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Capillary electrophoresis–mass spectrometry as a characterization tool for therapeutic proteins

Lynn A. Gennaro *, Oscar Salas-Solano, Stacey Ma

Late Stage Analytical Development, Genentech Inc., South San Francisco, CA 94080, USA

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Abstract

With the increasing use of capillary electrophoresis (CE) in the biotechnology industry, there is a demand for analytical tools and methodology that can be used to characterize CE profiles. This article describes the implementation and optimization of a robust online CE–mass spectrometry (CE–MS) system used for the characterization of several CE assays developed at Genentech Inc. These assays include CE as a complement to reverse-phase peptide mapping for the identification of small peptides eluting in the void volume, profiling N-linked glycopeptide heterogeneity, and determining O-linked site occupancy. In addition, CE–MS was used to confirm major 8-aminopyrene-1,3,6-trisulfonate (APTS)-labeled glycans released from recombinant antibodies that are routinely profiled by CE–laser-induced fluorescence (CE–LIF). For each study, CE–MS was able to successfully identify components seen in UV or LIF electropherograms, thereby expanding the capability of CE and CE–MS for profiling biomolecules.
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Capillary electrophoresis (CE)[1] has become widely accepted for the analysis of protein therapeutics because it provides valuable information about the physicochemical characteristics of proteins such as charge, hydrophobicity, and size [1]. This methodology offers high sensitivity, high resolution, fast analysis times, simple sample introduction, and reproducibility [2]. In particular, CE has been shown to be a replacement for slab gel electrophoresis methodologies such as sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and isoelectric focusing (IEF) gels in routine analysis because of the higher separation efficiencies and direct on-column detection that facilitates quantitative analysis and automation [3–5]. In addition, capillary zone electrophoresis (CZE) has been shown to be widely used for the analysis of proteins by peptide mapping and for the assessment of the charge heterogeneity of proteins as an orthogonal technique to HPLC [6,7].

One limitation to the use of CE in a biotechnology setting is the ability to identify peaks seen in UV or laser-induced fluorescence (LIF) electropherograms. For biomolecules, identification usually is made by comigration with difficult-to-obtain standards. This process often can be particularly time-consuming and labor-intensive, and it does not provide direct structural information on migrating components. Therefore, it is becoming increasingly desirable to use detection methods that offer unambiguous compound identification.

Coupling CE to mass spectrometry (MS) presents one possible solution to this characterization issue. CE–MS as

* Corresponding author. Fax: +1 650 225 3554.
E-mail address: lgennaro@gene.com (L.A. Gennaro).

[1] *Abbreviations used:* CE, capillary electrophoresis; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; IEF, isoelectric focusing; CZE, capillary zone electrophoresis; LIF, laser-induced fluorescence; MS, mass spectrometry; LC, liquid chromatography; HPLC, high-performance liquid chromatography; MALDI, matrix-assisted laser desorption/ionization; APTS, 8-aminopyrene-1,3,6-trisulfonate; MAb, monoclonal antibody; PVA, polyvinyl alcohol; EDTA, ethylenediaminetetraacetic acid; TFA, trifluoroacetic acid; ESI, electrospray ionization; MS/MS, tandem MS; EOF, electroosmotic flow; RSD, relative standard deviation; PGC, porous graphitic carbon.

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an analytical tool has undergone several advances during the past decade, mainly due to better understanding of CE–MS technology, an increase in commercially available systems, and the need for more orthogonal methods to conventional chromatography [8]. Although CE–MS technology is advancing rapidly, UV and LIF remain the detection modes of choice for CE assays used in industrial settings. Therefore, it is imperative that online CE–MS electropherograms retain the same peak profile so that peak identifications can me made accurately. To date, there have been relatively few reports evaluating CE–MS as a characterization tool for routine CE applications used in the biotechnology industry. Here we present the implementation of a robust online CE–MS system used for the characterization of several CE assays developed at Genentech Inc.

One of the most powerful techniques for characterizing primary protein structure is reversed-phase liquid chromatography (LC) peptide mapping [9,10]. Although these methods typically provide more than 95% sequence coverage, reversed-phase separations often encounter difficulty with respect to identification of small hydrophilic peptides eluting in the void volume and separation of glycosylated peptides. The small peptides (one to three amino acids) that are not well retained on typical reversed-phase packing materials present a particular characterization challenge, mainly due to the high salt content present in enzymatic digests. When MS is employed for unambiguous identification, the void volume containing these peptides typically is not analyzed to avoid contamination of the MS ion source [11,12]. For some recombinant antibodies, these sequences occur in the variable region, making their characterization imperative. Therefore, additional analyses become necessary to obtain information on these species and confirm 100% of the protein sequence. In the current work, CE and CE–MS are explored for the analysis of small hydrophilic peptides unretained on C8 or C18 media to provide increased separation capability and protein sequence coverage.

The successful launch of therapeutic glycoproteins has also drawn more attention to carbohydrate structures that may be present on these molecules. Protein glycosylation often is monitored by chromatographic analysis of glycopeptides derived from enzymatic digests. Although reversed-phase LC–MS analysis usually is able to identify major glycosylated peptides [13,14], these signals typically are broad and low intensity such that additional information on minor components is difficult to obtain without further studies [15]. In addition, multiple glycoforms arising from a single peptide usually are not well resolved, making UV-based quantification difficult. Due to the high separation efficiencies attainable and the increasing use of CE in the biotechnology industry for monitoring protein glycosylation, CE and CE–MS are explored for their utility in the characterization of N- and O-linked glycopeptides isolated from typical reversed-phase Lys-C peptide maps.

