Cogent Chemistry

ISSN: (Print) 2331-2009 (Online) Journal homepage: https://www.tandfonline.com/loi/oach20

Intact NIST monoclonal antibody characterization—Proteoforms, glycoforms—Using CE-MS and CE-LIF

Chien-Hsun Chen, Huatao Feng, Rui Guo, Pingjing Li, Anna Karen C. Laserna, Ya Ji, Bao Hui Ng, Sam Fong Yau Li, Shaheer H. Khan, Aran Paulus, Shiaw-Min Chen, Achim E. Karger, Michael Wenz, Daniel Lopez Ferrer, Andreas F. Huhmer & Andreas Krupke |

To cite this article: Chien-Hsun Chen, Huatao Feng, Rui Guo, Pingjing Li, Anna Karen C. Laserna, Ya Ji, Bao Hui Ng, Sam Fong Yau Li, Shaheer H. Khan, Aran Paulus, Shiaw-Min Chen, Achim E. Karger, Michael Wenz, Daniel Lopez Ferrer, Andreas F. Huhmer & Andreas Krupke | (2018) Intact NIST monoclonal antibody characterization—Proteoforms, glycoforms—Using CE-MS and CE-LIF, Cogent Chemistry, 4:1, 1480455, DOI: 10.1080/23312009.2018.1480455

To link to this article: https://doi.org/10.1080/23312009.2018.1480455

© 2018 The Author(s). This open access article is distributed under a Creative Commons Attribution (CC-BY) 4.0 license

View supplementary material

Published online: 17 Jun 2018.

Submit your article to this journal

Article views: 1520

View related articles

View Crossmark data

Citing articles: 6 View citing articles

Full Terms & Conditions of access and use can be found at
https://www.tandfonline.com/action/journalInformation?journalCode=oach20

Chen et al., *Cogent Chemistry* (2018), 4: 1480455
https://doi.org/10.1080/23312009.2018.1480455

cogent chemistry

ANALYTICAL CHEMISTRY | RESEARCH ARTICLE

Intact NIST monoclonal antibody characterization—Proteoforms, glycoforms—Using CE-MS and CE-LIF

Chien-Hsun Chen[1,2], Huatao Feng[3], Rui Guo[3], Pingjing Li[3], Anna Karen C. Laserna[3], Ya Ji[3], Bao Hui Ng[3], Sam Fong Yau Li[3], Shaheer H. Khan[2], Aran Paulus[1], Shiaw-Min Chen[2], Achim E. Karger[2], Michael Wenz[2], Daniel Lopez Ferrer[1], Andreas F. Huhmer[1] and Andreas Krupke[2*]

Received: 27 March 2018
Accepted: 21 May 2018
First Published: 25 May 2018

*Corresponding author: Andreas Krupke, Thermo Fisher Scientific, Life Science Solutions, 180 Oyster Point Parkway, South San Francisco, CA 94080, USA
E-mail: andreas.krupke@thermo-fisher.com

Reviewing editor:
Kishore Cholkar, Custopharm Inc, USA

Additional information is available at the end of the article

Abstract: Determining and linking the structural heterogeneity of recombinant antibodies to function is critical in the biopharmaceutical industry. We introduce a new microfluidic capillary electrophoresis—mass spectrometry (μCE-MS) approach to characterize intact monoclonal antibody (mAb) and simultaneously quantifying distinct variants. Our MS analysis of intact NIST mAb (RM8671) shows 18 variants identified amongst proteolytic and glycolytic modifications with a range of relative abundances between 0.1% and 100%. In order to verify our quantitative MS results, we used an established system based on capillary electrophoresis—with laser induced fluorescence (CE-LIF) for profiling the N-glycans. All major glycans were identified and further substantiated by exoglycosidase digestion. Interestingly, the μCE-MS analysis of intact NIST mAb consistently yielded higher amounts of G2FG2F-Hex glycoform (~3.4%), whereas the CE-LIF analysis indicates that only 1.4% of G2F-Gal is present. Therefore, the additional hexose adduct observed in μCE-MS may

ABOUT THE AUTHOR

The work reported here consists of combined research efforts by two groups from industry (Thermo Fisher Scientific) and one group from an academic institution (Singapore National University). Our research interests include the development of capillary electrophoresis instrumentation, reagents, software, and analytical methods for analysis of glycans from biopharmaceuticals. In addition to separation techniques we are also developing other tools such as mass spectrometry for complete characterization of biomolecules.

PUBLIC INTEREST STATEMENT

Monoclonal antibodies (mAbs) have emerged as important bio-therapeutics on the market. Since glycosylation is one of the important quality attributes of mAbs that impact efficacy, safety, and potency of the drug, regulatory agencies imposed stringent requirements for glycan analysis. High throughput glycan analysis can often be the rate-limiting step in the analytical characterization of biopharmaceuticals. Manufacturers of recombinant biopharmaceuticals have to deliver products with consistent quality attributes in order to assure reproducible clinical performance.

We introduce microfluidic capillary electrophoresis—mass spectrometry (μCE-MS) approach to characterize intact mAb and simultaneously quantifying distinct proteoform and glycoform variants. We also report the application of a high throughput glycan analysis method utilizingcapillary electrophoresis with laser-induced fluorescence detection. A simple streamlined workflow in combination with a multi-capillary CE instrument offers a high resolution and high throughput glycan analysis platform for glycan and can address the current unmet needs of the industry.

cogent oa

© 2018 The Author(s). This open access article is distributed under a Creative Commons Attribution (CC-BY) 4.0 license.

have been the glycation product of the mAb. Further analysis of deglycosylated mAb with μCE-MS made it possible to reveal the glycation with 10.5% of one hexose product and 4.9% of two hexose product in the intact deglycosylated antibody. An integrated solution using two orthogonal and complementary techniques for characterizing antibody glycosylation is provided here.

