Characterization of Pharmaceutical IgG and Biosimilars Using Miniaturized Platforms and LC-MS/MS

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Abstract

Therapeutic monoclonal antibodies (mAbs) have made a tremendous impact in treating patients with various diseases. MAbs are designed to target specifically a cell and illicit a response from the immune system to destroy the cell. As originator mAb drug patents are coming to an end, generic pharmaceutical companies are poised to replicate and produce so-called biosimilar drugs. MAbs are significantly more complicated than small drugs to analyze and produce. The mAb proteoform and glycoform must be as similar to the original drug as possible to be a viable replacement. The mAb proteoform is well characterized but can be altered through various undesirable reactions such as deamidation. The mAb glycoform is harder to replicate as the glycan formation is a complicated template-less one; it is proving difficult for the originator companies to produce a homogenous population of mAbs from batch to batch. Severe side-effects have occurred in patients taking mAbs with immunogenic glycans, highlighting the importance of quality control mechanisms.

The complex nature of mAbs requires sensitive and robust tools amenable to the high-throughput analysis required by a manufacturing setting. Miniaturized analytical platforms for complex biosimilar analysis are still in their infancy but have shown great promise for sample preparation. Capillary electrophoresis-laser induced fluorescence remains a powerful and fast technique for routine glycan analysis. Mass spectrometry is the method of choice for the analysis of mAb proteoforms and is emerging as a powerful tool for glycoform analysis.

Keywords

IgG; Microscale immobilized enzyme reactors; CHIPS; IgG; Biosimilars; LC-MS

Introduction

Glycosylation is one of the most common post-translational modifications (PTMs) found in living organisms as evidenced by their ubiquitous presence on cell surfaces and in the surrounding extracellular matrix [1, 2]. Extracellular glycosylation serves several vital functions including but not limited to cell adhesion, cell-extracellular matrix adhesion, signal transduction, immune responses, embryogenesis, and various adult physiological

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processes [3–12]. Glycosylation has a pronounced effect on protein folding, stability, and localization [13].

The importance of protein glycosylation is especially prominent in current disease models. The changes in individual proteoglycans are thought to mediate various pathophysiological events related to cancer tumor progression and have been suggested as biomarkers for prostate cancer (prostate-specific antigen), colon cancer (carbohydrate antigen CA-19-9), liver cancer (α-fetoprotein), and germ cell tumors (and β-human chorionic gonadotropin) [14–19]. Global changes in glycan and glycoproteins are correlated with: inflammatory conditions, age, pregnancy, following vaccination, infection, hereditary disorders, immune deficiencies, cardiovascular disease, and neurodegenerative disorders [20–23]. An increase in glycan fucosylation of haptoglobin has been observed in pancreatic and hepatocellular cancer [24, 25].

Immunoglobulin Gs (IgGs) are a high percentage of the antibodies present in humans [26]. IgGs are large (~150 kDa) glycoproteins comprised of two light and two heavy chains. The quaternary structure results in three distinct moieties: two identical antigen-binding fragment (Fab) regions composed of the light and heavy chains and one effector/crystallizable region (Fc) consisting of only the heavy chains. The Fab region binds foreign antigens while the Fc region alerts the various responses from the immune system by binding to Fc receptor types (FcγRI, FcγRII, FcγRIII, FcRn) or the complement fixation receptor c1q. Antibody activity and effectiveness depend on N-linked glycosylation at Fc asparagine 297 (N297); the Fab regions have also been reported to have glycosylation, but it does not seem to be as critical for antibody activity. Protein engineering has taken advantage of the immune response to modify monoclonal antibodies (mAb) for drug therapy. Most mAb therapeutics are IgG1 based. There are over 40 mAbs approved for use in the United States and Europe and an equal number in various stages of clinical trials [27, 28]. In addition to proteins, carbohydrates are also starting to be targeted for mAb therapy; the anticarbohydrate antibody Unituxin (dinutuximab) targeting GD2 ganglioside was approved for use in treating pediatric patients with high-risk neuroblastoma [28].

The utility of therapeutic mAbs lies in their hallmark ability to specifically bind to a target molecule, reducing side-effects and required dosage amounts. Some mAbs are engineered to bind to unique antigens present on various tumor cells and subsequently recruit the innate immune system to destroy the cancer cells via antibody-dependent cellular cytotoxicity (ADCC) or complement-mediated cytotoxicity (CMC) [29–32]. Other mAbs are designed to inhibit a specific transmembrane receptor; bevacizumab binds to and inhibits the vascular endothelial growth factor (VEGF) which results in angiogenesis inhibition and a decrease in nutrients and oxygen reaching the tumor [33, 34]. Antibody-drug conjugates (ADCs) are a hybrid of mAb and a small drug molecule; ADCs combine the specificity of an mAb with the potency of a highly toxic small molecule [35]. After ADCs bind to the outside of the target cell, they are endocytosed; the linker between the small molecule drug and the mAb breaks, typically pH or protease controlled, releasing the drug into the cell [36]. In addition to single mAb therapeutics, combinations of mAbs, antibody cocktails, can result in improved pharmacological profiles compared to a single mAb [37, 38]. Glycosylation has an immense impact on the efficacy and effectiveness of mAb therapeutics. All antibodies have

at least one N-linked glycan at asparagine 297 in the CH_2 domain of the crystallizable fragment (Fc) [39, 40]; some glycans are also present in the fragment antigen-binding (Fab) portion of some mAbs. The N-linked glycans found in the Fc domain are core fucosylated, biantennary oligosaccharides with up to two galactose residues whereas the Fab domain glycans are reported to be core fucosylated, biantennary oligosaccharides with one or two N-glycolylneuraminic acid (NeuGc) residues, have zero or one α-linked galactose, and zero or one β-linked GalNac [41]. Differences in mAb pharmacodynamics and pharmacokinetics can be attributed to glycosylation. The importance of glycosylation is further illustrated by the loss of binding activity and effector functions upon mAb deglycosylation [42, 43].

Molecular biology and cloning techniques, i.e. protein engineering, have allowed for better control over the production of mAb glycosylation that has improved the clinical efficacy of mAb therapeutics. It has been observed that the removal of fucose from the biantennary structure allows for an increase in binding to FCγRIIIa and subsequently an increase in cytotoxicity [44]. Dramatic clinical results were observed with chronic lymphocytic leukemia patients after treatment with an improved anti-CD20 antibody; a bisecting N-acetyl-D-glucosamine (GlcNAc) was added to the mAb [30, 31]. It has been suggested that mAb modifications could also aid in the treatment of HIV patients [45].

