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# Intact mass analysis of monoclonal antibodies by capillary electrophoresis—Mass spectrometry

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ABSTRACT

Characterization of monoclonal antibody (mAb) therapeutics by intact mass analysis provides important information on sequence integrity and post-translational modifications. In order to obtain domain specific information, monoclonal antibodies are reduced to heavy and light chain components or enzymatically digested into smaller portions or peptides. Liquid chromatography (LC) is widely used for separation of the antibody fragments in line with mass spectrometry (MS) for characterization. Capillary electrophoresis (CE) is an analytical technique with high separation efficiency, high sensitivity, and minimal inter-run sample carryover. Combining the resolving power of CE with electrospray ionization (ESI) MS has great potential in regards to accurate mass characterization of protein therapeutics and has been a long sought-after approach. However, the intrinsic technical difficulty in coupling CE to MS has hindered the broad application of CE-MS across the biopharmaceutical industry. Recently, a CE-MS interface has been developed [1] and commercialized. Herein, we report implementation of this technology for coupling CE to an Agilent time-of-flight (TOF) mass spectrometer. CE-MS provides an attractive complement to LC–MS for separation and intact mass determination of mAbs and antibody-based therapeutics.

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## 1. Introduction

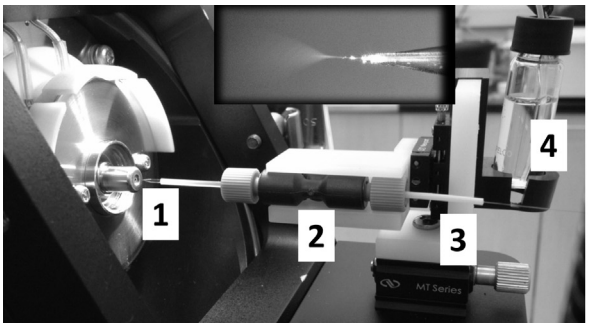
Protein therapeutics and monoclonal antibodies (mAbs) are the fastest growing therapeutic class of drug candidates and products developed by biopharmaceutical companies around the world [2–4]. Due to the complexity of the manufacturing process of mAbs, modest modifications in expression or purification conditions can result in production of heterogeneous mAb preparations consisting of individual species having altered primary sequences and varying posttranslational modifications, during cell culture expression, purification, formulation and storage [5,6]. Since heterogeneity can impact the overall stability and biological activity of mAbs, a comprehensive characterization of the preparation is required [7]. Among the extensive analytical techniques, mass spectrometry has played a key role for antibody characterization with its capability of high resolution and mass accuracy measurements. Protein intact mass analysis is an important component in the characterization of recombinant mAb therapeutics [5,7–10]. Direct mass measurement allows for the assignment of chemical modifications contributing to the product heterogeneity. Measuring whole and partial molecular species within a mAb preparation to characterize protein amino acid sequence, identify domain specific modifications, profile glycoforms and evaluate possible changes under in vivo conditions are all effective ways to identify protein features that may be susceptible to modification and that might be attributes critical to stability, efficacy and safety profiles [11].

In order to effectively characterize protein therapeutics, a sample introduction system is necessary for mass spectrometry analysis. To date, this has been largely carried out through liquid chromatography (LC) separation or direct-infusion [9,10]. The wide availability of LC columns, well-developed analytical methods, and system robustness have made LC the standard introduction system for the characterization of protein therapeutics by MS. The commonly used chromatography approaches separate proteins by their differences in hydrophobicity (reversed phase, RP, hydrophobic interaction chromatography, HIC), charge (ion exchange, IEX), or size (size exclusive chromatography, SEC) [9,10,12,13]. There are

*Abbreviations:* CE, capillary electrophoresis; CE-MS, capillary electrophoresis-mass spectrometry; LC–MS, liquid chromatography–mass spectrometry; IgG, immunoglobulin G; mAb, monoclonal antibody; TOF, time-of-flight; BGE, background electrolyte; BPE, mass spectrometry base peak electropherogram; LPA, linear polyacrylamide; PTMs, post-translational modifications; Fab, antigen-binding fragment; Fc, crystallizable fragment.

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1. Spray emitter
2. Interface tee
3. Separation capillary
4. Sheath liquid vial

**Fig. 1.** CE-MS interface installed on an Agilent TOF instrument. The outlet of the separation capillary was placed at the spray emitter tip through the interface tee. The mass spectrometer capillary voltage was set to be zero volts. The ESI voltage was provided by an external power supply (not shown), which generated an electrical field from the sheath liquid vial to the mass spectrometer. As a result, a steady electroosmotic flow was formed on the inner surface of the borosilicate glass spray emitter, providing nanoflow of sheath liquid which entrains the analytes into the mass spectrometer. The inset shows the spray plume.

inherent challenges in each of these chromatography-based techniques [9]. For example, in a typical setting of reversed phase LC–MS separation of reduced monoclonal antibody, the column temperature has to be heated (70 °C) in order to achieve baseline separation between heavy and light chains. The elevated temperature can introduce artificial degradation of these partial molecules [12]. In addition, protein retaining on the LC columns often leads to peak tailing and sample carry-over. Moreso, developing chromatography with consistent peak shapes and baseline separation of the heavy and light chains in an antibody can be time consuming with optimal conditions varying from antibody to antibody [13–15]. The impact of protein structure on pharmacokinetics has increased the need for higher protein separation efficiency combined with intact mass spectral analysis in order to characterize pharmacokinetics for biologics [9,11].