Subjects: Bioscience; Biochemistry; Biotechnology

Keywords: NIST mAb; proteoform; glycoform; glycation; capillary electrophoresis-mass spectrometry (CE-MS); capillary electrophoresis-laser induced fluorescence (CE-LIF)

1. Introduction

Recombinant antibodies have become one of the most important biopharmaceuticals since their commercialization in 1986 (Buss, Henderson, McFarlane, Shenton, & De Haan, 2012; Ecker, Jones, & Levine, 2015) as high target specificity within the human body provides significant value in targeted therapy. During drug development and manufacturing, undesired mutations and in vitro modifications may introduce sample heterogeneity causing changes to the protein structure and potentially leading to the loss of drug efficacy (Kaltashov et al., 2012). Therefore, the availability of a quick and comprehensive method to detect and assess any variability that might occur during drug development is necessary. Mass spectrometry (MS) has become one of the most important monoclonal antibody (mAb) characterization methods in recent years (Beck, Sanglier-Cianférani, & Van Dorsselaer, 2012; Chen et al., 2011). Characterization of mAbs at the intact level requires minimal sample preparation, which can save time and reduce the possibility of degradation during sample handling. Matrix-assisted laser desorption/ionization (MALDI) MS is commonly used for fast screening of intact proteins as it requires simple sample preparation and easy identification due to the feature of singly charged ions (Lin, Campbell, Mueller, & Wirth, 2003). Although the detection efficiency of very large biomolecular ions has been much improved (Chen, Lin, Chu, & Chen, 2010; Wenzel, Matter, Schultheis, & Zenobi, 2005), small variants occurring within a given mAb cannot be resolved by MALDI-MS due to the insufficient resolution at very high m/z range. Electrospray ionization (ESI) MS is another way for large biomolecule analysis that generates multiple charges at low *m/z* range (Fenn, Mann, Meng, Wong, & Whitehouse, 1989), and its exact molecular weight can be obtained by advanced deconvolution algorithms. Following the development of high resolution MS, major post-translational modifications (PTMs) can be resolved at the intact level (Hao et al., 2015). Manufactured mAb products are heterogeneous due to a complex mixture of all possible PTMs (Walsh & Jefferis, 2006), and each variant might consist of different combinations of PTMs, such as glycosylation, N-terminal pyroglutamate formation, deamidation, oxidation and glycation (Li et al., 2015). Simultaneous introduction of all present variants into MS can hinder the accurate assignment of charged ions to the corresponding variant, especially given the multiply-charged nature of the ions generated using ESI. Proper upfront separation of the complex variant mixture will be very helpful for successful MS analysis.

The current and most commonly used technique for protein characterization is based on liquid chromatography—mass spectrometry (LC-MS). Here, the LC separation of protein variants is not efficient, especially when LC buffer has to be adjusted to MS compatible (Bobály, Sipkó, & Fekete, 2016; Regnier & Kim, 2018). In comparison, capillary electrophoresis (CE) has been described as an effective and gentle protein separation method (Schwartz & Pritchett, 1994) and can be performed at room temperature. Furthermore, charge variance amongst protein variants allows separation to occur in a predictable way (Carbeck, Colton, Gao, & Whitesides, 1998). Coupling this gentle and high efficient separation technology to MS has been pursued in recent years (Cai & Henion, 1995; Liu, Zhang, & Dovichi, 2005; Smith, Wahl, Goodlett, & Hofstadler, 1993). Several CE-MS platforms were introduced recently for intact protein separation and characterization (Han, Rock, Pearson, & Rock, 2016; Sarg et al., 2013; Zhao, Sun, Knierman, & Dovichi, 2016). The newly introduced microfluidic CE-MS (μCE-MS)

technology used in this study provides unprecedented short analysis time of 3 min by using higher electrical field strength than previously reported (Redman, Batz, Mellors, & Ramsey, 2015; Redman, Mellors, Starkey, & Ramsey, 2016; Wang et al., 2015). LC-MS and μCE-MS techniques have not been able to provide quantitative results on detectable variants of intact proteins.

Our study shows the feasibility and first results on quantifying variants of intact proteins. Further, it also determined the presence and relative abundance of non-enzymatic glycation in our study of NIST mAb that is known to occur in proteins (Hermodson, 1992, Takátsy et al., 2009). In order to validate the analysis of intact protein glycoforms with μCE-MS, we examined the glycosylation profile via capillary electrophoresis – laser-induced fluorescence (CE-LIF). The glycans released from the mAb by Peptide-N-Glycosidase F (PNGase F) digestion were labeled with 8-Aminopyrene-1,3,6-Trisulfonic Acid, Trisodium Salt (APTS) and analyzed via CE-LIF, resulting into profiles showing the relative abundance of each glycan peak. CE-LIF has been widely used in glycan analysis due to its unique capability for multiplex high throughput separation of glycan isomers, and sensitive LIF detection (Aich, Lakbub, & Liu, 2016; Feng et al., 2017).

2. Materials and methods

The mAb used in this study is a reference material known as NIST RM8671 (Lot Number 14HB-D-001) and was obtained from the National Institute of Standard and Technology (NIST; Gaithersburg, MD) (Formolo et al., 2015). NIST RM8671 is a recombinant humanized IgG1κ antibody, and process-related impurities have been removed through various purification steps (Tarlov & Choquette, 2016). NIST RM8671 is characterized by high abundance of PTMs of C-terminal lysine clipping, N-terminal pyroglutamination, and glycosylation, and low abundance of glycation, deamidation, and oxidation. GlycanAssure Core Reagents kit, GlycanAssureBeads, GlycanAssureAPTS kit, LIZ600 DNA Ladder (LIZ 600), POP-7 polymer and 16-hole magnet stand were acquired from Thermo Fisher Scientific (South San Francisco, CA, USA). PNGase F and cleavage buffers were provided in each dye labeling kit. Galactosidase S (P0745S) and Neuramididase A (P0722S) enzymes were purchased from New England Biolabs (Ipswich, MA).

2.1. Intact mAb μCE-MS

The CE separation of the intact mAb was performed using a ZipChip™ CE device (908 Devices/Thermo Scientific). The chip was first primed with background electrolyte solution (BGE) consisting of 0.2% acetic acid and 10% 2-propanol (pH 3.17) with 6 psi gas pressure supplied from the sheath gas of MS. Once a stable ESI was established, 10 μL of 0.5 μg/μL mAb was manually pipetted into the sample reservoir. Samples were injected into the CE separation channel with 2 psi pressure for 5 s, which corresponds to an injection volume of 0.65 nL. High voltage of ~20 kV was applied for CE separation within 22 cm channel length on the HR chip, and ~2 kV was used for the ESI. The separations were completed in 3 min. The intact mAb mass spectroscopy was analyzed using a Q-Exactive Plus MS (Thermo Scientific). The MS resolution of 17.5 k was used at the scan rate of 12 scans/s. In-source collision-induced dissociation (100 e.v.) was provided to assist desolvation. The mass to charge (m/z) range (2500–6000) was selected for this mild-denatured condition of mAb. The MS instrument provided 2 psi of sheath gas to support ESI generation. Pressure assistance with the pressure of 2 psi was used to support CE migration. BioPharma Finder 2.0 software (Thermo Scientific) was used for data analysis. The amino acid sequence, disulfide bond linkages, and PTM information of NIST mAb were set up in the database of protein sequence manager. The intact mass was calculated through the deconvolution algorithm of ReSpect™ (Positive Probability, Ltd. (PPL)) using sliding window feature. The identification of each variant was below a mass error of 25 ppm.

