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On-Line CE−LIF−MS Technology for the Direct Characterization of N-Linked Glycans from Therapeutic Antibodies

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Glycan characterization of therapeutic proteins is of utmost importance due to the role of carbohydrates in protein stability, half-life, efficacy and mechanism of action. The primary assay for characterization and lot release of N-linked glycans on glycoprotein products at Genentech, Inc., is a capillary electrophoresis (CE) based assay, wherein PNGase F-released, APTS-labeled glycans are separated by CE with laser induced fluorescence (LIF) detection. With the growing number of new molecular entities in the pipeline, a fast and direct characterization approach is of increasing importance. This paper describes the development of CE−MS technology with on-line LIF detection that allows identification of major and minor glycan species (1–5% of total glycans) by providing accurate mass information. Data is presented for therapeutic rMAbs which presented previously unidentified, minor peaks during routine CE−LIF analysis. CE−LIF−MS was then used to provide accurate mass on these species, identifying CE peaks corresponding to sialylated (G1 + NANA, G2 + NANA), afucosylated (G0-GlcNAc-fucose) and low-level isomers of major APTS-labeled glycans G0, G1, G1′ and G2.

Of the many post-translational modifications present on therapeutic proteins, glycosylation has received considerable attention in the field of proteomics. In the biotechnology industry, it is well-known that carbohydrates can play a significant role in the activity and efficacy of a glycoprotein,[1–3] and must be carefully monitored. Even minor changes in glycan distribution (such as increased afucosylation) can have effects on the activity of a biopharmaceutical product.[4–6] Therefore, advances are continually being made in the biotechnology industry to minimize glycan heterogeneity and improve analytical assays used to identify and quantify minor changes across products and/or batches.[7] However, the task of glycoprotein characterization remains an analytical challenge due to the immense heterogeneity of these species. Although N-linked glycans retain the same trimannosyl core structure ($GlcNAc_2$-Man_3), additional species built upon this core can include those with any number of monosaccharide, branching, linkage and/or anomeric differences. Several analytical techniques have been implemented to characterize these species, such as high-performance liquid chromatography (HPLC),[8] nuclear magnetic resonance (NMR),[9–11] capillary electrophoresis (CE),[12–14] mass spectrometry (MS)[15,16] and hyphenated techniques such as HPLC−MS.[17,18] Each technique provides complementary information which can be used to assign glycan structure, each one having its own advantages and disadvantages. The advantages of using CE for glycan profiling include automation, short analysis times and the ability to separate and quantify isomeric species. The coupling of CE with on-column laser induced fluorescence (LIF) detection also has the advantage of highly sensitive detection.[19,20]

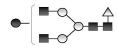
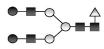
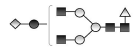
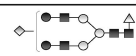
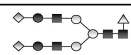
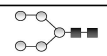
Genentech, Inc., has been using capillary electrophoresis (CE) based methods for profiling glycosylation for several years. The primary assay involves releasing N-linked carbohydrates with PNGase-F, labeling with the fluorophore 8-aminopyrene-1,3,6-trisulfonate (APTS), and analysis by CE−LIF. APTS was chosen due to the low-picomole sensitivity attainable for APTS derivatized carbohydrates.[21] An earlier version of this assay has been described by Ma and Nashabeh in 1999,[22] in which CE−LIF was used for profiling and quantification of rituximab glycans including major species G0, G1, G1′ and G2. Nomenclature and structures

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Table 1. Structure of the N-Linked Glycans Present on Typical rMAbs, with Corresponding APTS-Labeled Masses

Abbreviation	Structure	Expected Average Mass of APTS-Labeled Product	Expected m/z of Most Abundant Charge State
G0		1904.80	950.755 (-2)
G1, G1'		2066.94	1031.781 (-2)
G2		2229.09	1112.807 (-2)
G0-1		1701.61	849.215 (-2)
G0-F		1758.66	877.726 (-2)
G0-1-F		1555.47	776.186 (-2)
G1+NANA		2358.20	1177.330 (-2)
G2+NANA		2520.34	1258.355 (-2) 838.570 (-3)
G2+2NANA		2811.59	1403.905 (-2) 935.603 (-3)
Man 5		1676.56	836.699 (-2)
			

for the N-linked glycan present on typical rMAbs, as well as minor forms and corresponding APTS-labeled masses, are listed in Table 1. Currently, a modified form of this assay is validated and used routinely in a high-throughput format[23] for product characterization, clone selection and lot release of recombinant monoclonal antibody (rMAb) therapeutics at Genentech, Inc.

However, one disadvantage of CE-based methods is the lack of routine detection methods that can provide direct structural information on migrating species. For our assay, characterization was primarily done using one of two methods: (1) comigration with commercially available standards and (2) exoglycosidase treatment followed by coelution with standards or examination of migration shift. These methods can be particularly labor-intensive, and do not provide any direct information on glycan structure. Due to the more routine use of this assay in all stages of product development, we have been exploring new methods which can be used to characterize new or existing peaks that may be present.

Coupling CE to on-line mass spectrometry (MS) presents an attractive option, allowing direct mass information to be obtained on migrating components. The first on-line coupling of these two technologies was presented in 1987 by Olivares et al.,[24] wherein a metalized segment of the capillary outlet was inserted directly into an electrospray ionization (ESI) source prior to MS detection. In the past two decades, several additional types of CE−MS interfaces have been developed, including coaxial sheath[25] and liquid junction[26] interfaces. Although CE−MS technology continues to advance for both general applications[27,28] and carbohydrate analyses,[29–32] there have been relatively few reports on the robust application of this technology in the pharmaceutical or biotechnology industry. In 2006, our group performed numerous studies aimed at optimizing this technology toward routine use, and presented several relevant applications in the analysis of peptides, glycopeptides and carbohydrates.[33] Our current research expands on the capability of CE−MS and improved technology for the profiling of rMAb glycosylation.