Capillary electrophoresis (CE) represents an exciting complementary separation technique to LC for proteins and antibodies. CE offers selectivity, high resolving power, and fast separation speed [16,17]. CE is a widely used separation technique for the characterization of biotherapeutics for size, charge and glycan analysis [18–21]. Numerous robust assays have been established to analyze monoclonal antibodies, such as CE-SDS in the evaluation of product-related impurity, capillary isoelectric focusing (cIEF) for charge variants analysis, and CZE for released glycan analysis with either UV or Laser Induced Fluorescence (LIF) detection [18–21]. With the success of these optical detection based methods established, it is appealing that coupling CE separation to online electrospray ionization mass spectrometers as a detector to gain additional sensitivity and accurate mass identification [22–27]. However, due to the technical difficulties in coupling CE to MS, CE-MS has yet to be routinely paired with LC–MS as an alternative or complementary separation technique for protein characterization. A significant challenge for introducing samples from CE into the mass spectrometer involves the need of electrical contact between the electrophoretic separations from CE to the electrospray ion source. Currently, there are two major types of CE-MS interface—sheath liquid interface and sheathless interface [22,23,28–30]. Sheath liquid interface coaxially adds sheath liquid at the end of the CE separation capillary to provide electrical contact therefore assisting the spray. Sheath liquid can also be modified for improving electrospray efficiency [1,22,23]. However, the sensitivity is scarified due to the high dilution rate (10 to 100× fold) of analytes by sheath liquid. In addition, peak broadening might occur due to diffusion of analytes [29]. The sheathless interface overcomes the sample dilution issue by eliminating the sheath liquid. The CE separation capillary tip was etched and inserted into the stainless steel electrospray needle filled with conductive liquid [29,30] to establish the electrical contact improving the overall sensitivity. However, sheathless interfaces can lead to instability in the electrospray and needs to be carefully monitored during operation. While this system has definite advantages, the choice of CE instrument is limited [1,29]. Recently, a nanospray sheath flow interface was designed by Dr. Dovichi's lab [1] which utilize electroosmotic flow (EOF) on the inner surface (electrokinetic flow) of borosilicate glass emitter to drive the sheath liquid, has greatly enhanced the sensitivity and spray performance [1].

Current CE-MS interface technology has broadened the scope of the technology in proteomics, metabolomics, and protein characterization particular of complex proteins such as mAbs peptides, protein digest, protein complexes as well as some intact protein analysis [1,29–35]. Here, we describe coupling CE to a time-of-flight (TOF) mass spectrometer using the electrokinetic flow driven nanospray sheath liquid interface described above [1,32]. A mix of commonly used protein standards was utilized to identify optimal source conditions for introducing protein samples into the TOF instrument after separation by CE. With optimal source conditions defined, we tested the utility of this novel CE-TOF platform to measure the intact mass for some representative fully human mAbs and their fragments under native and reducing conditions. Under reducing conditions we were able to demonstrate baseline separation of antibody heavy and light chains with accurate mass determination by TOF MS without the need for elevated temperature during sample separation. CE-MS provides an attractive complement to LC–MS for separation and intact mass determination of mAbs and antibody-based therapeutics. The setup and use of CE-MS for protein characterization is discussed in detail.

## 2. Materials & methods

### 2.1. Materials and chemicals

Monoclonal antibodies were manufactured at Amgen (Thousand Oaks, CA). Myoglobin Ribonuclease A, Cytochrome C, Insulin and all other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA). The 1× PBS buffer (phosphate buffered saline) was purchase from Life Technologies (Grand Island, NY). PNGase F enzyme was obtained from New England BioLabs Inc. (Ipswich, MA). IdeS enzyme was purchased from Genovis Inc. (Cambridge, MA). Bio-Rad Bio-Spin P-6 column was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). LPA-coated capillaries and borosilicate glass spray emitters were purchased from CMP Scientific, Corp. (Brooklyn, NY). Ultra-pure water was generated in-house from a Milli-Q Plus system from Millipore.

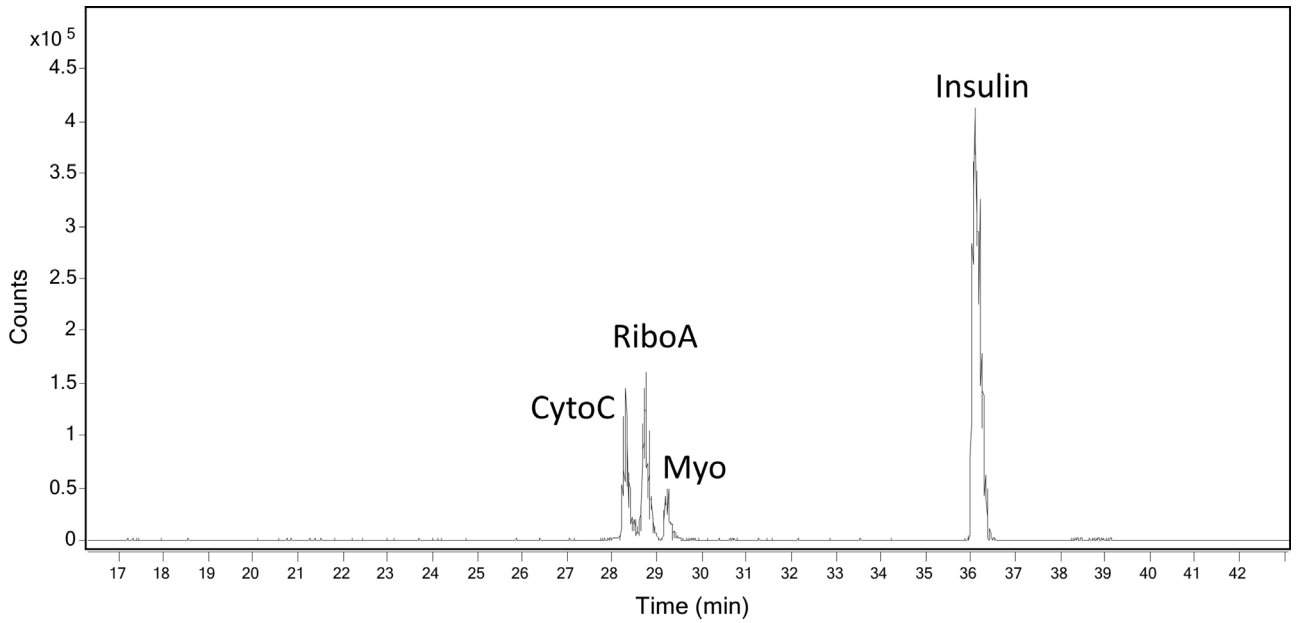



**Fig. 2.** Base peak electropherogram (mass spec) of standard protein mixture by CE-MS. Sample: 0.33 mg/mL of Cytochrome C, Ribonuclease A, Myoglobin, and Insulin in 0.1% formic acid, 30% acetonitrile. Conditions: capillary, LPA-coated fused silica (150 μm OD, 50 μm ID, 100 cm length); electrolyte, 30% acetic acid; sheath liquid, 0.5% formic acid, 50% methanol; injection, 2 s at 940 mbar; separation, 30 kV with 5 mbar pressure on capillary inlet.

