	50166.27	0.48	1.29	Sigma mAb HC	M5	4
3	50192.50	0.40	6.25	Sigma mAb HC	A1G0F	4
4	50249.38	5.23	1.94	Sigma mAb HC	A2G0	4
5	50395.13	41.52	4.34	Sigma mAb HC	A2G0F	4
6	50557.27	35.49	3.27	Sigma mAb HC	A2G1F	4
7	50719.61	6.16	2.52	Sigma mAb HC	A2G2F	4
8	51009.96	0.45	13.82	Sigma mAb HC	A2S1G1F	4
9	50847.93	0.22	5.69	Sigma mAb HC	A2S2F	4
	50337.06	2.04		Unmatched; -58.10 Da from A2G0F		
	50349.32	0.21		Unmatched; -45.84 Da from A2G0F		
	50351.98	1.35		Unmatched; -43.18 Da from A2G0F		
	50497.29	1.25		Unmatched; -60.06 Da from A2G1F		
	50499.45	0.56		Unmatched; -57.90 Da from A2G1F		
	50513.22	2.14		Unmatched; -44.14 Da from A2G1F		
	50674.43	0.57		Unmatched; -45.10 Da from A2G2F		
	50881.80	0.20		Unmatched; +162.27 Da from A2G2F		
10	22938.10	73.32	8.30	Sigma mAb LC	Pyroglutamic acid (Q)	2
11	23100.26	3.43	5.76	Sigma mAb LC	Pyroglutamic acid (Q) Glycation	2
12	22940.08	21.68	7.53	Sigma mAb LC	Pyroglutamic acid (Q)	1
	23054.08	1.57		Unmatched		

Table 1: Identified Sigma mAb subunit variants and their fractional abundances.

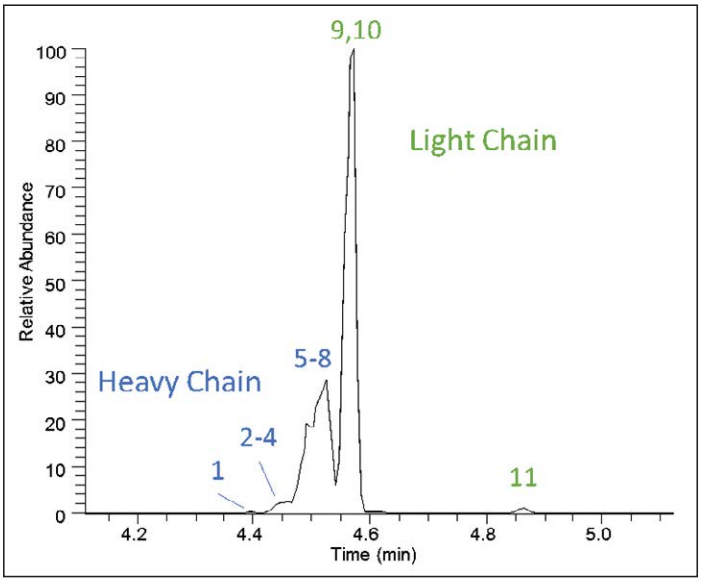

Figure 2: ZipChip separation of the reduced NIST mAb. The NIST heavy chain has C-terminal lysine variants that separate due to differences in net charge. A lower level of light chain with 1 intrachain disulfide bond is observed for the NIST mAb.

chain even though this variant was not resolved in the electrophoretic separation. Two light chain species are separated during the ZipChip separation that differ in the number of intrachain disulfide bonds present. The peak with a migration time of ~5.35 minutes corresponds to a light chain variant that has 1 interchain disulfide bond whereas the more abundant species at a migration time of ~4.8 min has two interchain disulfide bonds. The difference in disulfide bonds likely alters the hydrodynamic radius, and thus the electrophoretic mobility resulting in separation of the two species. Thus, with ZipChip analysis, variants of mAb polypeptide chains can be separated not just by differences in net charge, but also by differences in structure.