The patent protection on some therapeutic mAb are scheduled to expire soon [28], thus prompting the generic pharmaceutical market to start production of so-called biosimilar drugs, a biological compound highly similar in primary sequence to a reference drug with no clinically meaningful differences in safety, purity, and potency [46]. It has become readily apparent that replicating mAb drugs will be a very different and arduous process. Homogenous batches of small molecule drugs are routinely manufactured in large quantities through various well-controlled chemical reactions. This is in contrast to mAb manufacturing that utilizes biological processes. The mAb itself is disclosed in patents, but the manufacturing process remains undisclosed.

The numerous decisions made in manufacturing biosimilars have a profound effect on the final product, including the quaternary structure, proteoforms, glycoforms, aggregation, and ultimately the mAb activity. The proteoforms can be affected by either incorrect sequences in the cDNA used for mAb expression or in deamidation of asparagine/glutamine to eventually aspartic/glutamic; deamidated therapeutic mAb have been shown to have reduced [47–50]. The choice of cell line has a profound effect on the mAb glycoform with different cell lines yielding different glycoforms. Cetuximab, an mAb targeting EGFR approved for use with squamous-cell carcinoma of the head and neck and colorectal cancer, produced in murine SP2/0 cell line resulted in an anaphylaxis response in patients [51]. It was originally produced in Chinese Hamster Ovary (CHO) cells for clinical trials and scaled up for production in SP2/0 cells. The cause of the illicit immune response was the presence of galactose-α-1,3-galactose glyco-type; a foreign glycan in humans and diagnostic for SP2/0 cell production [51].

Some of the challenges associated with biosimilar production can be attributed to the heterogeneity associated with mAbs. Glycans attached at N297 of the Fc are of the complex biantennary type with a core pentasaccharide with variable addition of fucose, galactose,

bisecting N-acetylglucosamine and sialic acid to a lesser extent. Conversely, glycans derived from the Fab portion of IgGs are reported to be extremely heterogeneous with extensive galactosylation, substantial sialyation, complex biantennary, hybrid oligosaccharides, and N-glycolylneuraminic acid residues. The combination of Fc and Fab glycoforms has been estimated at 128 unique glycoforms. Maintaining glycoform fidelity at both sites has proved a daunting challenge in mAb manufacturing, much less biosimilar manufacturing. Analysis of four batches of Trastuzumab, an approved mAb targeting human epidermal growth factor receptor 2 (HER2) in breast cancer, revealed the batches had the same types of glycoforms but in different relative abundances. Analysis of an undisclosed IgG1 mAb from ImClone also revealed a range of isoforms [52].

In addition to the micro-heterogeneity of the mAb glycoform, the amino acid sequence can also be modified. As mentioned above, deamidation is a common issue in the biological drug manufacturing community. Other inadvertent protein modifications report include but are not limited to disulfide bridge formation between cysteines, N-terminal glutamine cyclization, C-terminal lysine clipping, oxidation, deamidation, non-enzymatic glycation, sequence truncation or point mutations resulting in single amino acid substitutions [53–56].

The extensive proteoforms and glycoforms of mAbs require a multitude of analytical techniques for complete compositional analysis and verification. Ayoub *et al.* [57] characterized Cetuximab employing several mass spectrometry-based (MS) strategies. The intact mAb was analyzed with MS[1] while the amino acid sequence and PTMs, especially the glycoform, was verified with middle-up, middle-down, and bottom-up proteomic approaches. This approach yielded a comprehensive glycosylation profile for the Fab and Fc domains as well as an error in the amino acid sequence of Cetuximab. Gahoual *et al.* [58] also characterized Cetuximab but utilized capillary zone electrophoresis coupled to a mass spectrometer with a sheathless interface (CESI-MS). A bottom-up strategy allowed for 100% amino acid sequence coverage in addition to a glycoform of 16 glycans. Henninot *et al.* [59] characterized an undisclosed mAb from cell culture media with liquid chromatography-mass spectrometry time of flight (LC-MS-TOF). A middle-down approach utilizing IdeS yielded large fragments that allowed for proteoform analysis in one experimental run; EndoS was employed for fucosylation yield analysis. The glycan information yield was equivalent to the gold standard technique, high-performance capillary electrophoresis-laser induced fluorescence (HPCE-LIF) but consumed significantly less media, 500 μL vs 200 mL.

The analytical techniques and examples highlighted in this review can be divided into several sections. The first section discusses advances in mAb sample preparation. We have chosen to focus on mAb for the examples cited in this review. Currently, there is no technique to analyze completely an intact mAb for sequence and glycoform elucidation in one experiment; this is an active area of research in the top-down mass spectrometry community. The mAb must be broken down into smaller pieces before primary sequence and glycoform identification. Typically, N-glycans are released with peptide-N-glycosidase F (PNGase F), an endoglycosidase, followed by digestion of the protein. The following sections highlight research that has allowed for comprehensive mAb proteoform identification and glycan determination. The combination of rapid, and efficient sample

preparation coupled to sensitive detectors yields a powerful approach for accurate mAb analysis.

Importance of Miniaturization in general

In the ultrasensitive measurements of biological and pharmaceutical samples, an extreme caution must be exercised in manipulating the minute quantities of such biomolecules. Biological samples at the low microgram scale, while becoming measurable with the modern instrumental techniques, can easily be adsorbed on the surface of glassware before such measurements. Sample loss during ultrafiltration, dialysis, lyophilization, *etc.*, can easily become a bottleneck of the entire analysis. Another problem with working at such a reduced scale is contamination (dust, solvent, reagent impurities, *etc.*). It is thus crucial to minimize the number of handling and transfer steps during the analysis. Miniaturized forms of separation, regarding reduced column diameters, solvent flow-rates, and the overall surface area that a pharmaceutical sample may encounter during analysis are becoming crucial in high-sensitivity work.

Microscale immobilized enzyme reactors

Microscale immobilized enzyme reactors (IMERs) are steadily gaining acceptance as a rapid means of sample preparation for peptide and glycan analysis. IMERs increase enzymatic efficiency by decreasing the substrate to enzyme ratio and by reducing the diffusion space between the enzyme and substrate. Enzymes are covalently bound to an immobilized monolith inside a capillary; the sample is passed through the capillary exposing the protein of interest to the enzyme. Some labs have reported dramatic improvements in sample preparation time with protease digestion and deglycosylation steps.