This paper describes the current assay for glycan screening by CE−LIF and development of CE−MS technology with an on-line LIF detector to aid in the identification of new peaks which may be observed. As the standard CE−LIF assay is being used routinely for all glycoprotein products, it is important to have additional characterization tools available for any issues which may arise due to the increasing pipeline of new molecular entities. In addition, this technology may provide insight into additional, unidentified glycan structures which may be present. For these studies, the ultimate goal was to achieve a similar separation profile with mass spectrometric detection as that obtained by the original LIF method. To accomplish this, the standard method was modified to allow MS coupling while ensuring that any differences could be attributed only to minor changes in resolution and/or efficiency. When performing on-line CE−MS analysis, an on-line LIF detector was added such that quantitative comparisons of LIF signal intensities from both assays remained consistent, ensuring that peak identifications are made accurately.

To illustrate the practical utility of on-line CE−LIF−MS, this technology was applied to two recombinant monoclonal antibody products exhibiting atypical peak profiles. In addition to major glycans, minor unknown species seen in the CE−LIF method were subsequently identified by CE−LIF−MS, wherein accurate mass information was obtained. These species included both sialylated and afucosylated glycans, which are of particular importance as they can have an effect on antibody clearance and/or antibody dependent cellular cytotoxicity (ADCC).[3] In addition, CE−MS was able to identify minor isomeric species for APTS-labeled rMAb glycans seen in this assay, which will be the subject of future investigations. These results demonstrate the utility of CE−LIF−MS as a complementary characterization tool for profiling N-linked glycans released from rMAbs.

EXPERIMENTAL SECTION

Materials and Reagents. All recombinant monoclonal antibodies (rMAbs) were produced by Genentech, Inc. Microcon-30 concentrators were obtained from Amicon (Billerica, MA). APTS labeling dye was obtained from Beckman Coulter (Fullerton, CA),

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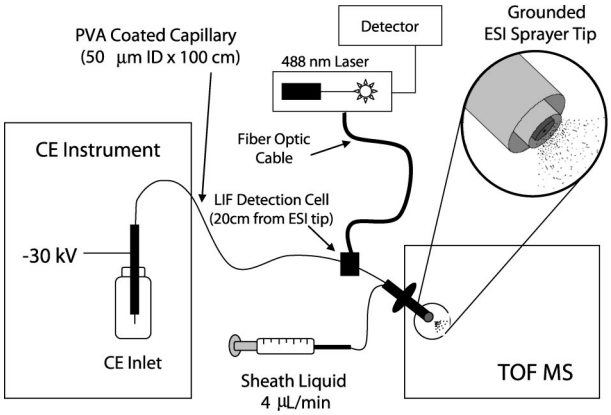


Figure 1. Schematic of the CE−MS system with on-line LIF detection.

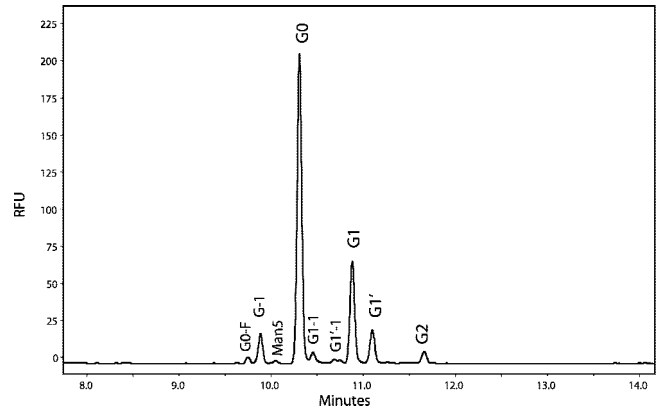


Figure 2. Standard CE−LIF analysis of APTS-labeled, N-linked glycans released from a recombinant monoclonal antibody. Identifications are based on enzymatic treatment with exoglycosidases and/or comigration with commercially available standards.

and labeling enzymes and buffers were obtained from Prozyme (San Leandro, CA). Centri-Spin 10 desalting cartridges were obtained from Princeton Separations (Adelphia, NJ). A solution of 1 M sodium cyanoborohydride in tetrahydrofuran was purchased from Aldrich (Milwaukee, WI). PVA coated capillaries were obtained from Agilent Technologies (Wilmington, DE).

Release and APTS-Labeling of Glycans. 750 μg of antibody in formulation buffer was exchanged into 1X reaction buffer (pH 7.5, supplied by the manufacturer) using a Microcon-30 concentrator. PNGase F diluted in reaction buffer 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 an excess solution of 15 μL of acidic APTS (5 mg in 0.5 mL of 15% v/v glacial acetic acid) and 5 μL of sodium cyanoborohydride. This solution was heated at 55 °C for 2 h. The solution was diluted with water to a final volume of 50 μL. Reaction components and excess APTS label were removed using Centri-Spin-10 desalting columns. For conventional CE−LIF analysis, samples were diluted 1:20 v:v with water prior to injection. For CE−MS analysis, samples were injected neat.