The NIST mAb was stored in formulation buffer with 12.5 mM L-histidine, 12.5 mM L-histidine HCL (pH 6.0) at −80°C until analysis. The mAb sample was diluted with deionized water to 0.5 μg/μL for the intact analysis. No desalting procedure was used. BGE was prepared in a glass bottle before the

experiments. Solvents of Optima LC/MS grade from Fisher Scientific (Fair Lawn, NJ) were used in this study including water (W6-500), acetic acid (A113-50), and 2-propanol (A461-1).

2.2. Deglycosylated mAb μCE-MS

For the investigation of glycation, the N-glycan has to be removed from the intact mAb. The deglycosylation was performed with combination of 10 μL of NIST-mAb (10 μg/μL), 5 μL of PNGase F (N-Glycanase Ultra, 400 mU), 5 μL of HEPES buffer (0.5 M, pH 7.9), and 80 μL of water at 37°C overnight. The deglycosylated mAb was then collected with 10k centrifugal filters (Amicon Ultra-0.5 mL) with a final concentration of 0.5 μg/μL.

2.3. Glycan CE-LIF

Glycan release, purification, and fluorescent labeling were performed by following the GlycanAssure kit protocol. The detailed procedure for glycan release, glycan purification, and fluorescent labeling were introduced in our previous work (Feng et al., 2017).

In specific, for glycan release, 10 μL NIST-mAb (10 μg/μL) combined with 25.5 μL HPLC grade water, and 4 μL PNGase F cleavage buffer to reach a volume of 39.5 μL. 0.5 μL recombinant PNGase F (500 U/μL) was added into the above mixture. The cleavage reaction was incubated at 50°C for 1 h. The released glycan further passed through the purification step by using magnetic beads and eluted to 50 μL elution buffer, and then 20 μL of released glycans were labeled with APTS reagent in the APTS Kit. The excess dye was removed by magnetic beads before the sample was collected.

For the exoglycosidase treatment of N-glycan released from NIST-mAb, 9 μL APTS labeled glycans were mixed with 1 μL respective enzyme, additional 1 μL 0.5 M sodium acetate, pH 5.5, and 1 μL 1 mg/mL BSA were added. The mixtures were then incubated at 37°C for 1 h (Feng et al., 2017).

DNA Analyzer (ABI 3130xl, Applied Biosystems) was used for glycan separation with 50 cm × 16-capillary array and POP 7 polymer. The separation module applied was the default module, Fragmentanalysis_POP_7. Electropherograms data were collected by GeneMapper 5.0 (Thermo Fisher Scientific). The peak areas and relative abundance were captured by using an in-house developed Matlab application (Table 2). A 96-well reaction plate (Thermo Fisher Scientific) was used to run the samples. In each plate well, 2 μL labeled glycan solution was mixed with 8 μL CE sample loading matrix. While the loading matrix was prepared by adding 8 μL of LIZ 600 into 200 μL CE loading buffer. The loading buffer matrix was incubated at 60°C for 5 min and cooled down to room temperature prior to use.

3. Results and discussion

The analysis and characterization of intact proteins is commonly conducted via LC-MS, but the LC separation of protein variants with MS compatible buffers is still challenging. In recent years, advancements in CE-MS technology, such as microfluidic chip based separation systems, have shown that these emerging tools are suitable for intact protein analysis and higher throughput. In this study, the intact mAb was first screened with μCE-MS. After the glycans were released, de-glycosylated mAb was analyzed with μCE-MS, and released glycan was analyzed by the CE-LIF (Figure 1).

3.1. Intact mAb

The intact mAb was directly analyzed via μCE-MS utilizing the ZipChip^{TM} CE interface technology, which is capable to separate mAb variants before entering the MS system (Supporting Information Figure S1). The intact NIST mAb was separated into several peaks within a 3-min separation window (Figure 2A). The separated variant forms include 0 additional lysine (0-Lys or 0K), 1 additional lysine (1-Lys or 1K) and 2 additional lysines (2-Lys or 2K) at the C-terminal of heavy chains. The positive charges from the additional lysines reduced the migration time. The mass spectra from 0-Lys, 1-Lys, and 2-Lys became distinguishable and clear after they were separated (Figure 2A,B). The charge distribution of the intact mAb mass spectrum remained within the mass range of *m/z* 4,600–5,800 with corresponding charge states (Figure 2B). Figure 2C shows the deconvoluted mass spectrum across all charge states for 0-Lys, 1-

cogent chemistry

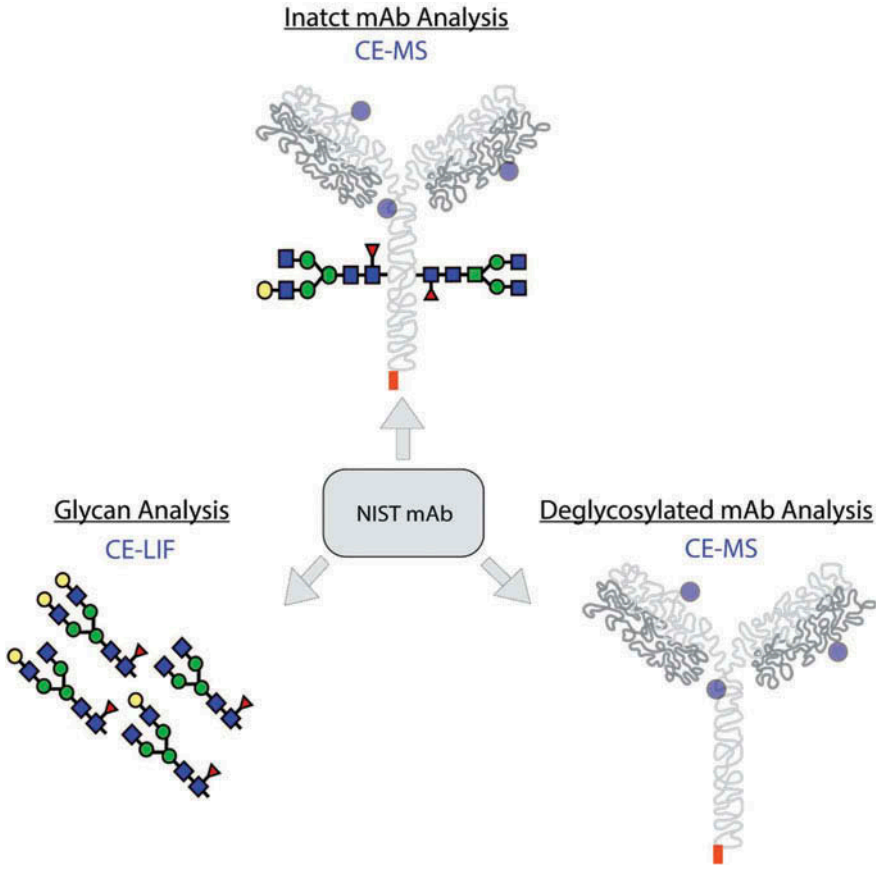


Figure 1. Workflow for the comprehensive characterization of NIST mAb includes the intact mAb analysis with μCE-MS, glycoform analysis with CE-LIF, and deglycosylated mAb analysis with μCE-MS.