Korecka *et al.* [60] utilized IMERs for the preparation and study of IgG fragments; chymotrypsin, trypsin, and papain were utilized to determine the presence of a heterogeneous population of IgG glycoproteins. Krenkova *et al.* [61]demonstrated an extremely efficient IMER with two proteases, trypsin, and endoproteinase Lys-C, coupled either online to ESI-TOF MS or offline to MALDI-TOF MS. An IMER digest of IgG for 6 minutes at room temperature produced comparable and in some cases, better peptide MS data as a soluble enzyme digest at 37°C for 24 hours. Krenkova *et al.* [62], also demonstrated efficient deglycosylation of IgGs with immobilized PNGase F coupled to MALDI-TOF-MS/MS; a 5.5-minute reactor deglycosylation was equivalent to a 24-hour solution based deglycosylation. Weng *et al.* [63] produced a PNGase F based IMER in conjunction with dimethyl labeling to identify 43 up regulated and 30 down regulated N-glycosylation sites in hepatocarcinoma ascites syngeneic cell lines; they also quantitated 11 N-glycoproteins related to tumorigenesis and tumor metastasis.

Microfluidic Devices/chips

Several microfluidic devices have been employed for the rapid analysis of glycans in academic labs. Zhuang *et al.* [64] were able to separate N-glycan positional isomers derived from standards ribonuclease B, and linkage isomers from asialofetuin; their method further

separated N-glycans derived from a cancer patient's serum. Mellors *et al.* [65] employed a more robust analytical system utilizing a hybrid capillary/microfluidic system for comprehensive online capillary electrophoresis liquid chromatography-electrospray ionization mass spectrometry. This allowed the identification of two different N-linked glycosylation sites from an mAb amid a complex mixture of tryptic peptides. Macchi *et al.* [66] was able to quantitate mAb derived glycans utilizing a chip-based nanoflow reversed-phase liquid chromatography coupled with the a time of flight mass spectrometer (nRPLC-Chip-TOFMS) approach.

Two commercially available chip kits are enabling the most widespread use of microfluidic devices. Both chips utilize an immobilized enzyme reactor for rapid deglycosylation, followed by capillary electrophoresis for glycan separation and subsequent detection by either LIF or MS. The Caliper LabChip GXII Microchip-CE platform has already been utilized by Amgen as a high-throughput assay to generate rapidly glycans derived from undisclosed mAb produced in cell cultures followed by LIF detection [67]. Agilent's mAB-glyco kit (Figure 1) has also been utilized for mAb sample preparation before MS-analysis [68–70].

mAb Characterization

After sample preparation and separation, a detector is required for a more detailed qualitative and quantitative analysis. The current approaches to glycoprotein analysis require separation of the glycan from the protein. Current methods are further limited by identification of only N-glycans; O-glycan identification remains a difficult and arduous task. MAb proteoform analysis is dominated by various mass spectrometry-based approaches while the current gold standard in routine glycan detection is high-performance capillary electrophoresis-laser induced fluorescence. Before analysis, glycans are labeled with a fluorophore, commonly commonly 9-Aminopyrene-1,4,6-trisulfonic acid (APTS). HPCE-LIF provides short analysis times, efficient separations and high sensitivity making it ideally suited for routine analysis of well-characterized glycoproteins.

Mass Spectrometry for mAb proteoform identification

Mass spectrometry (MS) is becoming a more widely adopted approach to glycoprotein and mAb analysis because it can characterize both the protein and glycan of glycoproteins through multiple experimental approaches. An MS[1] experiment, either denatured or natively folded (native MS), allows for intact mAb analysis allowing for initial qualitative and quantitative assessment of sample heterogeneity, a particularly useful technique in the emerging field of bispecific mAb and antibody cocktails/mixtures.

Native MS employs aqueous buffers and nano-electrospray to maintain a protein's tertiary and quaternary structure (Figures 2A and 2B). This results in a smaller charge-state envelope than a denatured protein; this allows for easier data interpretation and an increase in sensitivity because the ions are condensed into fewer charge states, improving the signal to noise ratio [71]. Native MS allows for rapid measurements (on the order of minutes for a single analysis), very sensitive (picomoles of mAbs) and minimal sample preparation (often

times a buffer exchange column is used right before MS injection) [72]. Several disadvantages handicap the native MS approach for mAb analysis. Native MS cannot differentiate small mass modifications (+1 Da for deamidation) and cannot determine the location of PTMs other than C-terminal lysine clipping, N-terminal glutamine cyclization, and glycosylation [72]. Work is being done to improve the mass resolution at the higher m/z observed in native MS as well as to improve fragmentation techniques for protein sequence and glycan identification.

Ion mobility mass spectrometry (IM-MS) provides shape to charge information along with mass to charge; this technique is well situated to identify differences in the shape of an isobaric mAb population. Bagal *et al.* [73] utilized IM-MS to resolve differences in disulfide structural isomers of IgG2 mAbs; these mAbs have the same m/z but differ in shape because of differences in the disulfide bonds formed. IM-MS is also being utilized to assess the structural flexibility of mAbs [74] and other large protein complexes [75, 76].

MS^1 experiments yield relevant information about the intact mAb but do not yield more detailed information about the protein of interest. To gain information about a protein's sequence or glycan information, tandem mass spectrometry (MS^2) experiments are performed. Several MS^2 experiments are currently used with differing results. Top-down MS, fragmentation of the intact protein in the MS with electron-transfer dissociation (ETD), electron-capture dissociation (ECD) or ultraviolet photodissociation (UVPD), is a very promising technique that has yielded tremendous information and insight into the histone biology field; numerous histone proteoforms have been identified utilizing this approach [77, 78]. The current top-down fragmentation techniques have resulted in poor sequence coverage for mAbs; approximately 33–34% of mAb was identified with either ECD- Fourier transform ion cyclotron resonance (FTICR) MS [79] and ETD-orbitrap Fourier transform mass spectrometry (FTMS) [80]. A top-down approach coupled with UVPD fragmentation has resulted in 100% sequence coverage of model monomer proteins [81] and 67% sequence coverage of two protein complexes, β-lactoglobulin and hexameric insulin [82].

Middle down mass spectrometry utilizes restrictive proteases, such as papain or IdeS for mAb, to generate large peptides for fragmentation. Fornelli *et al.* [83] utilized IdeS to generate large peptides and was able to observe 70% sequence coverage when several LC runs were averaged. Srzentic *et al.* [84] employed an 'extended bottom-up proteomics' approach using Sap9 for mAb analysis; this resulted in 100% sequence coverage of the light chain and 99–100% coverage of the heavy chain in a single collision induced dissociation (CID) LC-MS/MS run (Figure 2C).

Bottom-up mass spectrometry employs enzymatic digestion, usually with trypsin, to generate numerous peptides. Xie *et al.* [85] utilized this approach and was able to differentiate a difference of 2 amino acids of a biosimilar mAb as well the difference in relative abundance of biosimilar derived glycans and originator drugs. Mechref *et al.* [86] combined several methodologies including capillary electrophoresis (CE) and matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) to comprehensively characterize the glycans derived from murine immunoglobulin G_κ; tryptically generated

glycopeptides were essential to identifying the difference in glycan microheterogeneity and abundance between the conserved and variable domains (Figure 2D).