Capillary Electrophoresis. Off-line CE−LIF experiments (no MS) were performed using a Beckman P/ACE MDQ capillary electrophoresis system with LIF excitation at 488 and 520 nm emission filter. CE was performed with PVA coated capillaries (60 cm total length × 50 cm effective length × 50 μm ID × 360 μm OD). A running buffer of 40 mM ϵ-aminocaproic acid, pH 4.5 (adjusted with glacial acetic acid) + 0.02% hydroxypropylmethylcellulose (HPMC) with an applied voltage of –30 kV was used. Capillaries were kept at 20 °C and flushed with running buffer prior to each analysis. No additional conditioning was used. Injection was performed hydrodynamically at 0.5 psi for 10 s. Capillaries were stored in water when not in use.

On-Line CE−LIF−MS. A schematic of the on-line CE−LIF−MS is shown in Figure 1 Electrophoresis was performed using an Agilent CE system and CE−MS capillary cartridge allowing for external detection. PVA-coated capillaries were fitted with an ellipsoid by Picometrics (Toulouse, France) to allow for on-line LIF detection. Several electrophoretic conditions were modified to allow MS detection. The HPMC was removed from the running buffer due to MS incompatibility, and desalted samples were injected at 50 mbar for 30 s. The capillary length was extended to 100 cm due to physical constraints. For on-line LIF detection, a separate Picometrics ZetaLIF Discovery detection system was used. This system included a laser unit and LIF detection cell which was connected to the detector via fiber optic cable as shown in Figure 1. The Picometrics detection system was equipped with a 488 nm laser and 520 nm emission filter, and the signal was sent to Agilent Chemstation software for recording via an Agilent 35900E A/D converter. The capillary window (with attached ellipsoid) was installed into the LIF detection cell, which is supported in front of the MS electrospray source by an adjustable arm approximately 20 cm from the electrospray tip. Mass spectrometric detection was achieved using a Bruker MicrOTOf accurate mass MS instrument (Bruker Daltonics, Bremen, Germany), equipped with an orthogonal, at-ground electrospray source and time-of-flight mass analyzer. The MS instrument was supported by a table approximately 4 cm higher than that of the CE instrument. For MS interfacing, the end of the CE capillary was installed into a CE−MS sprayer (Agilent), allowing for a sheath liquid to be added coaxially. The tip of the CE capillary was aligned flush to the stainless steel capillary tip under 20× magnification. A sheath liquid of 50:50 isopropanol:water + 0.2% ammonia was then delivered at 4 μL/min. Mass spectra were acquired from 200 to 2000 m/z in the negative ion mode.

RESULTS AND DISCUSSION

Standard CE−LIF Assay. Using the CE parameters described above, a typical CE−LIF glycan profile for a rMAb is shown in Figure 2 In this profile, major glycan species G0, G1, G1′ and G2 can be easily resolved, as well as several minor peaks. The following sections will describe the adaptation of this assay (referred to as the conventional CE−LIF assay) for CE−MS such that minor peaks can be identified.

Adaptation for CE−MS. Due to the nature of mass spectrometric detection, there are several assay parameters which must be modified to utilize this technique. These include (1) removal of excess salts/label from APTS-labeled glycan samples which interfere with MS detection (data not shown), (2) modification of the running buffer to be MS-compatible and (3) increasing the length of the CE capillary such that it can exit the CE instrument and extend to the MS source. Therefore, the first part of our

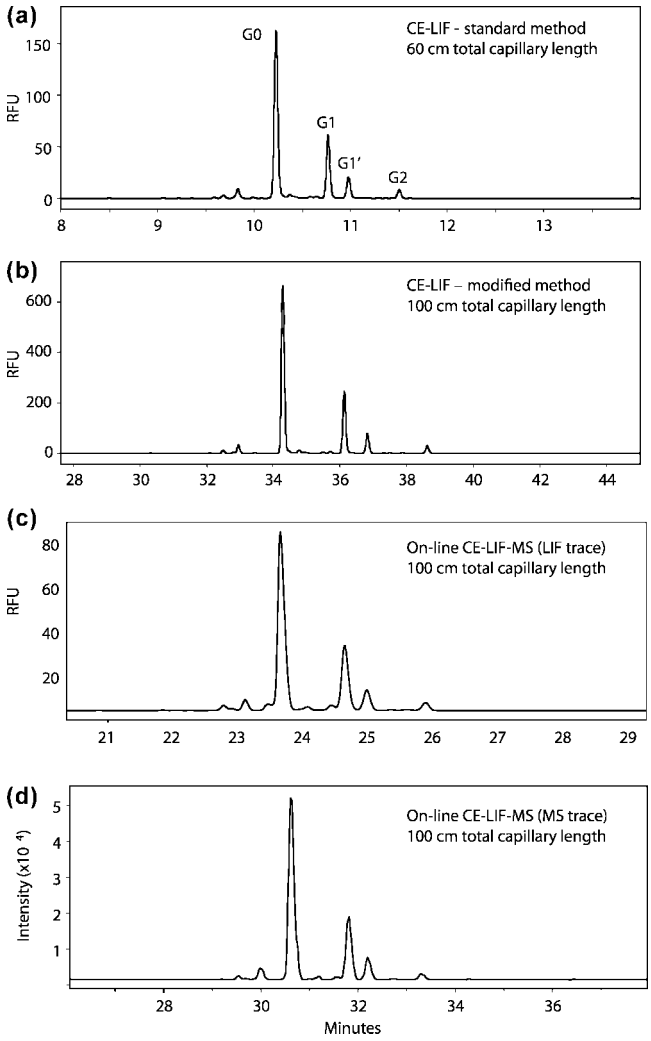


Figure 3. Full scale electropherograms of APTS-labeled rMAb glycans using (a) conventional CE−LIF with standard buffer [60 cm total capillary length] and (b) CE−LIF using modified conditions [100 cm total capillary length]. (c) LIF trace obtained on-line during CE−MS analysis with modified conditions [100 cm total capillary length] and (d) CE−MS base peak electropherogram using negative ion MS detection [100 cm total capillary length].