### 2.2. Sample preparation

#### 2.2.1. Standard protein mixture preparation

Myoglobin, Ribonuclease A and Cytochrome C were reconstituted with water to 2 mg/mL. Insulin stock solution was prepared at 3.76 mg/mL with 0.05% formic acid. Samples were diluted with 0.1% formic acid, 30% acetonitrile to make a mixture of proteins (0.33 mg/mL of each).

#### 2.2.2. Deglycosylation of IgG1

Deglycosylated IgG1 was made a total reaction volume of 250 μL by adding 83.3 μL of 30 mg/mL IgG 1 sample, 125 μL of PNGase F, 12.5 μL of 1 M Tris, pH 7.5, and 29.2 μL of water. The solution was mixed and incubated at 37 °C for 18 h.

#### 2.2.3. Sample desalting

Unless otherwise specified, all IgG samples were desalted for CE-MS analysis. BioRad P-6 desalting columns were used for desalting per manufacturer's procedure.

#### 2.2.4. Reduction of IgG1

Monoclonal antibody samples were diluted to 4 mg/mL with 50 mM Tris, pH 8.0 buffer in the presence of 4 M Guanidine

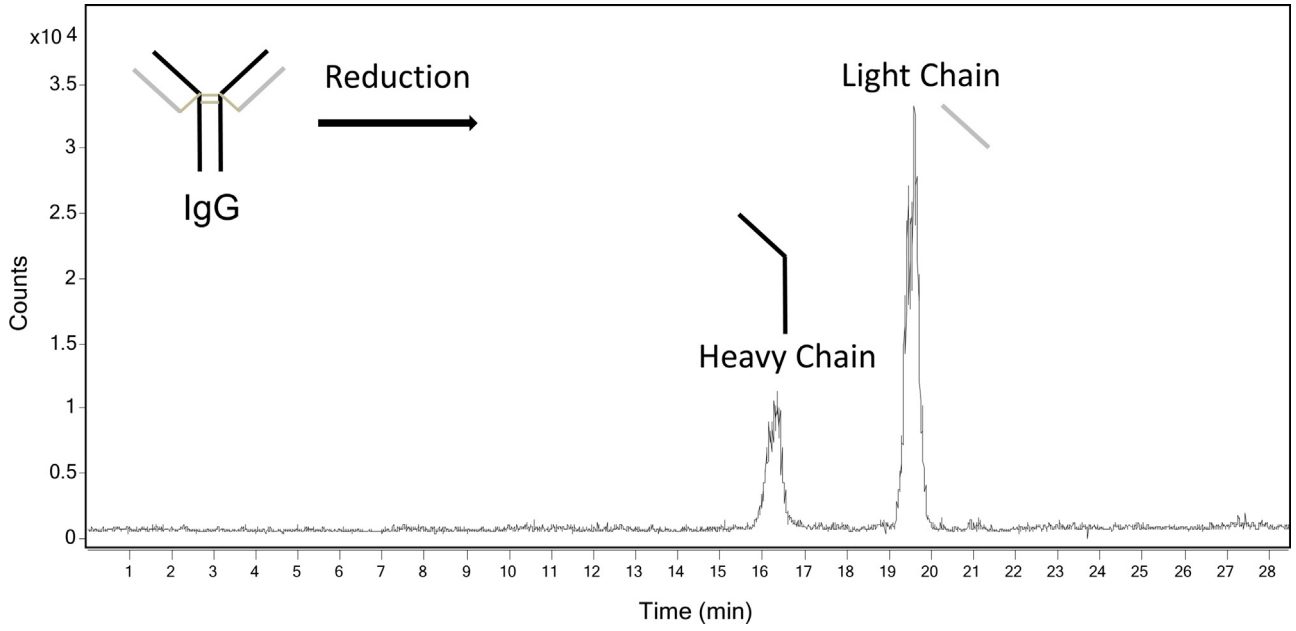



**Fig. 3.** Base peak electropherogram of reduced IgG1. Sample: 2.5 mg/mL of IgG 1 in 15% acetic acid, 15% acetonitrile. Conditions: capillary, LPA-coated fused silica (360 μm OD, 50 μm ID, 70 cm length), outlet etched to 180 μm OD; electrolyte, 10% acetic acid; sheath liquid, 0.5% formic acid, 50% methanol; injection, 5 s at 50 mbar; separation, 18 kV.