The second mAb chosen for analysis was the NIST mAb reference material. The NIST mAb is an IgG 1 mAb and has a kappa light chain. The separation of the NIST mAb molecule is provided in Figure 2. The electrophoretic mobility of the NIST heavy and light chains is more similar resulting in similar migration times. However, the two chains are resolved in a run time of ~5.0 minutes. Raw mass spectra were processed using BioPharmaFinder 1.0 SP1. Identified species and their modifications are listed in Table 2. The mass accuracy of all identified NIST mAb heavy chain and light chain species is less than 20 ppm.

Aglycosylated heavy chain (peak 1) is detected as the earliest migrating heavy chain species. As with the Sigma mAb, the presence of the glycan structures results in a decrease in electrophoretic mobility and all other heavy chain species detected bear glycans on the conserved region glycosylation site. Additionally, the NIST mAb is known to have C-terminal lysine variants

Peak	Average Mass	Fractional Abundance (%)	Matched Delta Mass (ppm)	Matched Sequence	Modifications	Intra-chain Disulfides
1	49453.33	0.35	7.07	NIST mAb HC		4
2	51026.80	3.41	7.56	NIST mAb HC	C terminal K, A2G0F	4
3	51188.75	3.11	11.36	NIST mAb HC	C terminal K, A2G1F	4
4	51351.09	0.74	7.53	NIST mAb HC	C terminal K, A2G2F	4
5	50695.65	2.02	3.46	NIST mAb HC	A1G0F	4
6	50898.69	39.13	6.40	NIST mAb HC	A2G0F	4
7	51061.01	35.51	2.95	NIST mAb HC	A2G1F	4
8	51222.88	6.30	8.29	NIST mAb HC	A2G2F	4
	50855.41	4.93		Unmatched; A2G0F -43.33 Da		
	51016.09	3.22		Unmatched; A2G1F -45.03 Da		
	50983.39	0.53		Unmatched		
9	23123.24	87.72	15.37	NIST mAb LC		2
10	23285.33	8.42	17.22	NIST mAb LC	Glycation	2
11	23125.38	2.20	10.13	NIST mAb LC		1
	23079.76	1.66		Unmatched; LC -43.50 Da		

Table 2: Identified NIST mAb subunit variants and their fractional abundances.

on the heavy chain and these species are resolved in the ZipChip separation due to the difference in net charge. Heavy chain species with a C-terminal lysine present (peaks 2-4) have an earlier migration time while heavy chain species lacking the C-terminal lysine have a later migration time due to a lower net charge (peak 5-8). Upon deconvolution, low levels of glycation on the light chain are detected. Similarly to the Sigma mAb, the NIST light chain separates into two peaks based on the number of intrachain disulfide bonds present. However, the amount of NIST light chain with 1 disulfide bond is much lower. For the NIST mAb this species is present at ~2% as compared to ~22% for the Sigma mAb. This is likely due to differences in how susceptible the intrachain disulfide bonds are to reduction for the two mAbs.

To evaluate the use of the ZipChip Platform for characterizing more complex mAb-based therapies, ADC mimics of both mAbs were analyzed. While there are several strategies used to create ADCs, the two explored here are cysteine based conjugation and lysine based conjugation. Cysteine based conjugation chemistry takes the strategy of partially reducing the molecule and functionalizing cysteine residues of the mAb. The result is an ADC with species generally appearing with the drug-to-antibody ratio (DAR) in multiples of 2 and ranging from 0 to 8. Using lysine based conjugation chemistry the primary amine of lysine side chains is functionalized with a small molecule drug. This type of reaction results in non-specific conjugation of the most solvent accessible lysine residues in the protein structure. Typically, this generates a very complex ADC where DAR values can range from 0 to 8+.