studies involved a systematic approach to examine the effects of modifying the sample preparation and electrophoretic conditions to be MS-compatible. First, experiments were conducted to determine if the desalting procedure necessary for CE−MS analysis had an effect on glycan distribution. In these studies, expanded-view CE−LIF electropherograms of APTS-labeled rMAb glycans using both standard and modified sample preparations were compared (data not shown). From these profiles, it was concluded that there was no change in glycan profile. Following sample cleanup, additional studies were performed to examine the effect of modifying the running buffer and increasing the total capillary length from 60 cm total length (50 cm effective) to 100 cm total length (90 cm effective). Figure 3 displays CE−LIF electropherograms of desalted, APTS-labeled rMAb glycans using (a) the standard buffer and 60 cm PVA-coated capillary and (b) removal of HPMC from the buffer and 100 cm capillary (modified conditions). As expected, an increase in migration time, intensity and resolution was observed using the modified buffer and longer 100 cm capillary. Increased intensity in Figure 3b is attributed to the removal of HPMC from the buffer, wherein additional material is injected (pressure injection) due to the lower viscosity of the buffer. Resolution (Rs) was examined for peaks corresponding to G1 and G1′ isomers and calculated to be 3.0 for the standard method (60 cm) and 3.9 for the modified method (100 cm, no HPMC). Most importantly, the overall peak profile remained consistent. These results suggest that the modified method is suitable for subsequent CE−MS analyses.

CE−LIF−MS Analysis of a Typical rMAb. In the above section, we have demonstrated that the standard CE−LIF method used for glycan profiling can be modified for MS-compatibility with minimal changes in overall electrophoretic profile. However, our group and others have noted that the addition of a MS detector can cause differences in the electrophoretic method and resulting profile. For example, CE−MS methods have no "outlet" buffer; they use a sheath liquid of different composition which can result in moving ionic boundaries.[34] In addition, it has been shown that CE−MS methods will exhibit some losses in both efficiency and resolution as compared to their off-line counterparts, mainly due to extra-column effects introduced by the sheath gas.[33,35,36] We have taken several steps to reduce the siphoning effect caused by the sheath gas pressure, including lowering the CE instrument 4 cm relative to the MS instrument and determining the minimum sheath gas pressure required to maintain a stable electrospray. Unfortunately, all of the effects could not be eliminated, and thus it is reasonable to expect that some changes in the electrophoretic profile will be seen when adding the MS detector. Since we are using CE−MS to identify peaks seen in CE−LIF electropherograms, we must have a means to ensure that peak comparisons can be made accurately prior to utilizing this technology. This can be accomplished by comparing peak areas between methods, provided that the detection method is identical. Although mass spectral peaks may have similar relative peak areas to those detected by fluorescence, this data cannot be used to confirm comparisons made on unknown peaks, as there may be significant differences in ionization efficiency between various glycan species.

For this reason, an on-line LIF detector was added to the CE−MS instrumentation to bridge the two techniques. The detector was placed approximately 20 cm from the electrospray tip, making the effective capillary length 80 cm. It should be noted that the MS sensitivity is expected to be significantly lower, mainly due to dilution from the sheath liquid while performing CE−MS. Therefore, the maximum range of the LIF detector used on-line was increased to avoid signal overloading (range value = 200). Regardless of absolute LIF signal intensity, the percent of total glycans represented by any specific peak will remain consistent under any electrophoretic conditions. Therefore, off-line (no MS) and on-line LIF traces can be compared quantitatively using peak intensities, whereas on-line LIF and MS traces acquired during the same run should have virtually identical separation profiles.

Following adaptation of the standard method to modified "MS-friendly" conditions as described in the previous section, both on-

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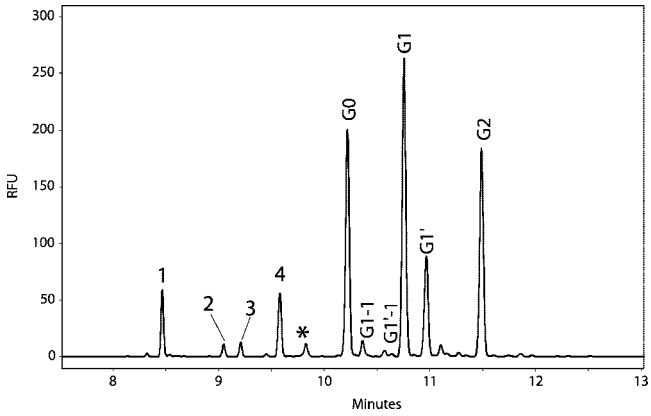


Figure 4. Standard CE−LIF analysis of APTS-labeled, N-linked glycans released from rMAb 1. Identifications are based on enzymatic treatment with exoglycosidases and/or comigration with commercially available standards.