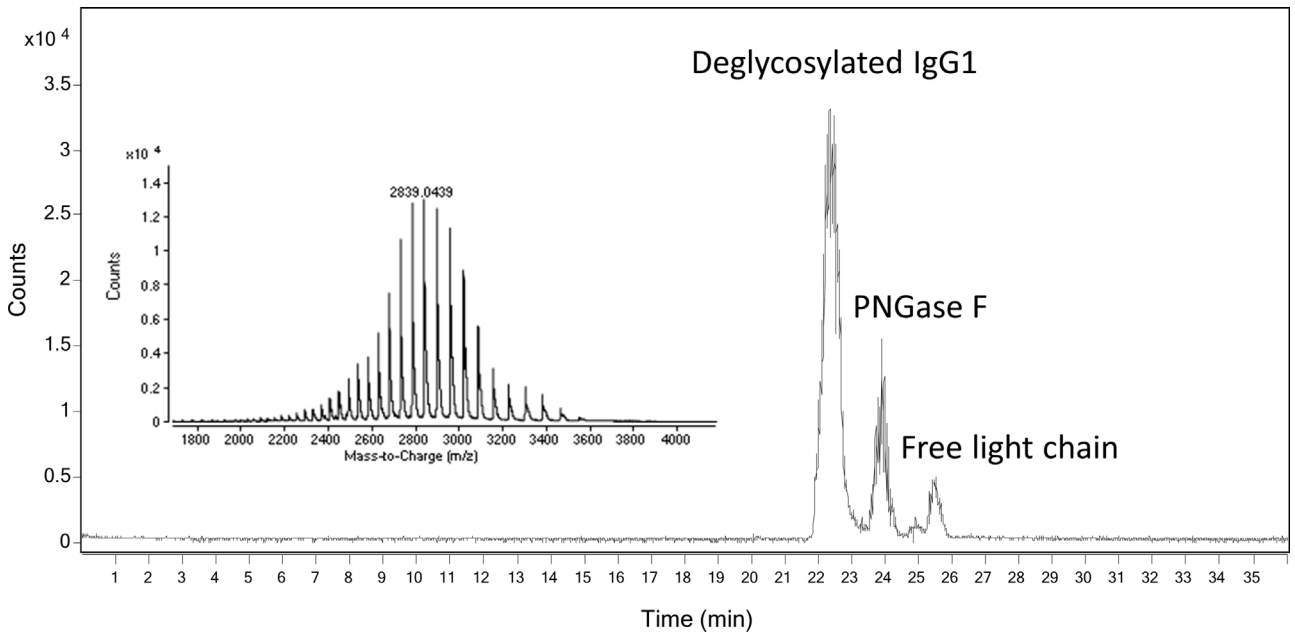



**Fig. 4.** Base peak electropherogram of deglycosylated IgG1. Inset: averaged mass spectrum of deglycosylated IgG1 peak. Sample: 1 mg/mL of IgG 1 in 15% acetic acid, 15% acetonitrile. Conditions: capillary, LPA-coated fused silica (360 μm OD, 50 μm ID, 70 cm length), outlet etched to 180 μm OD; electrolyte, 30% acetic acid; sheath liquid, 0.5% formic acid, 50% methanol; injection, 5 s at 100 mbar; separation, 18 kV with 1 mbar pressure on capillary inlet. Sample loading: 8.6 ng (58 fmol).

hydrochloride (GnHCl) and 50 mM dithiothreitol. The mixture was heated at 55 °C for 15 min.

*2.2.5. IdeS enzyme digestion*

60 μg of IgG samples were mixed with 60 units of IdeS enzyme. The digestion was kept at 1 × PBS buffer, pH 7.4 at 37 °C for 30 min.

*2.3. CE-MS analysis*

*2.3.1. Instrumentation*

All CE-MS analyses were performed on an Agilent 7100CE system coupled with an Agilent 6210 TOF mass spectrometer (Agilent Technologies, Santa Clara, CA) through an EMASS-II CE-MS interface purchased from CMP Scientific, Corp. (Brooklyn, NY) at room temperature. LPA coated fused silica capillary 50 μm ID × 360 μm OD × 70 cm length was used for the entire study unless otherwise noted. Mass range was chosen as *m*/*z* 700–4500 for intact mass and *m*/*z* 700–2500 for reduced intact mass analysis. Drying gas flow was set to 3.5 L/min with the temperature at 200–350 °C. Fragmentor voltage was set at 400–425 V. Skimmer voltage was set at 65 V. Capillary voltage was set at zero volts. The MassHunter Acquisition Software (B.04.00) for data acquisition and BioConfirm software for data analysis.

*2.3.2. CE-MS sample preparation*

All IgG samples were desalted into 5% acetic acid, and then mixed with equal volume of 30% acetic acid and 30% acetonitrile.

## 3. Results & discussion

*3.1. CE-MS interface installation on a TOF instrument*

The setup of the CE interface with the inlet to the mass spectrometer is critical for the success of CE-MS. Fig. 1 depicts the setup of the CE-MS interface on an Agilent 6210 TOF mass spectrometer. The Agilent LC–MS ion source was replaced with the CMP Scientific EMASS-II ion source (CMP Scientific Corp., Brooklyn, NY). The Agilent CE-MS cartridge outlet was used to provide the exit for the separation capillary. The capillary was thread through the EMASS-II interface tee, reaching to the spray emitter tip. The etched outlet end of the capillary was pushed slightly against the emitter tip. A sheath liquid vial was housed on the microstage, providing the primary electrical contact for external power supply to conduct electrospray ionization voltage. Under acidic pH conditions, the electrical potential between the electrode placed inside the sheath liquid vial and the mass spectrometer capillary generated a forward sheath liquid electroosmotic flow on the inner surface of the borosilicate glass emitter. The solvent shield cap on the TOF source was removed to allow monitoring the nanoflow electrospray ionization through a microscope camera (Fig. 1, inset). In order to adapt the need for quick switching between LC–MS and CE-MS projects, the nanoflow source adapter on the 6210 TOF was not utilized.

*3.2. System optimization and performance check using protein standards*

System optimization of CE-MS involved three major components: the CE-MS interface, CE conditions and MS parameters.

The CMP EMASS-II ion source was originally designed to be used on Thermo LTQ Orbitrap XL and other Thermo mass spectrometers. Here, we interfaced the CE instrumentation with Agilent 6210 TOF instrument. However, each instrument has significantly different design for ion transmission. To accommodate for these differences, several modifications to the setup had to be considered. First, the capillary voltage was set to zero to mimic the LTQ source design. The Agilent 6210 TOF also utilizing a drying gas at the inlet which cannot be turned off. This presented a challenge when large emitter tips (30–40 μm) were used, it was found that the drying gas impeded both sheath liquid and running buffer from reaching the emitter tip (not shown). Replacement with a smaller 20 μm tip was used throughout this study and drying gas was set at the minimum flow rate at 3.5 L/min on the 6210 TOF instrument. These modifications minimized the impedance produced from the drying gas.

For CE, sample injection condition, separation field strength, internal pressure application during separation as well as back-