A commercially available ADC mimic generated from the SILu™Lite Sigma mAb was analyzed. The ADC mimic is generated by conjugating a neutral small molecule and linker to cysteine residues in the hinge region of the mAb. The ZipChip separation of the reduced Sigma ADC mimic is provided in Figure 3a. The separation profile is like that of the base mAb but with an additional layer of complexity due to the conjugation. The heavy chain species migrate primarily as a single peak at ~4.55 min. Deconvolution of the mass spectra associated with this peak reveals the presence of heavy chain species conjugated with up to 3 small molecule drug mimics (Fig. 3b). The glycoforms of the base mAb are still visible despite the additional complexity. The light chain is conjugated with up to 1 small molecule drug mimic. The addition of the small molecule to the light chain structure causes the DAR 0 and DAR 1 species to begin to resolve in the CE domain. Since the small molecule is neutral and this conjugation chemistry does not impact the net charge of the light chain it can again be inferred that the electrophoretic mobility is primarily being impacted by a

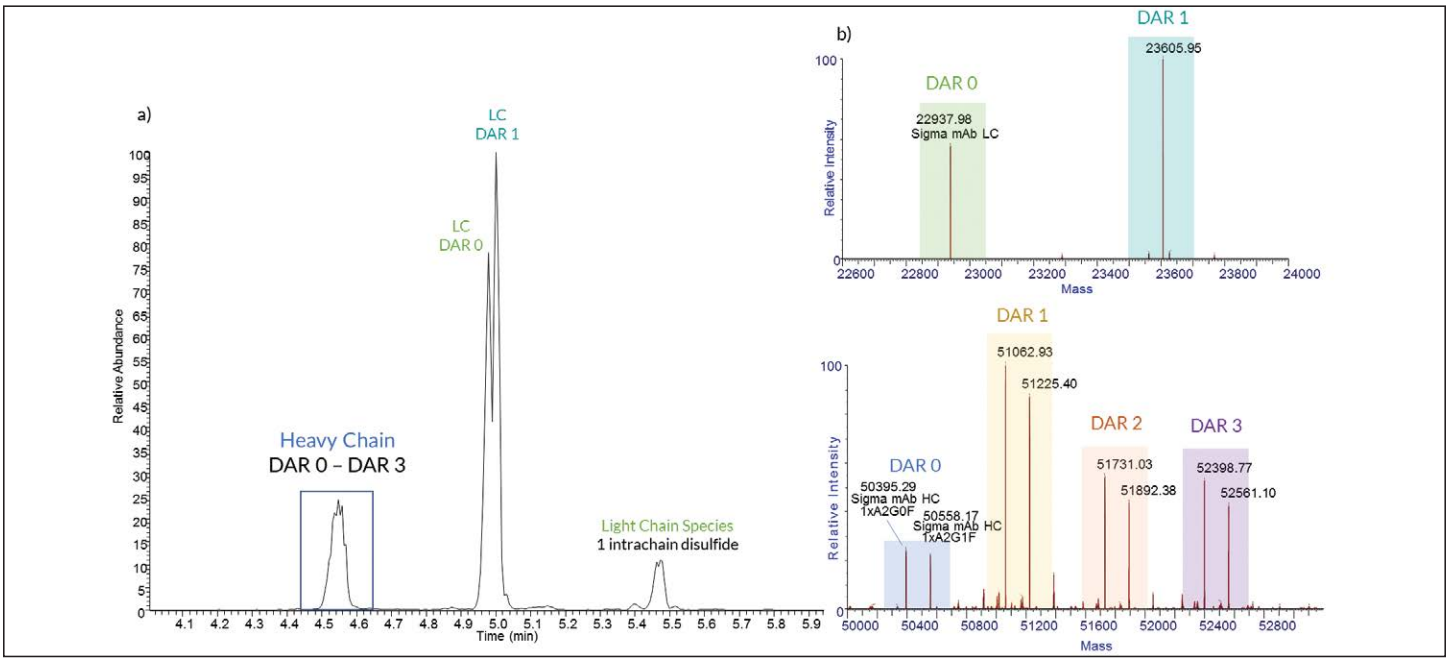

Figure 3: a) ZipChip separation of the reduced Sigma ADC mimic. Resolution between the different DAR species of the light chain is observed for this molecule. b) Deconvoluted mass spectra for the heavy and light chains. The various levels of conjugation can be determined after deconvolution.