Glycan analysis

Characterization of mAb also entails the analysis of the glycan moieties associated with the protein. Several analytical approaches have been adopted or developed to facilitate the characterization of the glycan moieties of mAb, including capillary electrophoresis (CE), capillary electrophoresis mass spectrometry (CE-MS), liquid chromatography ultraviolet (LC-UV), liquid chromatography fluorescence (LC-FL), and liquid chromatography mass spectrometry (LC-MS).

Capillary Electrophoresis and CE-MS

Capillary electrophoresis (CE) is a well-proven method for rapidly separating biological molecules; separation times are much faster than LC. The reduced sample and reagent requirements allow for a cost-reduction over a traditional LC separation approach. The small dimensions of CE are especially conducive to coupling with nanoflow electrospray ionization mass spectrometry (nESI-MS) as there is less solvent to evaporate before introduction into the instrument. Some laboratories have utilized CE for mAb analysis. Hunt and Nashabeh [87] developed a robust CE-LIF based assay to replace sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for routine mAb quality control experiments. The mAb is kept intact and labeled with 5-carboxytetramethylrhodamine succinimidyl ester, a neutral fluorophore, for low concentration detection. The method offered the sensitivity of a silver-stained SDS-PAGE gel as well as the advantages of a high-throughput assay: increased reproducibility, increased precision, and short analysis times. The same group [88] also developed an assay to assess better the N-glycans derived from the mAb, rituximab. It was known that the N-glycans affect the mAb's efficacy, but there was not a robust assay in use for routine glycan analysis. Their method derivatizes glycans with APTS followed by CE-LIF analysis. This approach is still used as a quick assessment for well-characterized glycans. Gahoual *et al.* [89] utilized sheathless capillary electrophoresis and tandem mass spectrometry (CESI-MS/MS) with a bottom-up proteomic approach for comprehensive mAb proteoform and glycoform analysis. The same group further showed their approach was well situated to discern differences between the biosimilar and original drug mAbs [58]. An automated offline approach was employed for the comprehensive analysis of an mAb; a novel capillary electrophoresis ultraviolet detection separation method was interfaced with matrix-assisted laser desorption ionization mass spectrometry (CE-UV/ MALDI-MS) [90]. A bottom-up proteomic approach was employed and resulted in 100% sequence coverage of the light chain and 92% of the heavy chain along with four major glycosylated peptides and their structural characterization.

Routine glycan analysis has typically been performed by HPCE-LIF because of the short analysis times, efficient separations, and relatively inexpensive equipment compared to MS. For these reasons, HPCE-LIF has been used as a high-throughput routine analysis to monitor mAb glycoforms. Glycans are labeled with a fluorophore, usually APTS, passed through a CE separation, excited with a laser and subsequent fluorescence detection. Ruhaak *et al.* [91]

optimized a gel-based CE method for mAb derived N-glycan profiling in a high-throughput format. This method was able to characterize glycans derived from a pregnant woman's serum; serum was collected during each trimester as well as 6 weeks, 3 months and 6 months post-partum. Szabo *et al.* [92] has utilized this approach to separate fucosylated and non-fucosylated glycans from several high mannose oligosaccharides derived from mAb (Figure 3). Szabo *et al.* [93] also utilized a CE-based method to detect the presence of the immunogenic α-1,3-Gal in mAb derived from mouse cell lines.

MS

Some different MS only-based approaches have been utilized for glycan characterization including but not limited to electrospray ionization-ion mobility-quadrupole-time of flight mass spectrometry (ESI-IM-Q-TOF MS) [94], matrix assisted laser desorption ionization-quadrupole- time of flight-mass spectrometry (MALDI-Q-TOF MS) and tandem mass spectrometry MS/MS [95], MALDI-TOF and LCQ®-Ion Trap MS [52]. Haselberg *et al.* [96] utilized CE coupled to TOF-MS via sheathless electrospray ionization to characterize the proteoform and glycoform of biological pharmaceuticals (Figure 4). The sheathless ESI source increased the ionization efficiency for intact proteins while simultaneously decreasing ionization suppression. This allowed for the identification of multiple glycoforms on interferon-β (18 glycans) and recombinant human erythropoietin (74 glycans) in addition to multiple PTMs on both proteins.

Combined Analytical Techniques to attain comprehensive characterizations of mAb

A combination of analytical techniques has resulted in a number of sensitive and high-throughput assays that can identify routine glycans and characterize novel or low abundant glycans. Gennaro *et al.* [97] developed a high-throughput glycan assay by combining the rapid separation of CE with the immediate information from LIF detection to a sensitive MS. This allowed for routine assays as well as more in-depth characterization should an anomaly appear on the LIF trace (Figure 5).

Maeda *et al.* [98] combined CE-LIF and ESI-MS to develop a high-throughput assay aimed at distinguishing immunogenic carbohydrates from human N-glycans in six commercial therapeutic mAbs. Hamm *et al.* [99] combined CE-LIF with off-line MS to characterize APTS-labeled-glycans derived from mAb produced in murine myeloma (NS0) cells. This approach allowed for routine analysis of well-characterized glycans with the more in-depth characterization of minor glycans utilizing MS analysis. Reusch *et al.* [100] also took a combined method approach but introduced a DNA analyzer for fluorescence detection. APTS-labeled glycans were initially identified with hydrophilic interaction liquid chromatography-ultra performance liquid chromatography-tandem mass spectrometry (HILIC-UPLC-MS/MS) analysis and subsequent capillary gel electrophoresis with laser-induced fluorescence (CGE-LIF) with a DNA analyzer. Mahan *et al.* [101] also coupled CE with a DNA analyzer to develop an inexpensive, routine, method for the high-throughput analysis of glycans derived from mAb. Wiegandt *et al.* [102] combined a high-throughput MS based assay to identify 37 N-glycan compositions derived from the therapeutic

originator mAb Cetuximab; the ten most abundant glycans were unambiguously characterized with proton nuclear magnetic resonance (^{1}H NMR). Redman *et al.* [103] integrated CE with MS in a microfluidic platform to characterize intact ADCs and their drug-to-antibody ratio (Figure 6).

Concluding Remarks

The sensitive and robust tools amenable to the high-throughput analysis required by a manufacturing setting are essential for the characterization of mAbs and biosimilars. Although miniaturized analytical platforms for complex biosimilar analysis are still in the early stages of development, they have demonstrated great promise for sample preparation. Capillary electrophoresis-laser induced fluorescence remains a powerful and fast technique for the routine analysis of glycans derived from mAbs and biosimilars. Mass spectrometry is the method of choice for defining the proteoforms of mAbs and biosimilars. MS is emerging as a powerful tool for glycoform analysis.