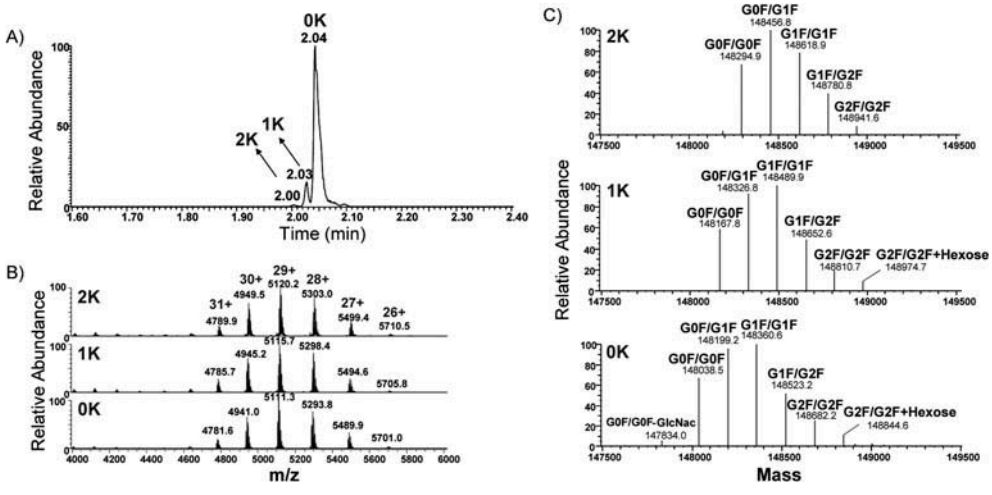


Figure 2. (A) Electropherograms of the NIST mAb in a 3-min run on a microfluidic CE coupled to a Q-Exactive MS. Variant forms having 0K, 1K and 2K attached to the C-terminal heavy chain of mAb were separated. (B) Three mass spectra were averaged over the widths from the three peaks on the electropherogram from left to right. (C) Deconvoluted mass spectra from three different migration time intervals including mAb with 2K adduct, mAb with 1K adduct, and mAb with 0K adduct.

Lys and 2-Lys. Here, seven clear peaks with a difference of 162 Da mass can be resolved after deconvolution with the sliding window ReSpect™ algorithm indicative of glycan variants. Deconvolutions were performed over each specific time window for 0-Lys, 1-Lys, and 2-Lys variants (Figure 2C). Five major glycoforms were observed at different lysine variants. Due to higher signal intensity from the 0-Lys and 1-Lys variants, two additional glycoforms were observed from the 0-Lys variant, and one from the 1-Lys variant. Overall, 18 described variants from the NIST mAb were identified on the μCE-MS platform and their relative abundances were quantified (Table 1A). CE-MS platforms are capable of separating multiple lysine variants and our study presents the first quantitative results on

protein variants. The presented μCE-MS analysis workflow provides a quick solution for intact mAb characterization. Upfront CE separation of charge variants reduces the complexity of heterogeneous mAbs, and the detection of lower abundant variants becomes possible via MS analysis. Presented data provide three orders of dynamic range to detect compounds down to 0.09% relative abundance (e.g. glycosylation form of G2F/G2F and the lowest abundance of lysine clipping form of 2-Lys). The identified variants were confirmed despite narrow migration times (Supporting Information Figure S2). The same 18 variants are also presented as the fractional abundance relative to total peak area for each lysine variant (Table 1B). The intact mAb μCE-MS analysis has shown all major glycoforms, as well as all three possible lysine variants and their relative quantification. This method is a useful tool for quick screening of therapeutic mAbs, including PTM information of proteoforms and glycoforms.

3.2. Released glycan

In order to verify our quantitative μCE-MS results, we used the established CE-LIF method for verification for deep glycan profiling and quantitative analysis. Released glycans from the NIST mAb were labeled with APTS and analyzed via CE-LIF (Supporting Information Figure S3). Obtained electropherograms show 17 peaks of resolved APTS-labeled glycans (Figure 3). All major glycan structures were identified and labeled. Only one peak was not identified due to lack of glycan standards and very low relative quantity to positively determine the structure. The observation of G0F-GlcNAc, G0F, G1F, G1F′, G2F, and G2F+Gal aligns with the intact mAb glycoforms in the μCE-MS intact mAb analysis. Ten additional glycans including G0-GlcNAc, G1F-GlcNAc, G2F+2Gal, and other minor fucosylated and sialylated glycans, can be identified from the released glycans using CE-LIF but not in the intact mAb μCE-MS analysis. Identification of the glycans by CE-LIF was performed by using a parallel exoglycosidase approach using two enzymes (Supporting Information Figure S4 and Table S2). Further confirmation of individual glycans was done by spiking of the respective individual standards (data not shown). CE separation system showed the separation of structural isomers of G1F (Peaks 10 and 11) and G2F+Gal (Peaks 15 and 16), arising from α1-3 and α1-6-arm galactosylation. The peak assignment was made based on the literature precedence, the peaks with α1-6-arm galactosylation have been reported to migrate faster than the peaks with α1-3-arm galactosylation (Reusch et al., 2015). The relative quantity of each of these glycans was determined from three separate glycan releases and labeling experiments. Each labeled glycan sample was analyzed by three consecutive injections on a DNA Analyzer. Repeatability of NIST mAb APTS analysis using DNA Analyzer is evaluated by analyzing the peak area ratios of all the peaks above 0.1% for different IgG input amounts (Supporting Information Figure S5). For data evaluation all peaks above 0.1% relative abundance were numbered and peak areas were added-up to 100% and respective standard deviations (SD) were calculated (Table 2 and Supporting Information Table S1). The excellent reproducibility of the method corroborated by the SD values make the sample preparation method very robust and precise. Small SDs obtained across various input amounts of NIST mAb resulted in high confidence in the relative quantitation of each glycan found in the antibody (Supporting Information Table S1). The resulting relative quantitation is shown in Table 2. As expected, the most abundant glycans found in this NIST mAb are the G0F, G1F and G2F, where the G0F and G1F both in the range of 40% of total N-glycan released from the NIST mAb and the G2F is about 7% of the total glycan. This finding is in agreement with the μCE-MS analysis of the intact NIST mAb. The CE-LIF based analysis offers higher sensitivity compared to μCE-MS. Therefore, multiple other glycan structures with <5% abundance of total glycan were identified via CE-LIF. The relative quantity of each major glycoform is highly similar between the intact mAb μCE-MS method and the released glycan CE-LIF analysis. The relative quantities of the APTS labeled glycans reported here are in agreement with recently reported values (Hilliard et al., 2017; Prien et al., 2015). Interestingly, we detected the G2F(+Gal) glycan structure with 1.4% fractional abundance, but we detected more than twice as much of G2F(+Hex) in 0- and 1-lysine variants in the intact NIST mAb via CE-MS analysis. This difference in abundance indicates that only half of the +Hex structure reported by our intact NIST mAb analysis stems from the +Gal, and half of the unknown +Hex may be attributed to glycation at the protein backbone. Therefore, we aimed to analyze deglycosylated mAb via CE-MS to validate the presence of glycation.