Chain	Average Mass	DAR	Fractional Abundance (%)
LC	23605.95	1	63.94
LC	22937.98	0	36.06
HC	50395.29	0	10.42
HC	51062.93	1	43.28
HC	51731.03	2	23.50
HC	52398.77	3	22.80

DAR=4.45

Table 3: Identified DAR species of the Sigma ADC mimic and their fractional abundances.

change in hydrodynamic radius. The enhanced resolution between the DAR light chain species is likely due to the smaller size of the subunit as compared to the heavy chain. The small molecule and linker is proportionally a greater percentage of the total mass of the light chain than the heavy chain. It is worth noting that if the small molecule drug and linker were charged, the resolution achieved in the ZipChip separation would be even greater since the net charge of the molecule would also be affected.

A summary of the heavy chain and light chain DAR species detected is provided in Table 3. The DAR and fractional abundances are listed in addition to the average mass of the species. An average mass shift of 667.86 Da was seen for the addition of a small molecule drug mimic and linker which agrees with the reported structure of the conjugate in the product literature. The following equation was used to calculate the average DAR:

$$DAR_{Avg} = 2 \times \frac{\sum n\,FA_{LC} + \sum n\,FA_{HC}}{100}$$

where n is the number of conjugations on the subunit and FA is the fractional abundance of that subunit species. An average DAR of 4.45 was calculated from the ZipChip analysis of the reduced ADC mimic. This value agrees with the reported DAR of 4.0(±0.8) in the product literature.

Because a commercial lysine linked ADC mimic was not available, a mimic was created using the NIST mAb and a commercially available NHS-functionalized small molecule. Depending on the properties of the small molecule, this conjugation approach can impact the net charge of the mAbs due to neutralization of the positively charged lysine side chains. This would induce a significant change in electrophoretic mobility of the subunits. However, the small molecule used to create the NIST ADC mimic is positively charged at the pH of