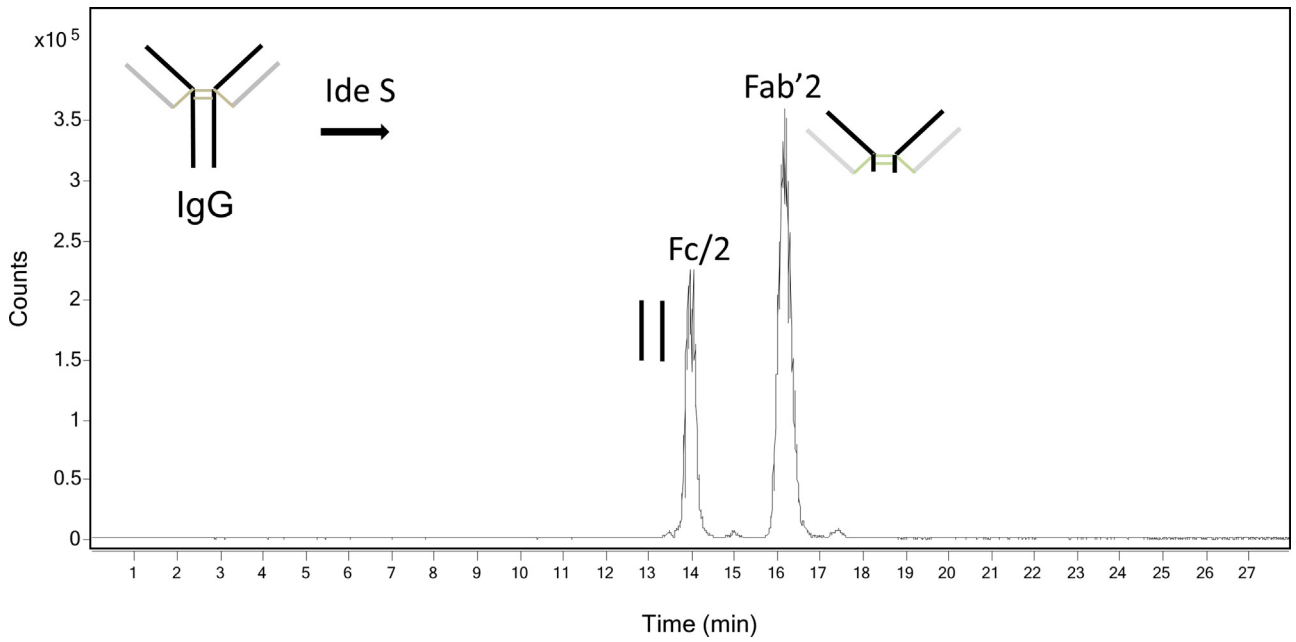



**Fig. 5.** Base peak electropherogram of IdeS enzyme digested IgG1. Sample: 2 mg/mL of IgG 1 in 15% acetic acid, 15% acetonitrile. Conditions: capillary, LPA-coated fused silica (360 μm OD, 50 μm ID, 70 cm length), outlet etched to 180 μm OD; electrolyte, 10% acetic acid; sheath liquid, 0.5% formic acid, 50% methanol; injection, 5 s at 50 mbar; separation, 18 kV with 1 mbar pressure on capillary inlet.

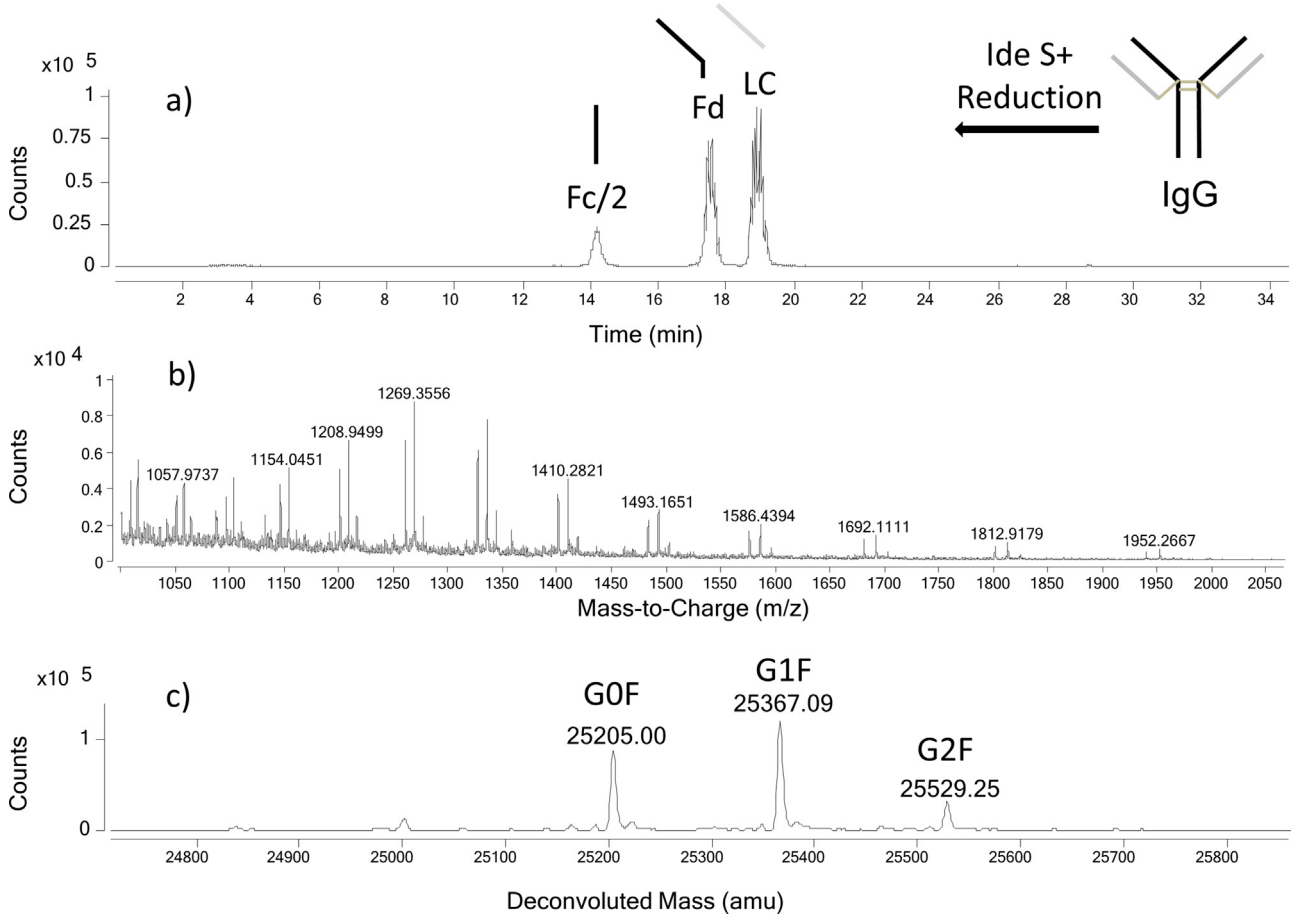



**Fig. 6.** CE-MS analysis of reduced IdeS enzyme digested IgG1. (a) Base peak electropherogram; (b) averaged mass spectrum of Fc/2 peak; (c) deconvoluted mass spectrum showing G0F, G1F, and G2F forms of Fc/2. Sample: 0.5 mg/mL of IgG 1 in 15% acetic acid, 15% acetonitrile. Conditions: capillary, LPA-coated fused silica (360 μm OD, 50 μm ID, 70 cm length), outlet etched to 180 μm OD; electrolyte, 5% acetic acid; sheath liquid, 0.5% formic acid, 50% methanol; injection, 5 s at 50 mbar; separation, 18 kV.