Additional methods used to characterize N-linked carbohydrate moieties include enzymatic release of glycans followed by high-performance liquid chromatography (HPLC), matrix-assisted laser desorption/ionization (MALDI) MS [16,17], or CE analysis of glycans labeled at the reducing terminus. One commonly used approach for carbohydrate profiling involves CE separation of released carbohydrates labeled at the reducing terminus with 8-aminopyrene-1,3,6-trisulfonate (APTS) [18–20]. CE–LIF analysis of APTS-labeled glycans released from a recombinant monoclonal antibody (MAb) has been optimized previously and is able to separate major species, including G0, G1, and G2 [18]. The glycans typically are identified by comigration with difficult-to-obtain (and often impure) standards, and a more direct characterization approach would be very beneficial. In the current work, CE–MS is explored for use as a characterization tool to support the routine LIF detection-based assay. For these experiments, CE–MS was used to confirm the identity of the major species G0, G1, and G2 and to determine whether this assay could be used to assign minor peaks seen in LIF-based electropherograms.

Materials and methods

Chemicals and supplies

All proteins used were produced by Genentech, Inc. Angiotensins I and II were obtained from Sigma. Endoproteinase Lys-C was obtained from Roche Diagnostics. C8 solid-phase extraction cartridges (1 ml) were obtained from Supelco. APTS labeling dye was obtained from Beckman Coulter (Fullerton, CA, USA), and labeling enzymes and buffers were obtained from Prozyme (San Leandro, CA, USA). Centri-Spin 10 desalting cartridges were obtained from Princeton Separations (Adelphia, NJ, USA). Microcon-30 concentrators were obtained from Amicon. A solution of 1 M sodium cyanoborohydride in tetrahydrofuran was purchased from Aldrich (Milwaukee, WI, USA). In-house purified water was used in all reactions and experiments, and all organic solvents were of HPLC grade. Polyvinyl alcohol (PVA)-coated capillaries were obtained from Agilent Technologies (Wilmington, DE, USA).

Endoproteinase Lys-C digestion

Prior to digestion, approximately 0.5- to 1-mg protein samples were subjected to denaturing conditions, and free cysteine residues were modified using either carboxymethylation or sulfitolysis. Then proteins containing O-linked glycans were desialylated using 5 U/ml sialidase A (Prozyme) and incubated at 37 °C for 3 h. Following buffer exchange into 25 mM Tris and 1 mM ethylenediaminetetraacetic acid (EDTA) at pH 8.3, 500-μl to 1-ml protein samples were digested with 0.1 mg/ml endoproteinase Lys-C at 37 °C for 3–5 h. The reaction was quenched by the addition of 15 μl of 10% trifluoroacetic acid (TFA).

Isolation of small, hydrophilic Lys-C peptides

Lys-C digests were prepared as above with the following exceptions. Ammonium bicarbonate was substituted for Tris in the digestion buffer at the same molarity and pH. Following Lys-C digestion, 500 µl digest was aliquoted and quenched with 1 µl concentrated formic acid and was immediately loaded onto 1-ml C8 SPE cartridges conditioned with 0.1% formic acid. The C8 flow-through was dried and reconstituted in water for CE analysis.

Isolation of glycopeptides

C8 or C18 reversed-phase HPLC analysis was performed on 50–100 µl of the Lys-C digest. The glycopeptide fractions were collected, dried, and reconstituted in water for CE analysis.

Release and APTS labeling of glycans

After 25 mg/ml antibody solution in formulation buffer was exchanged into PNGase incubation buffer solution using a Microcon-30 concentrator, PNGase F was added and the sample was incubated for approximately 15 h at 37 °C. The deglycosylated protein was heated and precipitated by centrifugation. The supernatant was dried and reconstituted in a solution of acidic APTS and sodium cyanoborohydride. This solution was heated at 55 °C for 2 h. The solution was diluted with water and subjected to desalting/APTS removal with CentriSpin-10 cartridges.

Capillary electrophoresis

CE–UV experiments were performed using a Beckman P/ACE MDQ capillary electrophoresis system with UV detection at 214 nm. CE was performed with 70–100 cm PVA-coated capillaries (75 µm i.d. × 360 µm o.d. unless otherwise noted). Capillaries were kept at 20 °C and flushed with running buffer at 30 psi for 5 min prior to each analysis. Separations were performed with an applied voltage of 20–30 kV. A running buffer of 100 mM acetic acid (pH 3.1) with an applied voltage of +25–30 kV was used for CE analyses of small peptides and glycopeptides. Injection of these samples was performed hydrodynamically at 1 psi. A running buffer of 25 mM formic acid (pH 3.5) with an applied voltage of −20 kV was used for released glycan analyses. Injection of these samples was performed electrokinetically at −30 kV.

Capillary electrophoresis–mass spectrometry

CE–MS was performed using a separate P/ACE MDQ system with electrodes reconfigured to allow injection and voltage application to be performed at the outlet end. MS analyses were performed using a ThermoFinnigan LCQ ion trap mass spectrometer with the standard electrospray ionization (ESI) interface. The interface was modified slightly using a commercially available CE–MS kit (ThermoFinnigan) that includes a larger bore needle and micrometer for fine adjustment of the capillary tip. Physical constraints of this system require a minimum capillary length of 68 cm, and the CE capillary tip was sanded flat and aligned flush to the sheath liquid needle. Electrophoretic conditions for each experiment, including capillary length, running buffer, and applied voltage, were the same as those used for UV and LIF experiments (see above sections). A sheath liquid composed of methanol/water/running buffer (80:15:5, v/v/v) was delivered via syringe pump at 2–3 µl/min. MS data collection was triggered by contact closure, with the ESI voltage off during injection. The CE–MS system was tuned and optimized by angiotensin (angiotensins I and II, 125 µg/ml each in H_2O). Full scan mass spectra were recorded from 200 to 2000 mass units (100–600 mass units for dipeptide samples), and data-dependent tandem MS (MS/MS) analyses were performed with a relative collision energy of 30. The ESI voltage was set at +3.5 or −4.0 kV (depending on polarity), and the heated capillary was maintained at 200 °C.