Acknowledgments

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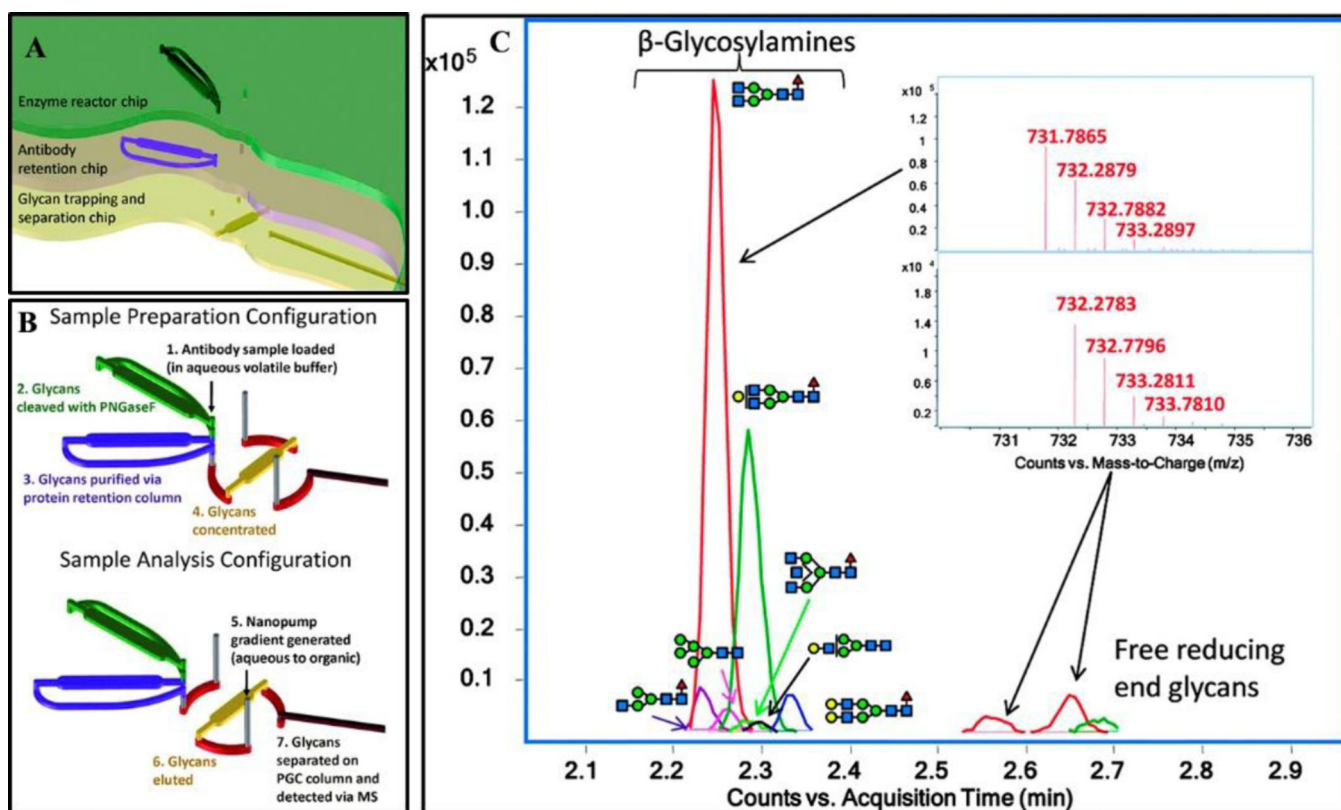
Figure 1. Illustration of a commercially available microfluidic device, Agilent's Mab-glyco chip
(**A**). Schematic showing the location and orientation of the deglycosylation enzyme reactor,
antibody retention region and the glycan trap to concentrate. (**2**). Diagram illustrating the
sample loading and handling scheme. (**B**). Samples are initially sent to the enzyme reactor
for deglycosylation with PNGase F. The deglycosylated proteins are trapped in the protein
retention column while the glycans pass through to the glycan trap where they are
concentrated. A rotor shift allows for exclusive elution of the glycans from the glycan trap;
the proteins remain on the retention column. The eluted glycans pass through an analytical
column followed by ESI-MS analysis (**C**). Figure reproduced with permission [68].
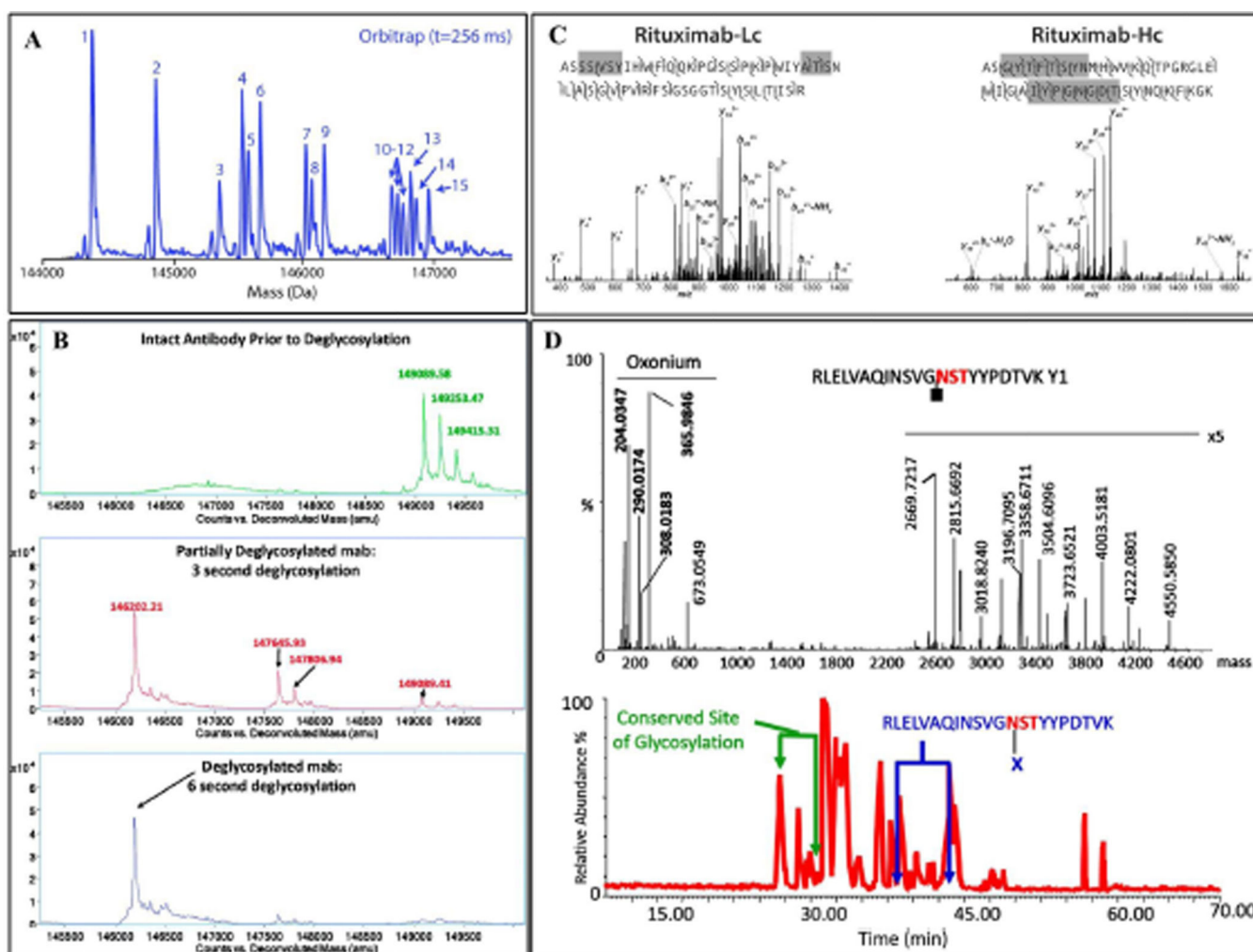
Figure 2. The different MS-based proteomics approaches to mAb proteoform identification