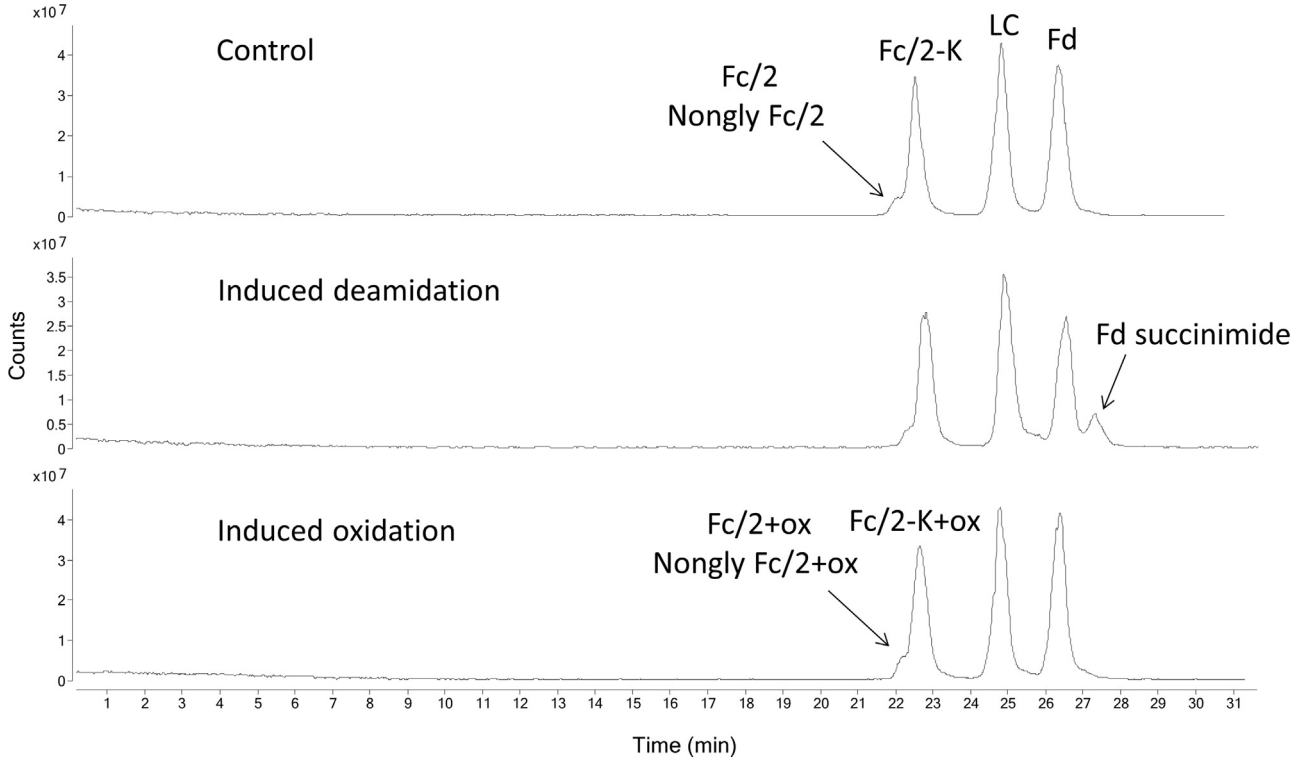



**Fig. 7.** Total ion electropherogram (smoothed) of reduced IdeS enzyme digested IgG1 samples. Samples: 0.5 mg/mL of IgG1 in 15% acetic acid, 15% acetonitrile. Conditions: capillary, LPA-coated fused silica (360 μm OD, 50 μm ID, 70 cm length), outlet etched to 180 μm OD; electrolyte, 30% acetic acid; sheath liquid, 0.5% formic acid, 50% methanol; injection, 5 s at 100 mbar; separation, 18 kV with 1 mbar pressure on capillary inlet.

ground electrolyte (BGE) composition were studied in order to develop a robust method.

Since CE injection volume is typically at nanoliter range, electrokinetic injection for protein analytes is not optimal due to the presence of minute residue salt component in sample buffer. Therefore, pressure injection was utilized throughout the study. Considering that the inner diameter (ID) tolerance of commercially available CE capillaries is a couple of micrometers (μm), the 50 μm capillary was used rather than 20–30 μm ID capillary in order to reduce the differences in injection amount between capillaries. Regarding the capillary outer diameter (OD), a 360 μm OD capillary was used. This type of capillary is also typically used in commercially available CE instrument for CE-UV analysis.

The Agilent 7100CE system used here allows for two modes of pressure injection, one is using regulated range from 0 to 100 mbar; the other is non-regulated higher pressure (920–950 mbar) which is normally used for capillary rinses. The non-regulated pressure was initially used for standard protein injection, and soon realized the inconsistency of the pressure level and low regulated pressure injection was utilized in the following studies. Field strength is another factor which is crucial to achieve optimal separation efficiency and peak shape. Although higher field strength could potentially lead to better separation, it was noted that when 30 kV was applied for monoclonal antibody samples, the high CE current generated increased system instability. Adjusting the field strength to 18 kV greatly increased stability and maintained good separation and was used for the rest of the study. The capillary was cut from 100 cm to 70 cm to compensate for some of the field strength loss. In order to get the optimized resolution, sample loading time was studied to avoid column overloading. The optimized sample loading time was determined by the peak shape and how well the peaks were separated. Unlike using uncoated capillary for CE separation, LPA coated capillaries have reduced electroosmotic flow; therefore even low pressure applied on CE separation capillary during electrophoresis process can affect the separation. Different internal pressure (0–100 mbar) during separation was investigated and revealed that lower internal pressure (<10 mbar) resulted in more improved, well defined peak shape.