Results and discussion

CE–MS system optimization

Although HPLC and MS are ideally suited for each other, this is not the case with CE–MS. Because CE methods typically are not pressure driven and require a grounding mechanism at the capillary outlet, coupling to MS is quite challenging. Several interface types have been developed for CE–MS coupling, including coaxial sheath [21], liquid junction [22], and sheathless interfaces [23].

In the current work, a CE–MS system employing a coaxial sheath interface was examined with a specific goal of matching CE–UV separation to facilitate peak identification for routine CE methods used in protein characterization. It is known that MS-compatible CE methods require several additional criteria, including a suitable interface and a volatile buffer system. Due to the electrospray process, buffer salts must be volatile, excluding high concentrations of common running buffers such as phosphates and borates. Although the coaxial sheath interface has higher flexibility in salt selection than do other interfaces [24], the concentration of buffer salts must be sufficiently low because high currents are not desirable for use with MS. Therefore, MS-compatible salts, including ammonia, triethylamine, acetate, citrate, and formate in moderate concentrations, were used in these studies.

Consideration must also be given to protein–wall interactions and expected electroosmotic flow (EOF) generated with the use of uncoated fused silica capillaries. Given the absence of a traditional outlet buffer reservoir, CE conditions generating an EOF moving away from the mass spectrometer typically will encounter problems with current stability. In addition, the negatively charged surface on these capillaries is known to interact with the positive charge on proteins, causing band broadening and/or material deposits [1]. To avoid such issues, all CE and CE–MS

analyses were performed with coated capillaries under acidic conditions, virtually eliminating EOF and analyte–wall interactions.

During CE–MS system optimization, emphasis was placed on obtaining ion electropherograms that closely resembled the profile obtained by UV detection. Among the various parameters studied, those that had the most significant impact on CE separation and MS sensitivity included sheath gas pressure, sheath liquid flow rate, sheath liquid composition, and tip positioning. Additional parameters, including ESI voltage, MS inlet temperature, and CE coolant tubing length, were optimized but had a less significant impact on separation and MS sensitivity. For example, increasing the sheath gas pressure decreased sample migration times significantly with a loss in resolution. CE–MS analysis of the angiotensin mixture using a sheath gas pressure of 20 psi (Fig. 1A) allowed separation of these components with a resolution of $R_s = 10$, whereas a setting of 30 psi (Fig. 1B) gave a resolution of $R_s = 2.5$. This effect has been observed previously [25] and attributed to a hydrodynamic bulk flow inside the CE capillary created by the high velocity of the nebulizing gas [26]. For all experiments reported here, the sheath gas pressure was set at a value of 20 psi, the minimum value required to maintain a stable electrospray. The sheath liquid flow rate was also optimized in the range of 1–8 µl/min. Electrospray instability has been observed previously at flow rates less than 0.5 µl/min with a conventional sheath–liquid interface [27]; however, careful consideration must be given to minimize the flow rate due to losses in sensitivity from excessive dilution [28,29]. It was found that the optimal flow rate for maintaining electrospray stability while optimizing MS signal in this CE–MS system was 2–3 µl/min.

The importance of capillary tip positioning and shape has been noted by several researchers [30–33]. The system used here has been equipped with a commercially available micrometer for fine adjustment of the capillary tip, and it was found that the optimal position of the CE capillary tip was flush to the sheath liquid tubing, providing a stable electrospray under various CE modes of operation.

The significant impact of the liquid sheath composition on CE separations has also been explored previously [34]; therefore, it is important to optimize the sheath composition for each application. In our studies, it was found that the absence of running buffer in the MS sheath decreased separation efficiency; high concentrations of running buffer reduced sensitivity. For most analyses, it was observed that migration times and resolution are decreased when 0% running buffer is present; this can be a significant disadvantage for particularly demanding separations. When the percentage running buffer is increased to 20%, a decrease in signal is observed, and this can be a disadvantage for sensitive analysis of less abundant species. As a compromise between resolution and sensitivity,

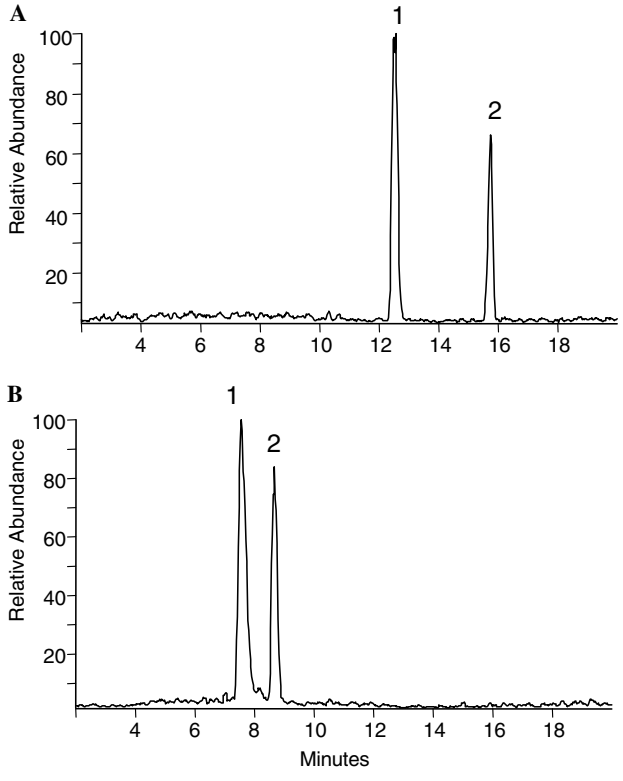


Fig. 1. CE–MS analysis of a synthetic peptide mixture to examine the effect of MS sheath gas pressure. (A) CE–MS with sheath gas pressure at 20 psi. (B) CE–MS with sheath gas pressure at 30 psi. For both experiments, applied voltage = +30 kV; injection = electrokinetic, 10 kV for 10 s; capillary = 75 µm i.d. × 68 cm.