line LIF and base-peak MS electropherograms can be obtained. Figure 3 illustrates the glycan profile of the same desalted rMAb sample using (a) standard CE−LIF conditions, (b) CE−LIF analysis using modified method, (c) a LIF trace obtained on-line during CE−MS analysis with modified conditions and (d) a base peak electropherogram using negative ion MS detection. These electropherograms indicate that CE−MS analysis can produce a similar peak profile to the original method, and has the potential to be used for characterization of glycan species separated by CE. As expected, differences in total migration time and resolution can be seen between all electropherograms. This is due to modified electrophoretic conditions, increased injection amount required for MS detection, differences in effective capillary length and extra-column effects cased by the sheath gas during MS analyses. In order to confidently compare peaks seen in all electropherograms, a simple quantitative comparison was performed using only the major species. For G0, G1, G1′ and G2, the total percent peak area using the standard method (Figure 3a) was approximately 65.1%, 23.8%, 8.0% and 3.0%, respectively. These areas can be directly correlated to those calculated from the on-line LIF trace shown in Figure 3c (approximately 65.3%, 24.1%, 7.6% and 3.0%, respectively), indicating that these peaks represent the same species in both conventional and MS based assays.

These studies confirm that CE−MS can be used to obtain accurate mass information on glycan species seen in the standard CE assay used for N-linked glycan profiling, and is capable of detecting species >3% of total glycans. In the next two sections, the glycan distribution of two therapeutic antibody products is examined by CE−LIF−MS with the goal of confirming peak identities using mass spectral data, as well as determining whether CE−MS can be used to provide information on unknown, minor peaks (<1–5%) which may be present.

CE−LIF−MS Analysis of rMAb 1. A CE−LIF profile of N-linked glycans released from recombinant monoclonal antibody 1 (rMAb 1) is shown in Figure 4 Peak assignments for major species were based on exoglycosidase treatment and/or comigration with synthetic standards. As expected for any rMAb, the major N-linked glycans can be identified as G0, G1, G1′ and G2. However, several minor species are observed in the region between 8 and 10 min that we have not typically observed on

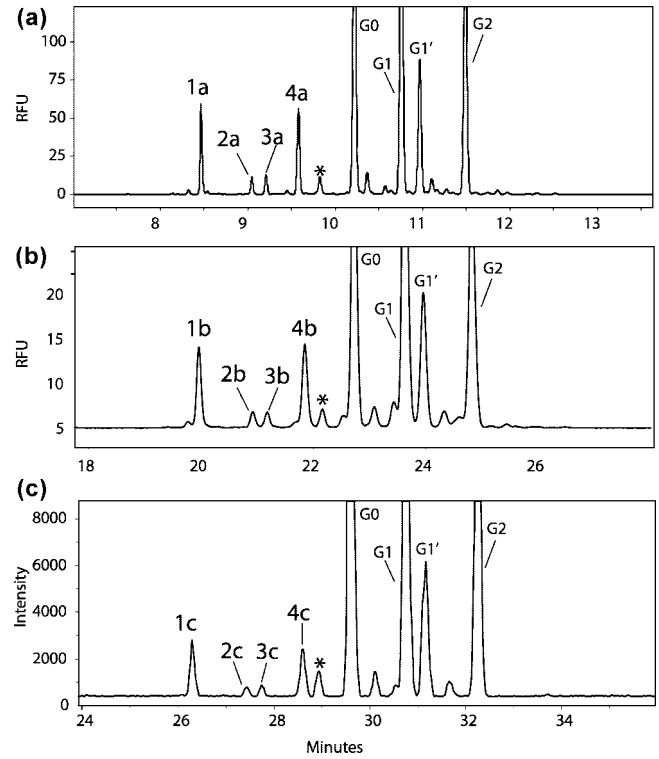


Figure 5. Expanded-scale electropherograms of rMAb 1: (a) standard CE−LIF electropherogram using a 60 cm capillary, (b) CE−LIF trace obtained on-line with MS detection and (c) CE−MS base peak electropherogram.

rMAbs, and are marked as peaks 1–4 (peak marked with asterisk * will be addressed later). It is important to note that this assay separates based on both charge and size. Therefore, due to the early migration of these species, it can be hypothesized that either (1) these species bear additional negative charges or (2) they are smaller than G0. These peaks were further investigated by CE−LIF−MS to confirm their identity.

Figure 5 displays expanded-scale views of (a) a conventional CE−LIF electropherogram using a 60 cm capillary, (b) a CE−LIF trace obtained on-line with MS detection and (c) a CE−MS base peak electropherogram. The four early migrating species can be seen clearly in both the on-line LIF and MS ion electropherograms. However, it is apparent that the MS-based assays (b and c) suffer from a shift in migration time and resolution loss due to the addition of the MS detector, as noted earlier. To ensure that peaks seen in both assays are the same species, a quantitative assessment of the total peak area was made between the LIF trace from the standard assay (peaks 1a−4a), and the LIF trace from the CE−MS based assay (peaks 1b−4b). The approximate percent peak area of the total glycans for peaks 1–4 is as follows: peak 1a = 4.5%, peak 2a = 1.0%, peak 3a = 1.1%, peak 4a = 5.7%, peak 1b = 5.1%, peak 2b = 1.0%, peak 3b = 1.0%, peak 4b = 5.7%. The correlation between peak areas allows the confidence to compare peaks from the standard (a) and MS-based assays (b and c).