(**A**) The intact mAb analysis yields rapid insight into the mAb population homogeneity and can be used to assess the deglycosylation efficiency of an enzyme reactor (**B**). Large peptide fragments result from a middle-out or 'extended bottom-up' approach. The large fragments can be used for determining the various components of the mAb, or they can be fragmented in the instrument for primary sequence elucidation. (**C**). Product ions from a fragmented glycopeptide illustrate a bottom-up proteomic approach which utilizes small tryptic peptides to verify sequence identity and in this example, glycan attachment site. Figure reproduced with permission [104] [68] [84] [86].
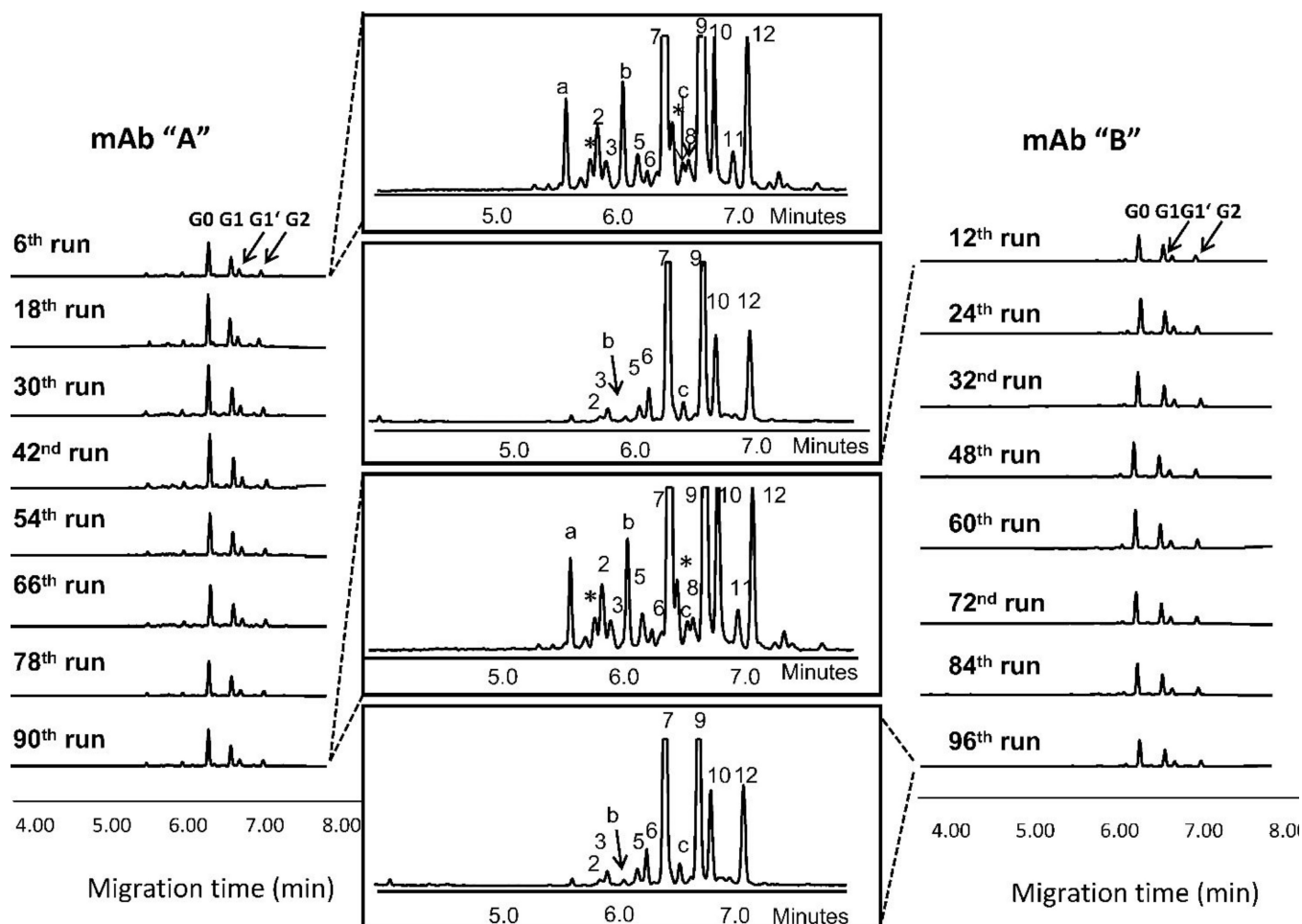
Figure 3. Representative Data for Capillary Electrophoresis
A 96-well plate based method was developed for the rapid analysis of N-glycans derived from two different mAbs. Figure reproduced with permission [92].
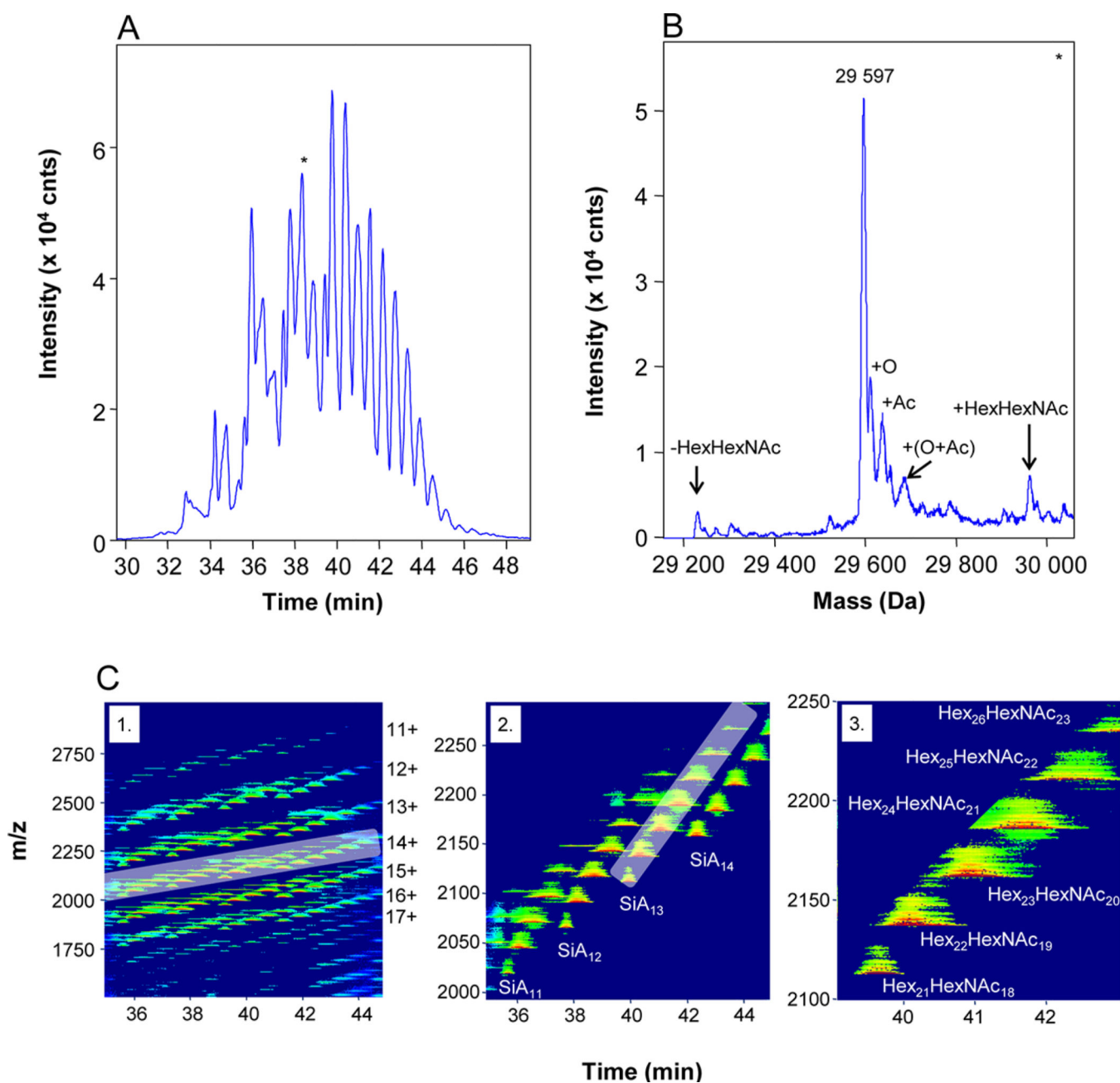
Figure 4. Sheathless capillary electrophoresis for extensive glycoform analysis of recombinant human erythropoietin (rhEPO)