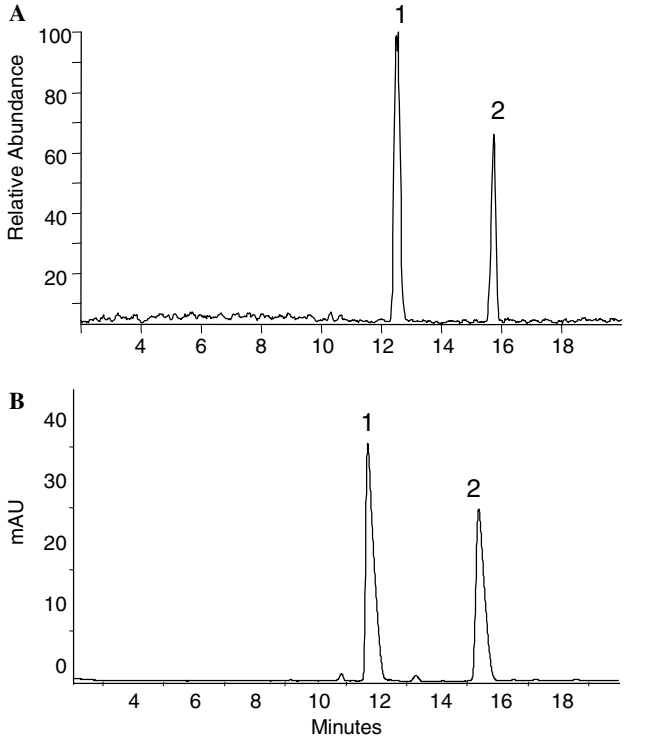


Fig. 2. CE–MS and CE–UV analysis of a synthetic peptide mixture for comparison of electrophoretic profile: (A) CE–MS; (B) CE–UV. For both experiments, applied voltage = +30 kV; injection = electrokinetic, 10 kV for 10 s; capillary = 75 µm i.d. × 68 cm total length.

a sheath liquid including 5% running buffer was used in our studies. In addition, running buffer was the only salt-containing component in the sheath liquid because previous studies have indicated that counterions can diffuse into the CE capillary, causing moving ionic boundaries and migration shifts [34,35].

The CE–MS total ion electropherogram for the mixture of angiotensins I and II under optimal conditions is shown in Fig. 2A. Following optimization of CE–MS parameters for separation and detection, this system was compared with CE–UV results of an identical mixture under similar conditions (Fig. 2B). Table 1 summarizes the results from these experiments. The capillary length was approximately 70 cm for both systems; however, the CE–UV system had an effective length of 60 cm (detection occurs ~10 cm from the capillary outlet). All other parameters were held constant. CE–MS produces an electropherogram similar to that produced by CE–UV with a small loss in resolution (R_s). This loss has been attributed to a siphoning effect created within the CE–MS interface caused by both the sheath gas pressure and the vacuum within the MS source. It should also be noted that the relative standard deviation (RSD) in migration time for peak 1 was less than 1% in all experiments, demonstrating migration reproducibility within the CE–MS system. This parameter is especially important considering that migration behavior along with separation profile and MS spectra will

Table 1
Results of CE–UV compared with CE–ESI–MS under optimized conditions

Method	MT Peak 1 (min)	RSD MT (%)	$R_{s_{1,2}}$	RSD $R_{s_{1,2}}$ (%)
CE–UV[a]	11.5	~1	12	3
CE–MS[b]	12.6	~1	9	11

Note. MT, migration time; R_s, resolution; RSD, relative standard deviation.
[a] $n = 7$.
[b] $n = 5$.