Table 1. (A) 18 variants identified from the NIST mAb in a 3-min run of μCE-MS, including the information of relative abundance and migration time. (B) Five major glycoforms were listed in the three groups of lysine variances including 0K, 1K, and 2K. Their fractional abundance were calculated from the group of 0K, 1K and 2K

(A)

	Proteoform	Glycoform	Observed mass (Da)	Theoretical mass (Da)	Mass error (ppm)	Relative abundance	Migration time (min)
1	mAb	G1FG1F/G0FG2F	148360.57	148361.35	5.2	100.00	2.032–2.142
2	mAb	G0FG1F	148199.22	148199.21	0.1	98.63	2.032–2.134
3	mAb	G0FG0F	148038.50	148037.07	9.7	67.84	2.004–2.115
4	mAb	G1FG2F	148523.22	148523.49	1.8	45.46	2.032–2.134
5	mAb	G2FG2F	148682.15	148685.63	23.4	16.06	2.044–2.138
6	mAb+Hex	G2FG2F	148844.60	148847.77	21.3	11.84	2.036–2.127
7	mAb	G0FG0F-GlcNAc	147834.03	147833.88	1.1	4.98	2.036–2.103
8	mAb plus 1K	G0FG1F	148326.84	148327.41	3.8	9.31	2.012–2.035
9	mAb plus 1K	G1FG1F/G0FG2F	148489.86	148489.55	2.1	8.39	2.012–2.035
10	mAb plus 1K	G0FG0F	148167.80	148165.27	17.1	6.47	2.015–2.035
11	mAb plus 1K	G1FG2F	148652.55	148651.69	5.8	4.27	2.015–2.035
12	mAb plus 1K	G2FG2F	148810.70	148813.83	21.0	1.62	2.021–2.035
13	mAb plus 1K+Hex	G2FG2F	148974.68	148975.97	11.0	1.09	2.015–2.038
14	mAb plus 2K	G0FG1F	148456.81	148455.61	8.1	1.03	1.988–2.014
15	mAb plus 2K	G1FG1F/G0FG2F	148618.85	148617.75	7.4	0.81	1.984–2.014
16	mAb plus 2K	G0FG0F	148294.88	148293.47	9.5	0.70	1.988–2.014
17	mAb plus 2K	G1FG2F	148780.83	148779.89	6.4	0.41	1.988–2.014
18	mAb plus 2K	G2FG2F	148941.58	148942.03	3.0	0.09	1.992–2.014

(B)

Lysine variance	Glycoform	Sum intensity	Fractional abundance (%)
	G1FG1F/G0FG2F	4.81E+09	29.0
	G0FG1F	4.74E+09	28.6

(*Continued*)

cogent chemistry

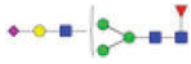
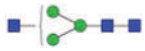
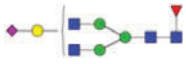
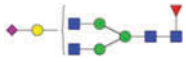
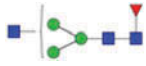
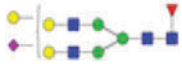
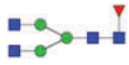
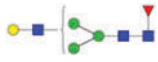
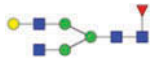
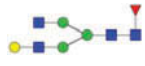
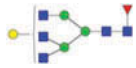
Table 1. (*Continued*)

(B)

Lysine variance	Glycoform	Sum intensity	Fractional abundance (%)
0K	G0FG0F	3.26E+09	19.7
	G1FG2F	2.19E+09	13.2
	G2FG2F	7.72E+08	4.7
	G2FG2F+Hex	5.69E+08	3.4
	G0FG0F-GlcNAc	2.40E+08	1.4
	G0FG1F	4.47E+08	29.9
	G1FG1F/G0FG2F	4.04E+08	27.0
1K	G0FG0F	3.11E+08	20.8
	G1FG2F	2.05E+08	13.7
	G2FG2F	7.79E+07	5.2
	G2FG2F+Hex	5.22E+07	3.5
	G0FG1F	4.95E+07	33.9
	G1FG1F/G0FG2F	3.89E+07	26.6
2K	G0FG0F	3.35E+07	22.9
	G1FG2F	1.98E+07	13.5
	G2FG2F	4.30E+06	2.9

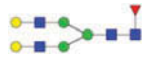
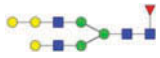
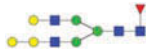
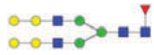
cogent chemistry

Table 2. Detected glycan signals at 100 µg injected sample with relative peak area ratios and correlating standard deviations (SD)

		Rel. peak area	
Peak no.	**Glycan ID**	**Average (%)**	**STD (%)**
1		0.77	0.04
2		0.31	0.04
3		0.12	0.03
4		0.17	0.07
5		2.67	0.12
6		0.25	0.03
7		38.96	1.86
8		2.40	0.11
9	Unknown	0.46	0.05
10		31.08	0.66
11		9.80	0.38
12		0.99	0.05

(*Continued*)

Table 2. (*Continued*)

		Rel. peak area	
Peak no.	**Glycan ID**	**Average (%)**	**STD (%)**
13		1.64	0.08
14		7.43	0.28
15		1.63	0.09
16		0.30	0.03
17		1.02	0.08