(A) TIC of eluted glycans and (B) deconvoluted MS of 38 minute LC window. (C) Contour plot of glycans illustrating the intensity of each m/z value over the LC run. The authors highlighted the 14+ glycoforms in (2) and zoomed in (3) to show they could distinguish a subset, the 14+ SiA_{13} sialoforms. This sheathless CE-MS allowed the authors to identify 74 glycoforms derived from rhEPO. Figure reproduced with permission [96].
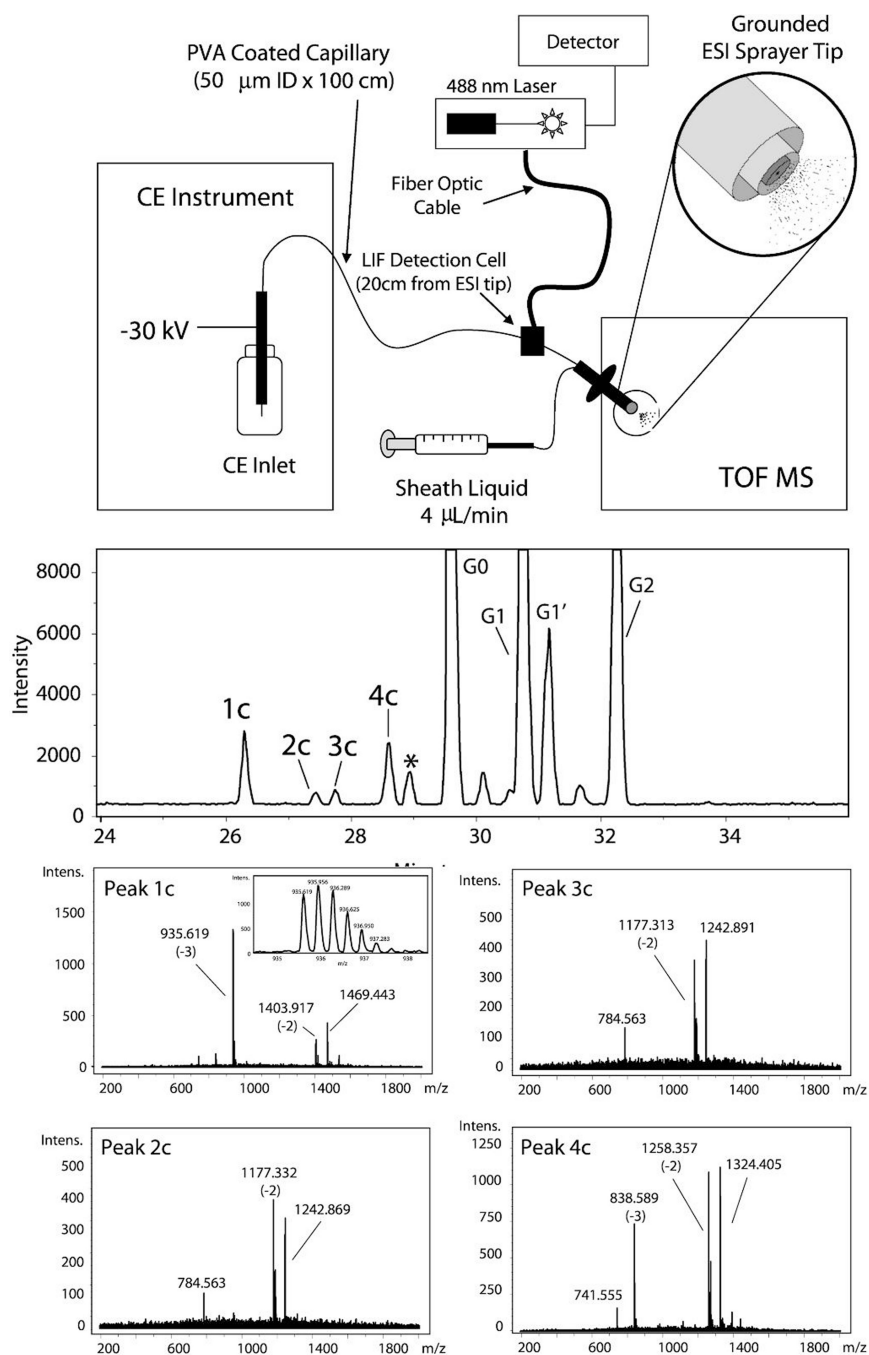
Figure 5. Schematic of a hybrid capillary electrophoresis-laser induced fluorescence (CE-LIF) method coupled to TOF-MS detection