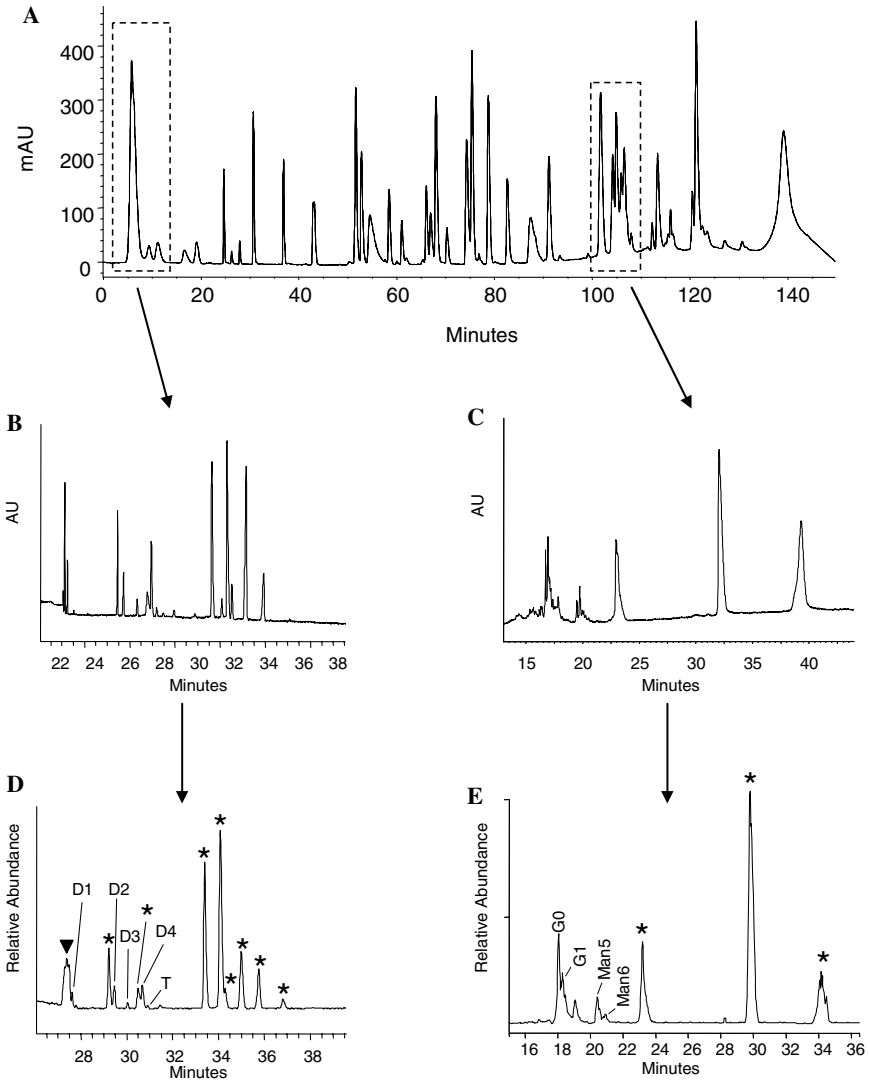


Fig. 3. (A) Lys-C peptide map of protein containing one N-linked glycosylation site. (B) CE–UV analysis of isolated void region. (C) CE–UV analysis of isolated glycopeptide region. (D) CE–MS analysis of isolated void region. (E) CE–MS analysis of isolated glycopeptide region. D, dipeptide; T, tripeptide; ▼, salt; *, unrelated peptides.

be used to confirm the identity of peaks eluting in a conventional CE–UV system.

In the following sections, several applications are presented in which CE is used as an orthogonal tool to conventional HPLC. Particular emphasis was placed on the separation capability of CE and the ability of CE–MS to provide structural information for characterizing the CE profile. These applications include (i) small hydrophilic peptides eluting in the reversed-phase void volume of LC peptide maps, (ii) glycopeptides from peptide digests, and (iii) APTS-labeled released glycans. For these applications, CZE methods employing both UV and LIF detection and different polarities are transferred to CE–MS for characterization of eluting species.

CE as a complement to reversed-phase peptide mapping

Reversed-phase peptide mapping continues to be an invaluable tool for characterization and quantification of peptides from enzymatic digests of intact protein. With excellent separation capability and amenability to MS, nearly all peptides can be assigned from a single digest. For the two glycoproteins studied herein, Lys-C reversed-phase peptide maps are presented in Figs. 3A and 4A. Two drawbacks to the reversed-phase separations of protein digests illustrated here include the inability to retain smaller hydrophilic species that coelute in the void volume and suboptimal separation of glycosylated peptides.

For the Lys-C peptide map shown in Fig. 3A, the void region is expected to include five dipeptides and one tripeptide based on the theoretical protein sequence. As mentioned previously, compounds eluting in this region can be particularly challenging to identify by reversed-phase LC–MS because this region typically is diverted to waste due to the high salt content. There have been several attempts to separate and characterize these peptides using other chromatographic stationary phases prior to MS analysis, including porous graphitic carbon (PGC). In one study,

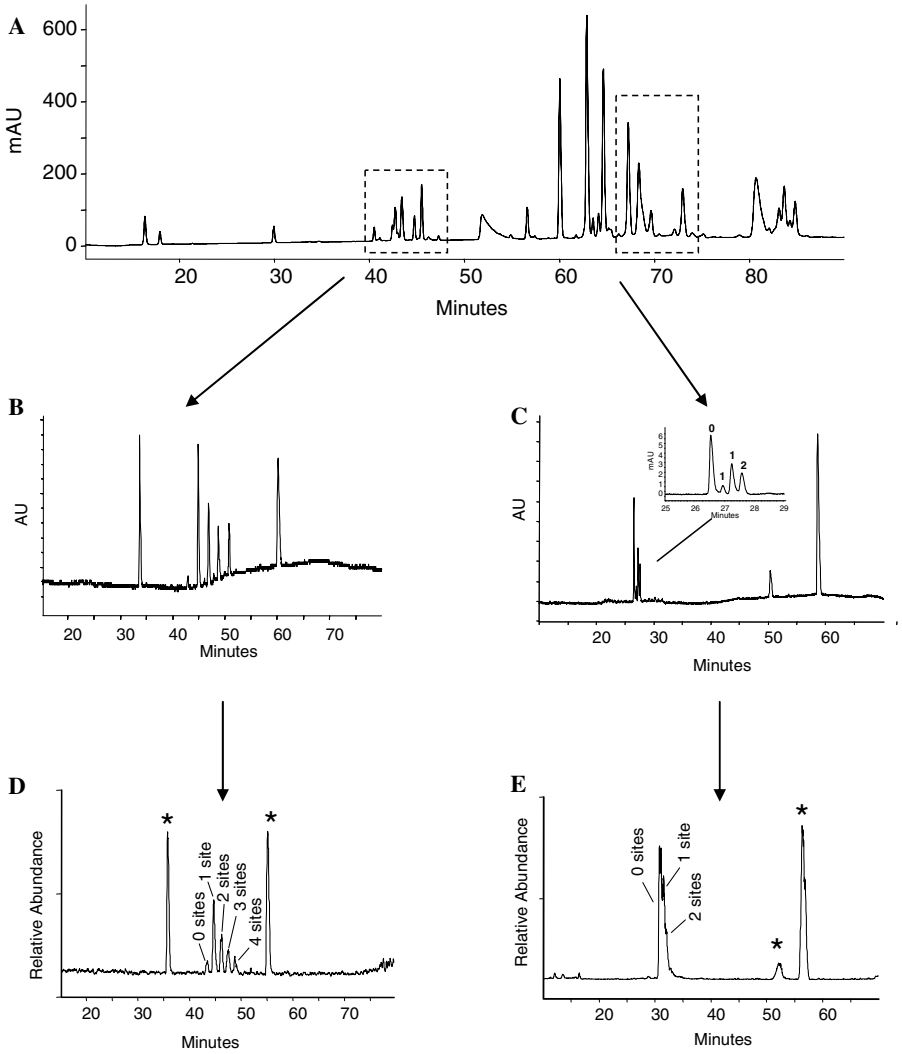


Fig. 4. (A) Lys-C peptide map of protein containing two peptides with multiple O-linked glycosylation sites. (B) CE–UV analysis of isolated peptide 1 fraction with zero to four O-linked glycans. (C) CE–UV analysis of isolated peptide 2 fraction with zero to two O-linked glycans. The inset shows an expanded view of the region between 25 and 29 min. (D) CE–MS analysis of isolated peptide 1 fraction. (E) CE–MS analysis of isolated peptide 2 fraction. *, unrelated peptides.