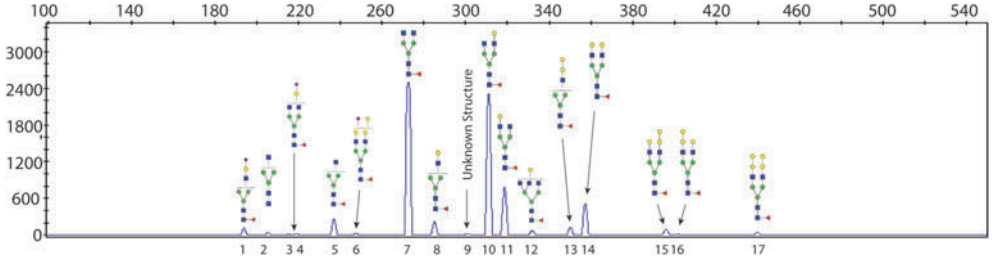


Figure 3. CE-LIF electropherogram for APTS-labeled N-glycans released from NIST mAb (IgG input 100 mg, 10× dilution).

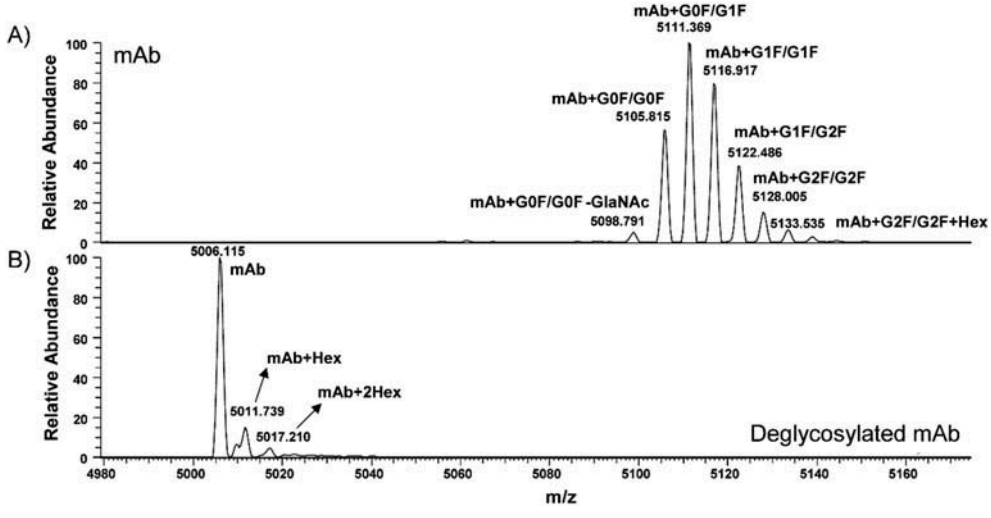


Figure 4. Investigation of glycation by comparing the raw mass spectra from (A) intact mAb and (B) deglycosylated mAb. Spectra comes from 0-Lys variance under 29+ charge state.

Table 3. Identified variants from the deglycosylated NIST mAb

Proteofrom	Glycoform	Observed mass (Da)	Theoretical mass (Da)	Mass error (ppm)	Sum intensity	Relative abundance (%)	Fractional abundance (%)
mAb	No	145148.04	145146.43	11.1	1.31E+11	100.0	79.9
mAb + Hexose	No	145308.87	145308.57	2.1	1.73E+10	13.2	10.5
mAb + 2Hexose	No	145467.56	145470.71	21.7	8.01E+09	6.1	4.9
mAb plus 1K	No	145275.80	145274.63	8.1	6.98E+09	5.3	4.2
mAb plus 2K	No	145404.51	145402.83	11.6	7.91E+08	0.6	0.5

3.3. Deglycosylated mAb

After the glycans were removed by PNGase F, the deglycosylated mAb was analyzed by μCE-MS. In comparison to the raw mass spectra from the 0-Lys part of the intact mAb and deglycosylated mAb, a clear shift from the higher *m/z* to the lower *m/z* region, which comes from the release of glycan was observed (Figure 4A,B). After glycan release, seven different glycoforms were reduced to 3. All N-linked glycans were removed by PNGase F, and only one pure compound from the deglycosylated mAb was expected. The extra two peaks with the mass shift of 162 and 324 Da were observed and were recognized as one and two hexose adducts from glycation. The quantitative values of hexose adducts combined with lysine variants are listed in Table 3. The quantity of mAb0K(+1Hex) with 10.5% and mAb0K(+2Hex) with 4.9% is greater than the estimated relative quantity of the G2F(+Gal) with 1.4% from the glycan analysis. And finding of the deglycosylated mAb (+Hex) adduct indicates that there may be some level of glycation in this lot of mAb.

4. Conclusion

The presented μCE-MS analysis workflow provides a quick solution for intact mAb characterization, and our study presents the first quantitative results on protein variants. The quantitative MS results have been corroborated by the established CE-LIF method. In addition, the μCE-MS measurements detected a glycation event, which was further verified by CE-LIF examination. Overall, the high-throughput μCE-MS and CE-LIF analyses provided comprehensive information on the proteoforms, glycoforms, and glycation of the mAb of interest.

Abbreviations

NIST, National Institute of Standard and Technology; mAb, monoclonal antibody; PNGase F, Peptide-N-Glycosidase F, 8-Aminopyrene-1,3,6-Trisulfonic Acid, Trisodium Salt APTS, CE, capillary electrophoresis; MS, mass spectrometry; LIF, laser induced fluorescence; MALDI, matrix-assisted laser desorption ionization; ESI, electrospray ionization; PTMs, post-translational modifications; BGE, background electrolyte solution; LC, liquid chromatography; μCE-MS, microfluidic capillary electrophoresis-mass spectrometry

Supplementary material

Supplemental data for this data article can be accessed here.

Acknowledgements

The authors gratefully acknowledge the help and support of Scott Mellors, Erin Redman from 908 Devices Inc., and Zoltan Szabo, Stephane Houel, Jennifer Sutton, and Darren Bauer from Thermo Fisher Scientific Inc.

Funding

The authors received no direct funding for this research.

Competing interests

No potential conflicts of interest were disclosed.