The CE background electrolyte (BGE) solvents were acetic acid solution in various concentrations, ranging from 5% up to 30% (v/v) in water. The sieving matrix effect of acetic acid-based BGE increased with percentage of acetic acid. Better separation can be achieved with higher acetic acid concentration. However, due to the increasing viscosity of acetic acid buffer, using a BGE of 30% acetic acid significantly elongated the analysis time. Optimization on the percentage of acetic acid in the BGE was hence necessary for each individual molecule to balance between separation efficiency and total analysis time.

Sample with and without desalting was also studied and compared, with the concern of the strong drying gas flow from mass spectrometer, it was found sample desalting/buffer exchanging to volatile buffer in this instrumentation set up is necessary to avoid salt deposit at the emitter tip. Therefore, samples were desalted before analysis.

A sample stacking technique was employed by using high organic solvent in sample buffer to focus protein analytes and enhance separation efficiency [36,37]. After the sample was injected by pressure, the charged protein analytes migrated faster than the sample buffer matrix (which is composed of high organic solvent) under electrical field resulting in a narrow sample zone formed in the buffer region. The benefit of reducing protein diffusion is to obtain sharper elution peaks which aids in mass spectrometry analysis. Fig. 2 shows the CE-MS electropherogram of a four standard protein mixture using this stacking approach. The baseline resolved electropherogram of proteins with similar sizes (Cytochrome C, 12,370 Da; Ribonuclease A, 13,690 Da; Myoglobin, 16,964 Da) demonstrated that this instrument setup combined with the sample stacking technique allowed for efficient protein

analysis. In contrast to LC–MS, no major optimization is required after the initial CE-MS system set up is completed.

Due low sample amount and low flow rate from CE, to minimize band broadening, it is recommended that background electrolyte buffer at CE side is at the same height as the capillary outlet or higher toward the electrospray interface to avoid the counter flow from the outlet end of capillary due to the siphoning effects which could reduce the resolution [38]. It is worth to point out that in this application, both CE and mass spectrometer was from the same vendor and it was optimized designed for CE-MS application, the CE capillary outlet is slightly higher than the MS entrance, prevented the sheath liquid flow entering into the separation capillary to mix with the eluent, which is a key factor for obtaining optimal resolution [38].

### 3.3. Reduced monoclonal antibody (heavy chain and light chain)

Antibodies are composed of two heavy and two light chains, which are linked together by Cys–Cys disulfide bonds [7,9]. Reducing these disulfide bonds allows for dissociation of the intact IgG molecule to its heavy chain and light chain components. Measuring the molecular weight of heavy chain and light chain provides separate sequence information, which facilitates the identification of sequence variations, post-translational modifications (PTMs) as well as glycosylation patterns [7,39]. The reduced-IgG intact mass analysis has become a routine analysis for recombinant monoclonal antibody therapeutics to confirm sequence integrity [7,39,40]. In Fig. 3, the CE-MS base peak electropherogram (BPE) of the CE-MS run shows a two minutes wide separation between the heavy chain and light chain from antibody A. The degree of separation between heavy chain and light chain could potentially be valuable for the identification and characterization of degradation forms. The heavy chain and light chain peak widths are both less than one minute. Another IgG was also tested using this method and baseline resolution was also achieved (data not shown). This suggests that the performance of reduced mAb CE-MS analysis using this platform is now equivalent and comparable to that of the LC–MS methods.

It should be noted that 10% of acetic acid was used as the BGE and was sufficient to separate heavy chain from light chain. The decrease in solvent viscosity explains why the light chain molecule was eluted much earlier (Fig. 3, 19–20 min) than that in 30% acetic acid (Fig. 4, 25–26 min).

### 3.4. CE-MS of deglycosylated IgG1

Monoclonal antibodies are highly glycosylated molecules [7]. In order to reduce sample complexity and increase signal intensity for IgG intact mass analysis, the N-linked glycans on the monoclonal antibodies are usually removed enzymatically by PNGase F [7,41,42] resulting in a simplified species for the intact mass analysis. Here we used an IgG1 molecule (antibody A) to evaluate the performance of our CE-MS platform. The CE-MS base peak electropherogram (BPE) of the deglycosylated IgG1 sample is shown in Fig. 4. The inset showed the averaged mass spectrum of deglycosylated IgG1 peak with a charge state envelope spread between $m/z$ 2200–3600. After cleaving the *N*-glycans from the antibody, the PNGase F enzyme stayed in the sample. We can see that PNGase F peak was eluted after the deglycosylated IgG1 peak. A small amount of free light chain of the antibody A was also observed at 25–26 min.

The excellent separation between these three species with molecular weights ranging from ~20 kDa to 150 kDa further emphasizes that this CE-MS system is a valuable tool for intact protein mass analysis. Baseline separation of IgG whole molecule from other small proteins is important in that ion suppression due to co-eluting analytes is a common challenge in protein mass analysis. In addition, characterization of degraded forms such as heavy chain clips and monitoring the presence of free light chain is more likely with this baseline resolution.

### 3.5. IdeS enzyme digested monoclonal antibody (Fab2 **and Fc/2)**

IdeS is a bacterial cysteine proteinase with specific recognition of IgGs and will cleave IgG1 molecules right after the hinge region [43,44], resulting into two domains—Fab'2 and Fc/2. Fab'2 contains each of two light chains linked to each of two partial heavy chains ($V_H$—$CH_1$) and hinge region connected by intra-chain and inter-chain disulfide bonds, Fc is the remaining $CH_2$—$CH_3$ domains of the heavy chain [43,44]. These domains are much different than the heavy chain and light chain partial molecules. Turning the IgG1 whole molecule into Fab and Fc domains, and analyzing them separately can generate important information relevant to the antibody efficacy (Fab) and *N*-glycan profile (Fc), which has become a useful approach for investigating product quality attributes [43], such as glycan form profiling, Fc oxidation, N-terminal PyroE formation and C-terminal Lysine processing. Fig. 5 shows the BPE of IdeS digested IgG1. The first eluting peak Fc/2 was well separated from the second eluting peak Fab'2. This treatment and separation can add value in characterizing protein integrity and heterogeneity.