researchers were able to obtain MALDI–MS data on small peptides (two to five amino acids) derived from a Lys-C proteolytic digest by using volatile mobile phases combined with a PGC column [11]. However, in our studies with graphite solid-phase extraction of an antibody Lys-C digest, several dipeptides are still unretained (data not shown). Other characterization methods for these compounds include N-terminal sequencing, amino acid composition, and characterizing other protein digests using an alternate enzyme such as Asp-N. These methods can be time-consuming and labor-intensive, and a direct method for characterization of these small hydrophilic peptides is becoming increasingly desirable.

Considering the hydrophilic nature of these small peptides and the ability of CE to tolerate and disperse salt ions, CE is a very attractive method for the analysis of "void" peptides. In combination with LC–MS, these methods could then provide 100% sequence coverage of a single enzymatic digest. Initial experiments suggested that a small reduction in sample conductivity was still necessary for subsequent CE analyses to avoid significant ionic boundaries when injecting large sample volumes. Therefore, a simple change in the digest procedure was made to include ammonium bicarbonate buffering reagent as a replacement for Tris. Following digest optimization and isolation of the void volume using C8 SPE cartridges, samples were analyzed by CE–UV and CE–MS for characterization and identification of peptides. The results of these analyses are shown in Figs. 3B and D, respectively, where the CE–UV trace can be compared with the MS total ion electropherogram. The UV data were comparable to the MS data with respect to separation profile and resolution, allowing MS confirmation of peak assignment and quantification based on UV peak areas. It should also be noted that one dipeptide of interest eluted much later in the electropherogram (~80 min) due to a sulfitolyzed cysteine residue (data not shown). Additional peaks seen in these electropherograms included several larger peptides also seen by LC–MS (Fig. 3D, labeled with asterisk [*]) and minor UV-absorbing components not identified by CE–MS. In these studies, CE–MS was able to consistently identify the components of interest not assigned by LC–MS. These components included all five dipeptides and one tripeptide. This example illustrates that CZE and CE–MS can be used as a complementary technique to LC–MS peptide maps for protein characterization, facilitating confirmation of the protein sequence.

In addition to the peptides eluting in the void region, reversed-phase peptide maps often provide insufficient resolution of glycosylated peptides. A typical Lys-C peptide map of an antibody with one N-linked glycosylation site is shown in Fig. 3A, with the glycopeptides eluting in the region between 97 and 110 min. Separate LC–MS analysis of this region (data not shown) indicates that multiple species are present, including both glycosylated and nonglycosylated peptides. Unfortunately, the very broad peaks corresponding to the glycosylated species gave little information on attached glycoforms, so CE analysis of the collected peptides from this region was examined for its ability to assign these peptides.

Fig. 3C contains a CE–UV electropherogram of LC fractions collected from this region. Subsequent CE–MS analysis under these conditions (Fig. 3E) provides a similar profile, allowing facile identification of major components in the electropherogram.

MS analysis of the single species migrating at approximately 23, 30, and 34 min (Fig. 3E, labeled with asterisk) allows identification of these species as nonglycosylated peptides by data-dependent MS/MS fragmentation and database searching. These species, which coelute within a 6-min region of the LC chromatogram, are well resolved from the glycopeptides by CE.

Mass spectra of species in the region between 17 and 22 min (Fig. 3E) indicated several glycosylated species. The region between 17 and 19 min consisted of G0 and G1 glycoforms, and the region between 20 and 22 min contained two distinct species identified as high-mannose structures Man5 and Man6. It should be noted that minor well-resolved peaks in the CE–UV electropherogram were not detected as individual peaks by CE–MS, indicating that these species either are coeluting isomers or are below the detection limit of the CE–MS system used in this work. Separation and identification of minor components in this region currently are under way in our laboratory.

A Lys-C peptide map of a protein with six O-linked glycosylation sites on two Lys-C peptides is shown in Fig. 4A, with the glycopeptide regions present at 39–48 min (all forms of glycopeptide 1, containing four O-linked sites) and 65–74 min (all forms of glycopeptide 2, containing two O-linked sites). Separate LC–MS analysis of this region (data not shown) indicates that several species are present in each region, including both glycosylated and nonglycosylated peptides. Due to coelution of nonglycosylated species with the glycopeptides, quantification of glycosylation distribution is difficult. Therefore, CE was evaluated as an orthogonal method to HPLC for the profiling and quantification of isolated glycopeptide regions.

Following LC analysis, isolated fractions were collected, dried, and reconstituted in water for CE analysis. CE–UV analysis for each region was performed and is illustrated in Figs. 4B and C, where sufficient resolution was achieved for each migrating component to facilitate quantification. CE–MS analysis performed under similar conditions (Figs. 4D and E) was employed to further assign each peak. For the glycopeptides relating glycopeptide 1 (containing four possible O-linked sites), a distinct peak was identified for the nonglycosylated peptide, as well as those for one, two, three, and four sites occupied (Fig. 4D). Similarly, for glycopeptide 2, containing two O-glycosylation sites, peaks were obtained for the nonglycosylated peptide, one site occupied (two isoforms), and two sites occupied (Fig. 4E). In addition, nonglycosylated peptides coeluting in the LC region were identified to be

unrelated peptides from the protein digest and are marked with an asterisk.

In these experiments, CE has provided a complementary technique to HPLC for the analysis of O-linked glycopeptides. This method allowed quantification of the O-linked glycan occupancy and the relative distribution of major glycoforms, with CE–MS providing an essential characterization tool for the identification of UV peaks.