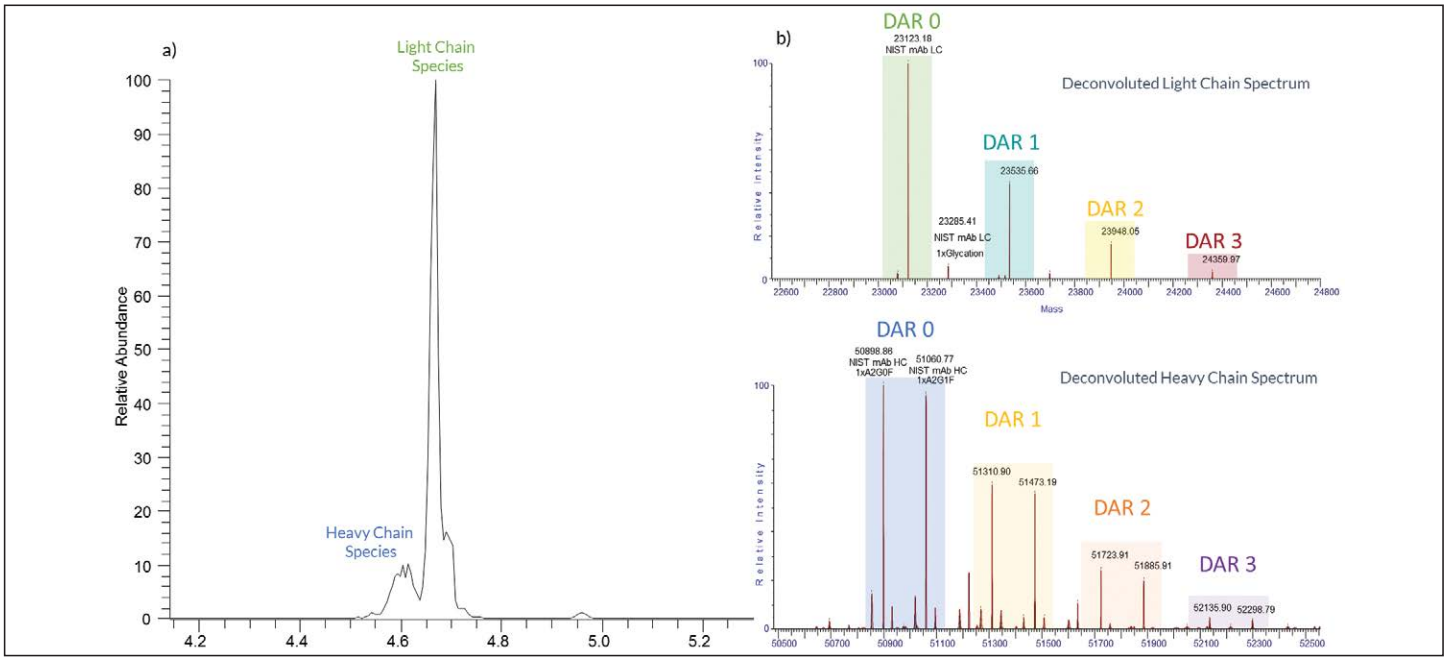

Figure 4: a) ZipChip separation of the reduced NIST ADC mimic. Due to the positive charge and lower mass of the small molecule drug mimic the difference in electrophoretic mobility of the conjugated subunits is minimal and they are not resolved in the electrophoretic separation. b) deconvoluted mass spectra of the NIST ADC mimic subunits. DAR 0 to DAR 3 was detected in the deconvoluted spectra for both subunits.

Chain	Average Mass	DAR	Fractional Abundance (%)
LC	23123.176	0	61.4
LC	23535.66	1	26.9
LC	23948.047	2	9.7
LC	24359.973	3	1.9
HC	50898.863	0	53.8
HC	51310.898	1	31.8
HC	51723.91	2	13.1
HC	52136.477	3	1.1

DAR=2.27

Table 4: Identified DAR species of the NIST ADC mimic and their fractional abundances.

the analysis conditions so there should be no significant change in net charge due to conjugation. Additionally, the mass of the small molecule used is ~412 Da which may not be sufficient to significantly impact the hydrodynamic radius of the heavy and light chains. Therefore, the shift in electrophoretic mobility between DAR species is expected to be quite low. The separation of the reduced NIST ADC mimic is provided in Figure 4a. The profile is like that of the unconjugated NIST mAb which supports this assumption. However, different DAR species are easily detected in the deconvoluted mass spectra (Figure 4b). For both the light and heavy chain, DAR species from 0-3 were detected. Table 4 lists the identified conjugated light and heavy chain species. The average mass and fractional abundance are also provided. The above equation was used to calculate the average DAR. For the NIST ADC mimic the average DAR was found to be 2.27.

The more complex ADC mimics were easily analyzed using the same ZipChip method that was used for reduced mAbs. Additional sample preparation, such as deglycosylation, was not needed to detect the various DAR species of each subunit after conjugation. An average DAR was calculated for both mimics and the value obtained for the Sigma ADC mimic agreed well with the average DAR reported in the product literature. The properties of the small molecule impact the separation of the conjugated subunit species. The small molecule used for the ADC mimics in the work are such that the impact on electrophoretic mobility is minimal. Larger shifts in mobility would be generated by larger molecules or conjugate molecules that affect the net charge.

CONCLUSIONS

In this study, the ZipChip Platform was used to analyze reduced mAbs and ADC mimics. Run times for both types of molecules were 6.0 minutes or less and the heavy and light chain species were resolved in the CE domain. Further, separation of subunit variants was achieved based on differences in both net charge and structural differences. Identification of the variants was facilitated by the high resolution accurate mass data generated from the mass spectrometer. For the ADC mimics, the DAR species of the subunits were detected and characterized without the need for additional sample processing, such as deglycosylation. The data generated using this method provides high resolution separations and high quality mass spectra to facilitate characterization of the large biomolecules. Overall, the ZipChip Platform is a simple way to rapidly analyze reduced mAbs and higher complexity ADCs. Although not performed in this work, intact mAbs and ADCs can also be analyzed. The ZipChip intact mAb assay can separate charge variants of intact mAbs, provide mass information about the different species and glycoforms, and allow for the average DAR calculation for ADCS. Together, the two assays allow users to perform multiple levels of mAb and ADC characterization using a single platform.

908devices

www.908devices.com