### 3.6. Reduced IdeS enzyme digested monoclonal antibody (Fd, LC and Fc/2)

The IdeS enzyme digested sample was further reduced to generate three partial molecules: Fd which is reduced form of partial heavy chain containing $V_H$—$CH_1$, light chain, and Fc/2 domain. Please note, a lower sample concentration was used in this study and was sufficient to generate good signal. In Fig. 6a, the Fc/2 domain was eluted at approximately the same time as in the non-reduced IdeS digest run (Fig. 5, ~14 min). The light chain was eluted at ~19 min, which is also the case in Fig. 4 (~19 min). These further indicate that the CE-MS platform reported here provides consistent results, which is critical for analytical assay development.

The averaged mass spectrum of the Fc/2 peak (Fig. 6b) clearly shows three major forms of Fc domain. The corresponding deconvoluted mass spectrum shown in Fig. 6C reveals the three major species with molecular weights of 25,205.00 Da, 25,367.09 Da, and 25,529.25 Da, which matches to the masses of Fc domain of this IgG1 molecule with attached G0F, G1F, and G2 F glycan forms.

### 3.7. Chemically Stressed IgG1 treated with IdeS enzyme followed by reduction and subsequent CE-MS

Post-translational modifications on monoclonal antibodies are complex and can have significant impact on the drug quality, safety and efficacy [6,45]. It is therefore important to monitor prevalent modifications such as oxidation, deamidation, and C-terminal processing. A workflow combining IdeS enzyme digestion with this CE-MS platform was then proposed to investigate the modifications on two accelerated stability study IgG1 samples (antibody B) and the results are shown on Fig. 7.

Four major species were observed in control sample (Fig. 7, top panel). The deconvoluted averaged mass spectrum (data not shown) indicated the peaks are: Fc/2 domain (C-terminal Lys not processed. MW = 25187.01) and with small amount of Non-glycosylated Fc/2, Fc/2 domain (C-terminal Lys processed, MW = 25058.98), light chain (MW = 24095.00), and Fd domain (MW = 24276.41). In the CE-MS runs using less than 30% of acetic acid as the BGE, the Fd and light chain peaks were not as well resolved as with 30% acetic acid BGE (data not shown). It should be noted that this IgG1 molecule (antibody B) in Fig. 7 is a different sample than that in Fig. 6 (antibody A). The light chain of this antibody even eluted earlier than the Fd domain (Fig. 7, top panel). This

suggests that method development using various concentration of acetic acid is necessary in order to obtain satisfactory resolution for the CE-MS intact mass analysis.

In the induced deamidated sample, an additional peak eluted right after the Fd domain (Fig. 7, middle panel). The molecular weight of that peak matched to succinimide form of the Fd domain. The electropherogram of the control and the induced deamidated runs can be easily distinguished from each other. The induced oxidized sample did not produce a distinct electropherogram when compared to the control sample. However, molecular weights of the peaks indicated that the Fc domain is oxidized, while the light chain and Fd domains remain unmodified. These results demonstrate that the workflow developed here is of great value in screening for even minor modifications in antibody domains.

The development of protein therapeutics demands a high degree of analytical characterization to identify critical attributes and assess product heterogeneity and stability. Capillary electrophoresis has been used for protein characterization over the past decades due to its high separation efficiency, fast analysis time and low sample consumption sample consumption [28,46] and minimum sample carry over [47,48]. High mass accuracy spectrometers have become enormously useful tools for accommodating the increasing need for protein sequence confirmation and post-translational modification analysis particularly enabled it for protein intact mass analysis, in which the monoclonal antibody molecules are either analyzed in its whole intact form or partial molecules (by either reduction or enzymatic digestion). The capability of measuring the masses of these molecules provides valuable information regarding molecule integrity and domain-specific modifications, which are critical attributes for product quality such as drug purity, safety and efficacy.

CE coupled to accurate TOF mass spectrometers offers a valuable complementary tool to LC–MS. Separation of charged and polar molecules by LC can be very challenging. The mechanism of separation by CE is based on the mobility of ions under an applied electric field and is therefore a robust and efficient technique for the separation of protein therapeutics for downstream characterization by MS. The CE-MS platform presented here seamlessly combines the ultrasensitive electokinetic flow driven sheath liquid interface with high-resolution Agilent 6120 TOF mass spectrometer. By using nanoliters per minute flow rate interface, the dilution factor was reduced by 10 to 100x therefore increased sensitivity. In addition, the fact that the electrode and the emitter are separated away from each other mitigates the bubble formation during the run and kept the electrical contact at all times. This interface design also combined the sheath liquid flow and electrode together to establish a stable electrospray [1]. Furthermore, no significant carryover of protein was observed between runs for up to 50 injections. Based on the robustness and ease of method development, CE-MS provides an attractive complement to LC–MS for separation and intact mass determination of mAbs and antibody-based therapeutics. Further work is in progress to improve separation speed for high throughput sample analysis to implement CE-MS into everyday workflow of mAb characterization.

## 4. Conclusion

In conclusion, we presented a proof-of-concept of extending the use of capillary electrophoresis in biopharmaceutical industry by coupling it with online electrospray ionization TOF mass spectrometer. The analysis of non-reduced intact antibody analysis could reveal potential degradation product, and reduced or IdeS digested partial molecule intact mass analysis could monitor the domain specific modifications as well as monitoring glycosylation forms. IdeS enzymatic digested sample followed by CE-MS work flow provided a fast screen of domain specific modifications. The well separated forms of succinimide in stability sample and able to monitoring oxidation has put it into a place of stability indicating assay with minimum sample preparation. Giving the fact that these studies were performed at room temperature compared to elevated temperature for RP separation, it is less likely to introduce artificial degradation induced by high temperature [12]. In addition, no significant sample carry over was observed with this platform which is a good alternative solutions for protein retaining on the LC columns often leading to peak tailing and sample carry-over issues. Furthermore, it is worth to point out that the reagent consumptions were very low (1 mL of BGE for up to 5 injections without noticeable CE-MS system change) which greatly reduced the hazardous waste generation, further reducing the long-term production cost.

CE-MS provides an attractive complement to LC–MS for separation and intact mass determination of mAbs and antibody-based therapeutics. This novel CE-MS platform will greatly benefit the biopharmaceutical industry for protein intact mass analysis in the years to come.

## Financial & competing interest disclosure

No potential conflict of interest was disclosed. The authors are currently Amgen employees and have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from the disclosed. No writing assistance was utilized in the preparation of this manuscript.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2015.12.045.

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