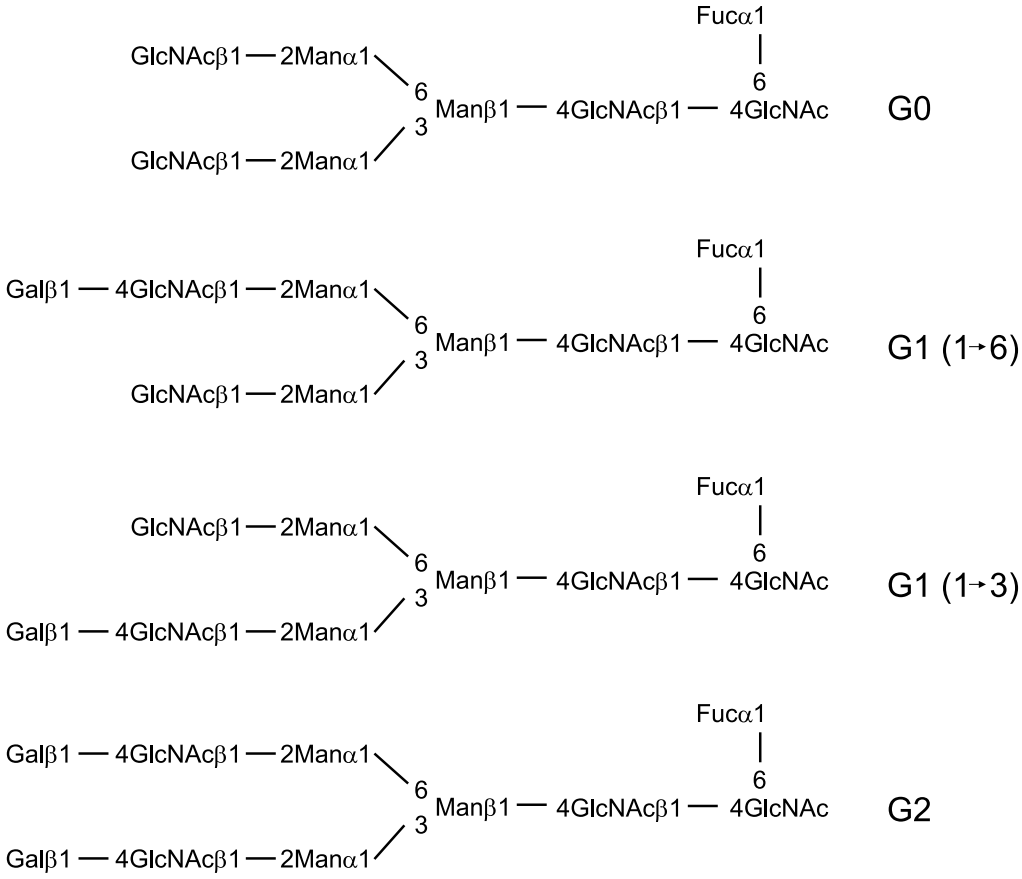


Fig. 5. Structures of N-linked glycans G0, G1, and G2.

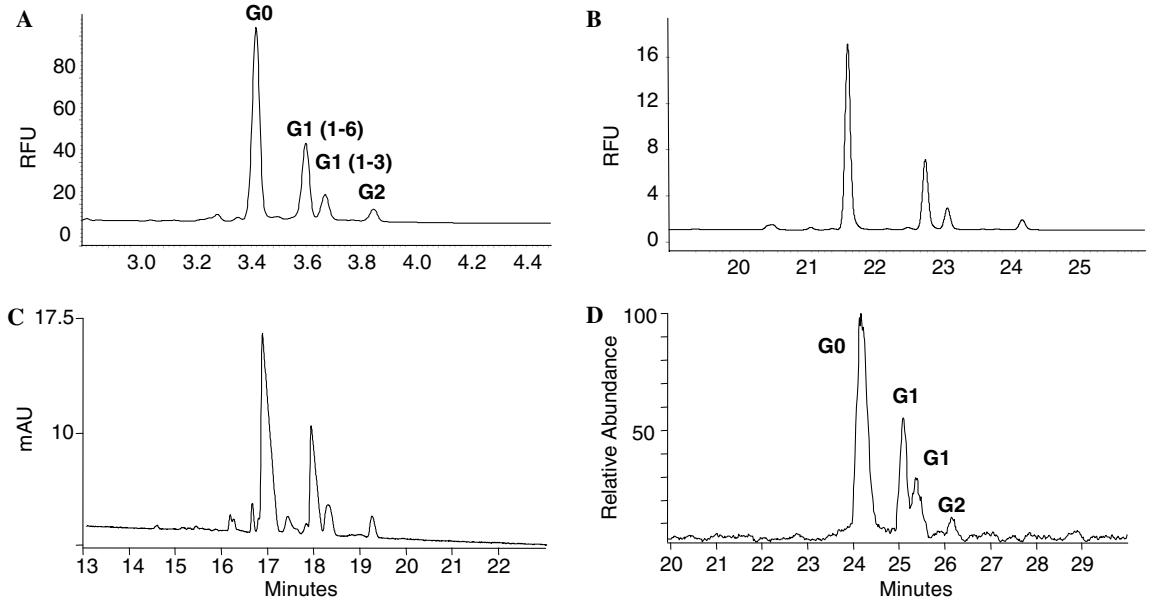


Fig. 6. CE analysis of released, APTS-labeled glycans from a rhuMAb. (A) CE–LIF assay for CZE profiling of APTS-labeled glycans on a 30-cm × 50-μm i.d. capillary, with identifications based on coelution with synthetic standards and exoglycosidase treatment. (B) CE–LIF analysis with original buffer system and extended length 70-cm × 75-μm i.d. capillary. (C) CE–UV analysis with MS-friendly buffers and 70-cm × 75-μm i.d. capillary. (D) CE–MS analysis on 70-cm × 50-μm i.d. capillary, with identifications based on mass spectra.