Author details

Chien-Hsun Chen[1,2]
E-mail: alexchen95131@gmail.com
ORCID ID: http://orcid.org/0000-0003-2620-5066
Huatao Feng[3]
E-mail: fhtfht@gmail.com
Rui Guo[3]
E-mail: ruiguo1980@gmail.com
Pingjing Li[3]
E-mail: erilp@nus.edu.sg
Anna Karen C. Laserna[3]
E-mail: chmakcl@nus.edu.sg
Ya Ji[3]
E-mail: jiya@u.nus.edu
Bao Hui Ng[3]
E-mail: e0267404@u.nus.edu
ORCID ID: http://orcid.org/0000-0003-4946-3623
Sam Fong Yau Li[3]
E-mail: chmlifys@nus.edu.sg
Shaheer H. Khan[2]
E-mail: Shaheer.Khan@thermofisher.com
Aran Paulus[1]
E-mail: aran.paulus@thermofisher.com

Shiaw-Min Chen[2]
E-mail: Shiaw-Min.Chen@thermofisher.com
Achim E. Karger[2]
E-mail: Achim.Karger@thermofisher.com
Michael Wenz[2]
E-mail: Michael.Wenz@thermofisher.com
Daniel Lopez Ferrer[1]
E-mail: daniel.lopezferrer@thermofisher.com
Andreas F. Huhmer[1]
E-mail: andreas.huhmer@thermofisher.com
Andreas Krupke[2]
E-mail: andreas.krupke@thermofisher.com
E-mail: Andreas.krupke@gmail.com
[1] Thermo Fisher Scientific, Chromatography and Mass Spectrometry, 355 River Oaks Parkway, San Jose, CA 95134, USA.
[2] Thermo Fisher Scientific, Life Science Solutions, 180 Oyster Point Parkway, South San Francisco, CA 94080, USA.
[3] Department of Chemistry, National University of Singapore, Science Drive 3, Singapore S117543, Singapore.

Citation information
Cite this article as: Intact NIST monoclonal antibody characterization—Proteoforms, glycoforms—Using CE-MS and CE-LIF, Chien-Hsun Chen, Huatao Feng, Rui Guo, Pingjing Li, Anna Karen C. Laserna, Ya Ji, Bao Hui Ng, Sam Fong Yau Li, Shaheer H. Khan, Aran Paulus, Shiaw-Min Chen, Achim E. Karger, Michael Wenz, Daniel Lopez Ferrer, Andreas F. Huhmer & Andreas Krupke, *Cogent Chemistry* (2018), 4: 1480455.

References

Aich, U., Lakbub, J., & Liu, A. (2016). State-of-the-art technologies for rapid and high-throughput sample preparation and analysis of N-glycans from antibodies. *Electrophoresis*, *37*, 1468–1488. doi:10.1002/elps.201500551

Beck, A., Sanglier-Cianférani, S., & Van Dorsselaer, A. (2012). Biosimilar, biobetter, and next generation antibody characterization by mass spectrometry. *Analytical Chemistry*, *84*, 4637–4646. doi:10.1021/ac3002885

Bobály, B., Sipkó, E., & Fekete, J. (2016). Challenges in liquid chromatographic characterization of proteins. *Journal of Chromatography B*, *1032*, 3–22. doi:10.1016/j.jchromb.2016.04.037

Buss, N. A., Henderson, S. J., McFarlane, M., Shenton, J. M., & de Haan, L. (2012). Monoclonal antibody therapeutics: History and future. *Current Opinion in Pharmacology*, *12*, 615–622. doi:10.1016/j.coph.2012.08.001

Cai, J., & Henion, J. (1995). Capillary electrophoresis-mass spectrometry. *Journal of Chromatography A*, *703*, 667–692. doi:10.1016/0021-9673(94)01178-H

Carbeck, J. D., Colton, I. J., Gao, J., & Whitesides, G. M. (1998). Protein charge ladders, capillary electrophoresis, and the role of electrostatics in biomolecular recognition. *Accounts of Chemical Research*, *31*, 343–350. doi:10.1021/ar970255q

Chen, C.-H., Lin, J.-L., Chu, M.-L., & Chen, C.-H. (2010). MALDI ion trap mass spectrometer with charge detector for large biomolecule detection. *Analytical Chemistry*, *82*, 10125–10128. doi:10.1021/ac102217e

Chen, G., Warrack, B. M., Goodenough, A. K., Wei, H., Wang-Iverson, D. B., & Tymiak, A. A. (2011). Characterization of protein therapeutics by mass spectrometry: Recent developments and future directions. *Drug Discov Today*, *16*, 58–64. doi:10.1016/j.drudis.2010.11.003

Ecker, D. M., Jones, S. D., & Levine, H. L. (2015). The therapeutic monoclonal antibody market. *MAbs*, *7*, 9–14. doi:10.4161/19420862.2015.989042

Feng, H.-T., Li, P., Rui, G., Stray, J., Khan, S., Chen, S.-M., & Li, S. F. Y. (2017). Multiplexing N-glycan analysis by DNA analyzer. *Electrophoresis*, *38*, 1788–1799. doi:10.1002/elps.201600404

Fenn, J., Mann, M., Meng, C., Wong, S., & Whitehouse, C. (1989). Electrospray ionization for mass spectrometry of large biomolecules. *Science*, *246*, 64–71.

Formolo, T., Ly, M., Levy, M., Kilpatrick, L., Lute, S., Phinney, K., … Schiel, J. (2015). Determination of the NISTmAb primary structure. In *State-of-the-art and emerging technologies for therapeutic monoclonal antibody characterization volume 2 biopharmaceutical characterization: The NISTmAb case study* (pp. 1–62). American Chemical Society.

Han, M., Rock, B. M., Pearson, J. T., & Rock, D. A. (2016). Intact mass analysis of monoclonal antibodies by capillary electrophoresis-mass spectrometry. *Journal of Chromatography B, Analytical Technologies in the Biomedical and Life Sciences*, *1011*, 24–32. doi:10.1016/j.jchromb.2015.12.045

Hao, Z., Zhang, T., Xuan, Y., Wang, H., Qian, J., Lin, S., … Cianférani, S. (2015). Intact antibody characterization using orbitrap mass spectrometry. In *State-of-the-art and emerging technologies for therapeutic monoclonal antibody characterization volume 3. Defining the next generation of analytical and biophysical techniques* (pp. 289–315). American Chemical Society.

Hermodson, M. (1992). *Foreword A2 - Angeletti, Ruth Hogue. Techniques in protein chemistry III* (p. xiii). Academic Press.