The time-of-flight mass spectra of each peak 1c−4c is shown in Figure 6, and accurate mass confirms their identity as sialylated species. The presence of sialic acid, or *N*-acetylneuraminic acid (NANA), adds an additional negative charge and is a common element of many glycoproteins. On this rMAb, unknown peaks 1–4 correspond to G2 + 2NANA, G1 + NANA, G1′ + NANA, and

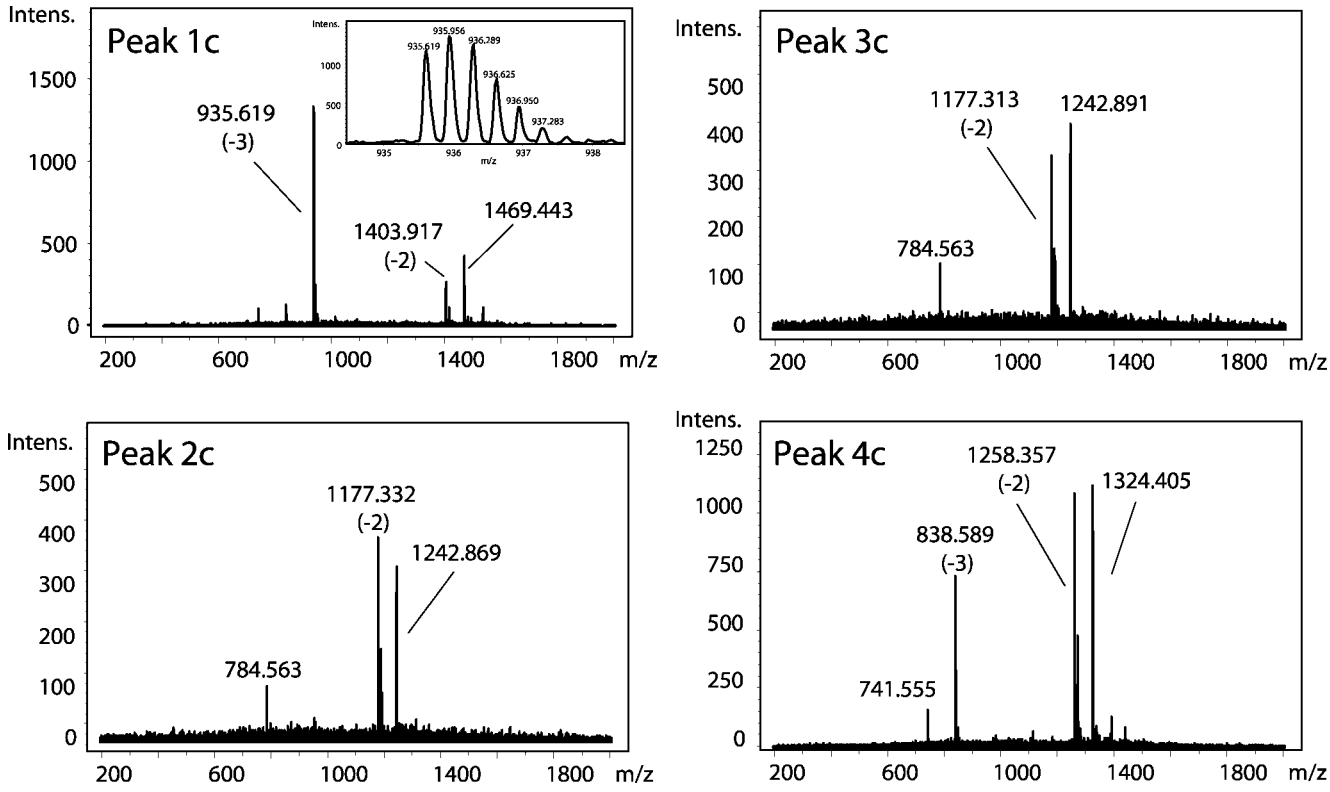


Figure 6. Mass spectra obtained for (a) peak 1c, (b) peak 2c, (c) peak 3c and (d) peak 4c present in Figure 5 confirming their identity as sialylated species.

G2 + NANA (observed m/z of most abundant charge state for unknown species 1–4 = 935.62, 1177.33, 1177.31, 1258.36, theoretical m/z for sialylated species = 935.60 [−3], 1177.33 [−2], 1177.33 [−2], 1258.36 [−2], respectively). The assignment of these peaks was then confirmed by sialidase treatment and CE−LIF analysis, wherein each peak 1–4 collapsed into its respective desialylated counterparts, G1 and G2 (data not shown). Further confirmation of sialylated species was also obtained by sialic acid analysis (acid hydrolysis and derivatization followed by reversed-phase HPLC), and the amount of sialic acid was determined to be 0.32 mol of NANA per mol of protein (data not shown). The total percent sialylated species as determined by CE−LIF (12%) also corresponds to the loss in acidic variants using ion-exchange chromatography of the intact rMAb following desialylation (13%, data not shown).

Although a small amount of sialic acid (0.045 mol of NANA per mol of protein) is present on typical IgG_1-derived rMAbs produced at Genentech, there are several conformational and/or sequence changes that can increase the likelihood of sialylation.[37,38] Since sialylation (and subsequent capping of exposed galactose) is known to affect clearance of therapeutic glycoproteins,[3] the identification of these species as sialylated was of particular importance to the overall characterization of this rMAb.