CE of APTS-labeled glycans

As described earlier, CE employing LIF detection is commonly used for profiling APTS-labeled glycan species released from recombinant MAbs. In this section, CE–MS is explored as a characterization tool that can be used in parallel with the routine LIF-based assay for mass confirmation of the N-linked structures G0, G1, and G2 (Fig. 5).

For CE analysis of protein-released glycans, a fluorophore such as APTS typically is added to the reducing terminus, enabling both electrophoretic mobility and fluorescence detection. When considering CE–MS, the derivatization reaction has an additional purpose. Native neutral carbohydrates typically do not electrospray well due to the lack of an easily ionizable functional group [36]. Based on previous CE–MS research on similar compounds [37,38] and its inherent ability to maintain a negative charge at low pH values, APTS glycans present an ideal candidate for CE–MS analysis.

The routine CE separation of APTS-labeled glycans typically involves the use of short capillaries (30 cm) and running buffers unsuitable for CE–MS analysis (Fig. 6A). To evaluate the feasibility of CE–MS analysis, the analysis of these compounds was explored using various "MS-friendly" alternatives.

Following removal of excess APTS, CE–LIF analysis was performed with an extended length capillary (70 cm) to evaluate the effect of the capillary length on the separation of APTS glycoforms (Fig. 6B). Because separation profile of APTS glycoforms was maintained, further studies were performed using a formic acid buffer system and UV detection (Fig. 6C). In this case, UV detection was used to determine whether baseline separation could still be achieved with larger injections while using a detection method more comparable to the sensitivity expected from MS. Subsequent CE–MS analysis using similar conditions and 50-μm i.d. capillaries is shown in Fig. 6D. In this case, the use of a smaller internal diameter capillary increases resolution, allowing the partial separation of G1 isomers differing in the site of terminal galactose linkage even with online MS detection. To our knowledge, this is the first report illustrating the separation and direct identification of G1 isomers by CE–MS.

For each CE–MS experiment, negative ion full scan mass spectra were recorded; individual spectra of the components (APTS G0, G1, and G2) are illustrated in Fig. 7. Although full scan MS data are sufficient to identify these species, MS/MS experiments provide additional structure conformation by providing the complete *y*-ion series while retaining intact APTS (data not shown). From these experiments, it can be seen that online CE–MS technology can be used to assign even structural isomers because it maintains a similar peak profile while providing mass information. Future work will be aimed at increasing MS sensitivity while retaining resolution, facilitating the detection of minor species. The results have shown that CE–MS provides a complement to CE–LIF analysis, with the advantage of mass confirmation and the ability for MS/MS structural characterization.

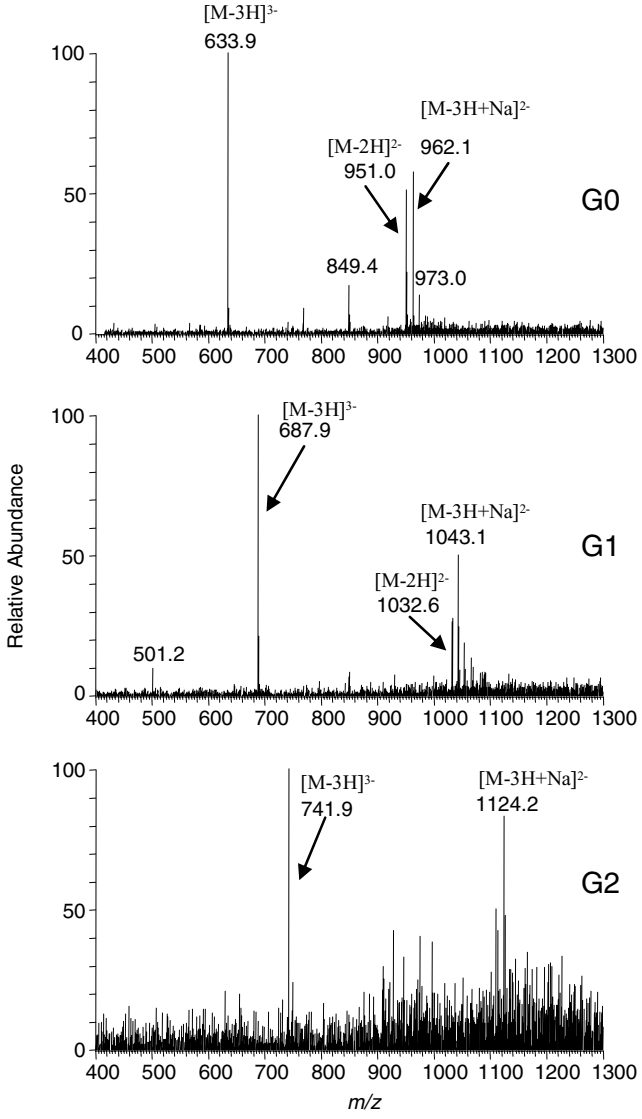


Fig. 7. Full scan mass spectra of major glycans in the negative ion mode.

Conclusions

With the increasing use of CE in the biotechnology industry, there is a demand on analytical tools and methodology that can be used to characterize CE profiles. In this article, we have reported the application of a robust CE–MS system used in parallel with CE–UV or CE–LIF for characterization of therapeutic proteins. This methodology was applied to several CE assays used for identifying small peptides eluting in the peptide map void volume, profiling N-linked glycopeptide heterogeneity, and determining O-linked site occupancy. In addition, CE–MS was used to confirm major APTS-labeled glycans released from recombinant antibodies that are routinely profiled by CE–LIF. In all experiments, CE–MS data were able to consistently identify migrating components, facilitating the application of CE assays for the development of biomolecules. Future

work will include efforts to increase CE–MS sensitivity to allow characterization of minor species and expand the versatility of our CE–MS technology to include other modes of CE such as CE–SDS and cIEF.

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