Hilliard, M., Alley, W. R., Jr., McManus, C. A., Yu, Y. Q., Hallinan, S., Gebler, J., & Rudd, P. M. (2017). Glycan characterization of the NIST RM monoclonal antibody using a total analytical solution: From sample preparation to data analysis. *MAbs*, *9*, 1349–1359. doi:10.1080/19420862.2017.1377381

Kaltashov, I. A., Bobst, C. E., Abzalimov, R. R., Wang, G., Baykal, B., & Wang, S. (2012). Advances and challenges in analytical characterization of biotechnology products: Mass spectrometry-based approaches to study properties and behavior of protein therapeutics. *Biotechnology Advances*, *30*, 210–222. doi:10.1016/j.biotechadv.2011.05.006

Li, W., Kerwin, J. L., Schiel, J., Formolo, T., Davis, D., Mahan, A., & Benchaar, S. A. (2015). Structural elucidation of post-translational modifications in monoclonal antibodies. In *State-of-the-art and emerging technologies for therapeutic monoclonal antibody characterization volume 2. Biopharmaceutical characterization: The NISTmAb case study* (pp. 119–183). American Chemical Society.

Lin, M., Campbell, J. M., Mueller, D. R., & Wirth, U. (2003). Intact protein analysis by matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry*, *17*, 1809–1814. doi:10.1002/rcm.1102

Liu, C. C., Zhang, J., & Dovichi, N. J. (2005). A sheath-flow nanospray interface for capillary electrophoresis/mass spectrometry. *Rapid Communications in Mass Spectrometry*, *19*, 187–192. doi:10.1002/rcm.1769

Prien, J. M., Stöckmann, H., Albrecht, S., Martin, S. M., Varatta, M., Furtado, M., … Schiel, J. E. (2015). Orthogonal technologies for NISTmAb N-glycan structure elucidation and quantitation. In *State-of-the-art and emerging technologies for therapeutic monoclonal antibody characterization volume 2. Biopharmaceutical characterization: The NISTmAb case study* (pp. 185–235). American Chemical Society.

Redman, E. A., Batz, N. G., Mellors, J. S., & Ramsey, J. M. (2015). Integrated microfluidic capillary electrophoresis-electrospray ionization devices with online MS detection for the separation and

characterization of intact monoclonal antibody variants. *Analytical Chemistry*, *87*, 2264–2272. doi:10.1021/ac503964j

Redman, E. A., Mellors, J. S., Starkey, J. A., & Ramsey, J. M. (2016). Characterization of intact antibody drug conjugate variants using microfluidic capillary electrophoresis-mass spectrometry. *Analytical Chemistry*, *88*, 2220–2226. doi:10.1021/acs.analchem.5b03866

Regnier, F. E., & Kim, J. (2018). Proteins and proteoforms: New separation challenges. *Analytical Chemistry*, *90*, 361–373. doi:10.1021/acs.analchem.7b05007

Reusch, D., Haberger, M., Maier, B., Maier, M., Kloseck, R., Zimmermann, B., … Wuhrer, M. (2015). Comparison of methods for the analysis of therapeutic immunoglobulin G Fc-glycosylation profiles–part 1: Separation-based methods. *MAbs*, *7*, 167–179. doi:10.4161/19420862.2014.986000

Sarg, B., Faserl, K., Kremser, L., Halfinger, B., Sebastiano, R., & Lindner, H. H. (2013). Comparing and combining capillary electrophoresis electrospray ionization mass spectrometry and nano-liquid chromatography electrospray ionization mass spectrometry for the characterization of post-translationally modified histones. *Molecular & Cellular Proteomics*, *12*, 2640–2656. doi:10.1074/mcp.M112.024109

Schwartz, H., & Pritchett, T. (1994). *Separation of proteins and peptides by capillary electrophoresis: Application to analytical biotechnology*. Beckman Coulter.

Smith, R. D., Wahl, J. H., Goodlett, D. R., & Hofstadler, S. A. (1993). Capillary electrophoresis/mass spectrometry. *Analytical Chemistry*, *65*, 574A–584A. doi:10.1021/ac00061a717

Takátsy, A., Böddi, K., Nagy, L., Nagy, G., Szabó, S., Markó, L., … Gjerde, D. (2009). Enrichment of Amadori products derived from the nonenzymatic glycation of proteins using microscale boronate affinity chromatography. *Analytical Biochemistry*, *393*, 8–22. doi:10.1016/j.ab.2009.06.007

Tarlov, M., & Choquette, S. (2016). *NISTmAb, humanized IgG1k monoclonal antibody. Report of investigation.* Naitonal Institute of Standards and Technology.

Walsh, G., & Jefferis, R. (2006). Post-translational modifications in the context of therapeutic proteins. *Nature Biotechnology*, *24*, 1241–1252. doi:10.1038/nbt1252

Wang, X., Yi, L., Mukhitov, N., Schrell, A. M., Dhumpa, R., & Roper, M. G. (2015). Microfluidics-to-mass spectrometry: A review of coupling methods and applications. *Journal of Chromatography A*, *1382*, 98–116. doi:10.1016/j.chroma.2014.10.039

Wenzel, R. J., Matter, U., Schultheis, L., & Zenobi, R. (2005). Analysis of megadalton ions using cryodetection MALDI time-of-flight mass spectrometry. *Analytical Chemistry*, *77*, 4329–4337. doi:10.1021/ac0482054

Zhao, Y., Sun, L., Knierman, M. D., & Dovichi, N. J. (2016). Fast separation and analysis of reduced monoclonal antibodies with capillary zone electrophoresis coupled to mass spectrometry. *Talanta*, *148*, 529–533. doi:10.1016/j.talanta.2015.11.020

cogent oa

© 2018 The Author(s). This open access article is distributed under a Creative Commons Attribution (CC-BY) 4.0 license.

You are free to:
Share — copy and redistribute the material in any medium or format.
Adapt — remix, transform, and build upon the material for any purpose, even commercially.
The licensor cannot revoke these freedoms as long as you follow the license terms.

Under the following terms:
Attribution — You must give appropriate credit, provide a link to the license, and indicate if changes were made. You may do so in any reasonable manner, but not in any way that suggests the licensor endorses you or your use.
No additional restrictions

You may not apply legal terms or technological measures that legally restrict others from doing anything the license permits.

***Cogent Chemistry* (ISSN: 2331-2009) is published by Cogent OA, part of Taylor & Francis Group.**

Publishing with Cogent OA ensures:

- Immediate, universal access to your article on publication
- High visibility and discoverability via the Cogent OA website as well as Taylor & Francis Online
- Download and citation statistics for your article
- Rapid online publication
- Input from, and dialog with, expert editors and editorial boards
- Retention of full copyright of your article
- Guaranteed legacy preservation of your article
- Discounts and waivers for authors in developing regions

Submit your manuscript to a Cogent OA journal at www.CogentOA.com