CE−LIF−MS Analysis of rMAb 2. A CE−LIF profile of N-linked glycans released from recombinant monoclonal antibody 2 (rMAb 2) is shown in Figure 7. Peak assignments for major species were based on exoglycosidase treatment and/or comigration with synthetic standards. Similarly, the major N-linked glycans can be identified as G0, G1, G1′ and G2. However, one unidentified minor species is observed at 9.3 min and labeled as peak 1. Similar to rMAb 1, it can be hypothesized that this peak is either sialylated or smaller than G0. However, the migration time of this peak does not correlate with previously identified sialylated species in rMAb 1 and was further investigated by CE−MS to confirm its identity. Figure 8 displays expanded-scale views of (a) a conventional CE−LIF electropherogram using a 60 cm capillary, (b) a CE−LIF trace obtained on-line with MS

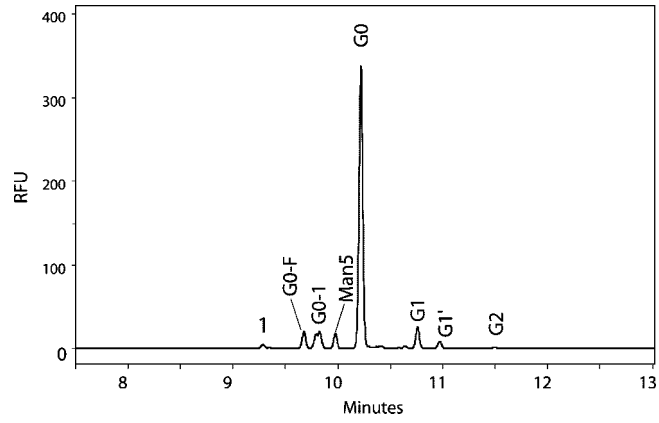


Figure 7. Standard CE−LIF analysis of APTS-labeled, N-linked glycans released from rMAb 2. Identifications are based on enzymatic treatment with exoglycosidases and/or comigration with commercially available standards.

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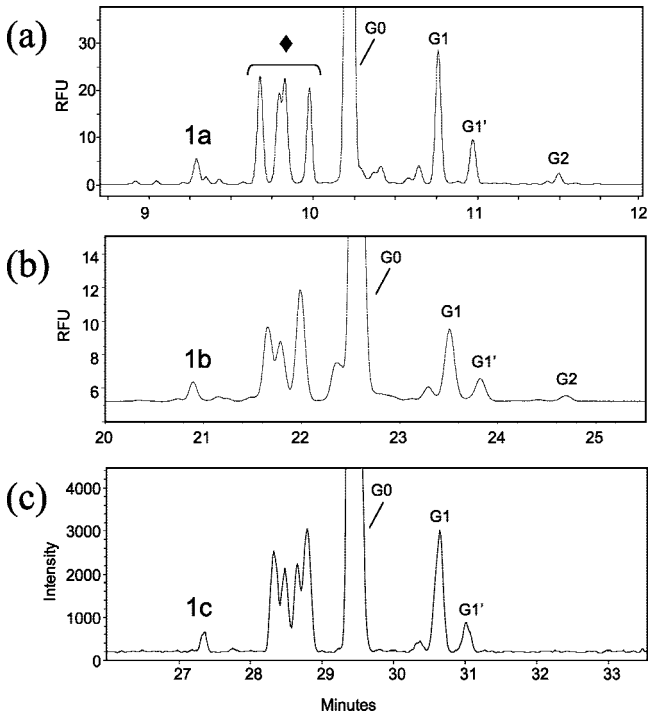


Figure 8. Expanded-scale electropherograms of rMAb 2: (a) standard CE−LIF electropherogram using a 60 cm capillary, (b) CE−LIF trace obtained on-line with MS detection and (c) CE−MS base peak electropherogram.

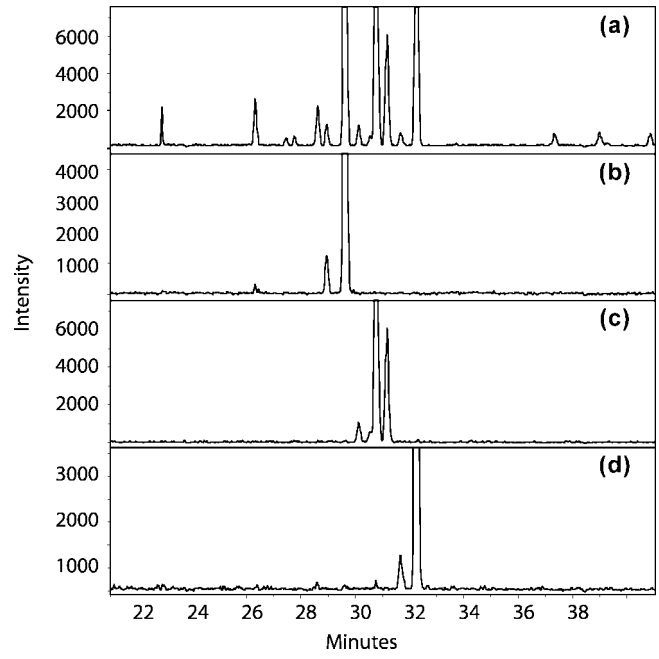


Figure 9. (a) Expanded-view base peak electropherogram of rMAb 1. (b) Extracted ion electropherogram of *m*/*z* 950.76, corresponding to doubly charged G0. (c) Extracted ion electropherogram of *m*/*z* 1031.78, corresponding to doubly charged G1. (d) Extracted ion electropherogram of *m*/*z* 1112.81, corresponding to doubly charged G2.

detection and (c) a CE−MS base peak electropherogram. The unidentified, early migrating species can be seen clearly in both the on-line LIF (peak 1b) and MS ion electropherograms (peak 1c). However, closer examination of other minor peaks in the three expanded-scale electropherograms presented in Figure 8 (marked ♦) suggests that the MS detector may be causing peak distortion, in addition to the change in migration time and resolution loss (this will be a subject of future research in our laboratory). Although this distortion does not appear to affect peak 1, it is imperative that quantitative results be examined between the standard and MS-based assay. The percent peak area was calculated for both the LIF trace from the standard assay (peak 1a) and the LIF trace from the CE−MS based assay (peak 1b). The percent peak area of the total glycans for peak 1a is ∼1.3% and peak 1b is ∼1.4%. Again, the correlation between peak areas allows the confidence to properly compare the peaks from the standard (a) and MS-based assays (b and c). Mass spectral data obtained on peak 1c (not shown) confirms its identity as afucosylated species G0-GlcNAc-fucose (G0-1-F) with a single MS peak observed at m/z 776.19 (theoretical m/z of doubly charged G0-1-F = 776.19). Following identification of this species by CE−MS, further investigations using MALDI mass spectrometry were able to confirm the presence of this additional afucosylated species (data not shown). Since fucosylation is known to affect antibody dependent cellular cytotoxicity (ADCC) activity of some therapeutic rMAbs,[4–6] it is important to account for all afucosylated species. The identification of this species as G0-1-F demonstrates the importance of having characterization tools to identify minor peaks observed in the CE−LIF assay.

Identification of G0, G1 and G2 Isomeric Species. During the course of CE−MS assay development, many antibody products and commercially available standards displayed minor electrophoretic peaks corresponding to isomeric species of each major APTS-labeled carbohydrate (G0, G1 and G2). These species migrated slightly earlier that their wild-type counterparts, and had yet to be observed by complementary assays typically used for glycan profiling in our group (HPLC and MALDI mass spectrometry). The most notable species, a minor peak eluting before G0, displayed the same accurate mass as G0 and was migrating within the cluster of G0-F, G0-1 and Man5 peaks. In the standard CE−LIF assay, it is hypothesized that this peak often comigrates with G0-1 and is thus unidentified. In the case of rMAb 1, assignment of this peak in the standard assay (Figures 4 and 5a, labeled *) is ambiguous as it has a comparable migration to G0-1. Using CE−MS, the identity of this peak (Figure 5c, labeled *) was confirmed as an isomer of G0 based on accurate mass. Figure 9 a illustrates the base peak electropherogram (expanded-view) of rMAb 1, with extracted ion chromatograms corresponding to G0, G1 and G2 in Figures 9b, 9c and 9d respectively. In each case, multiple peaks having the same mass were separated electrophoretically providing evidence of isomeric species present in APTS-labeled carbohydrate samples. Further studies were performed to determine if these species are artifacts from the APTS label and/or sample preparation procedure. NMR studies on the commercially available APTS showed no evidence of isomeric species (data not shown), confirming that these species are not due to label impurities. In addition, the APTS labeling procedure was performed on a commercially available maltoheptaose standard followed by CE−MS analysis (data not shown). Similarly, no evidence of isomeric species was evident. Both studies indicate that these species either (a) are not artifactual, and are present

on some rMAbs, or (b) result from terminal GlcNAc isomerization during the sample preparation. Although the base-catalyzed conversion of GlcNAc to ManNAc has been reported in the literature,[39] this conversion is not known to occur in conditions under pH 9.0.[40]

Although other researchers have reported isomeric species of G1 and G2 detected via mass spectrometric analysis and sequential fragmentation, putative structure included those with branching differences and terminal mannose.[41] Follow-up studies in our laboratory have indicated that isomeric species detected by CE analysis (including G0 and G0-F) are not affected by mannosidase treatment, suggesting that their structures are likely due to anomeric configuration and/or linkage differences. Further structural analysis of these species using complementary techniques will be the subject of future investigations in our laboratory.

CONCLUSIONS

The standard assay for N-linked glycan profiling of rMAbs at Genentech, Inc., is a CE-based assay with LIF detection. Due to the routine use of this assay and increasing number of molecules in the pipeline, it is important to develop characterization tools able to determine the identity of all peaks seen in this assay. In this report, CE−MS technology with on-line LIF detection has been developed to provide accurate mass information on major and minor peaks observed in the routine CE−LIF assay. The CE−MS methodology was developed with an emphasis on maintaining the CE profile obtained using the standard assay, such that comparisons could be made between the CE−LIF and CE−MS electropherograms. In addition, placing an on-line LIF detector before the electrospray source allowed better understanding of the effects of adding the MS, as well as a means to bridge the data. By utilizing the quantitative data provided by the on-line LIF trace, the user has increased confidence when assigning masses to CE peaks observed in the standard assay. The utility of CE−MS for identifying minor species has been demonstrated with two rMAbs, wherein previously unidentified peaks were determined by accurate mass to be sialylated and afucosylated species. Both sialylation and afucosylation can play an important role in the mechanism of action and/or clearance of therapeutic rMAbs, and it is important to accurately identify and quantify such species. In addition, CE−MS was able to identify several isomeric species which were comigrating with other components in the standard CE−LIF assay, and thus previously unidentified. This technology is now being used routinely as a complementary tool for glycan characterization.

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