The CE-LIF method allows for rapid separation and analysis of the glycans for routine quality control experiments while the TOF-MS allows for more detailed analysis should an anomaly appear in the CE-LIF experiment. (A) The instrumentation configuration shows the CE-LIF detection first followed by TOF-MS. (B). Representative data from a CE-LIF experiment. In this example, the 1c–4c peaks were not accounted for by the standards. (C) MS of the 1c–4c peaks allows for more detailed analysis of the peaks resulting in their identification as sialylated species. This approach combines the advantages of CE-LIF and

MS resulting in a robust assay amenable to high-throughput environments. Figure reproduced with permission [97].
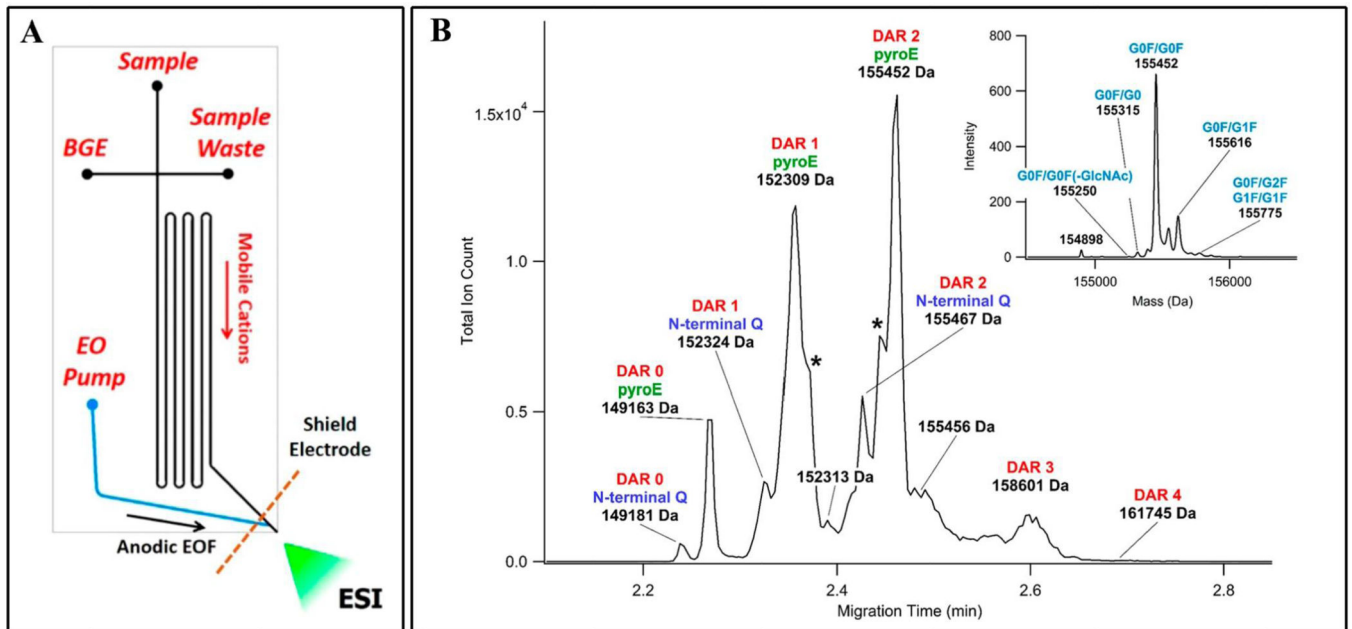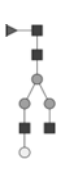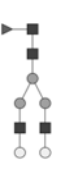
Figure 6. Intact ADC analysis with CE-MS in a microfluidic platform
A novel microfluidic CE-MS device (A) was used to rapidly analyze the products of the drug conjugation reaction (B). [103]

Table 1

Glycan identified by LC-MS/MS. Symbols Square: N-acetylhexosamine; triangle: fucose; diamond: N-glycolylneuraminic acid; dark grey circle: mannose; light grey circle: galactose. Reproduced from [102] with permission.

Glycan composition[a]	Identified by		Quant.	Glycan composition[a]	Identified by		Quant.
	MS [Da]	Acc. [ppm]	MS [%]		MS [Da]	Acc. [ppm]	MS [%]
	1624.604*	**3.7**	**31.4**		2475.888*	1.9	0.4
	1462.552*	3.7	19.6		2239.802	1.5	0.4
	2110.762*	2.7	13.9		1931.692	1.9	0.3
	2255.800*	2.3	7.7		2313.838	0.6	0.3
	1786.656*	2.7	7.5		2620.931*	1.5	0.3
	1234.436	2.2	2.9		2637.945*	1.6	0.3
	1259.465	1.0	2.0		1761.621	1.6	0.3
	1745.626*	1.7	1.9		2296.8198	0.3	0.2
	2094.763*	1.3	1.5		2458.868	0.6	0.1

Glycan composition[a]	Identified by		Quant.
	MS [Da]	Acc. [ppm]	MS [%]
	2093.746*	2.2	1.5
	1421.519	1.1	0.4
	1583.574*	1.3	1.0
	1478.542*	1.4	1.0
	2151.782	1.3	0.8
	2400.832*	1.9	0.6
	1989.734*	0.8	0.6
	1907.680*	1.5	0.6
	2782.984*	1.4	0.5
	1599.566*	1.4	0.5
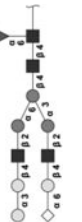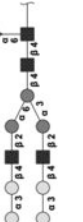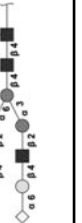
Glycan composition[a]	Identified by		Quant.
	MS [Da]	Acc. [ppm]	MS [%]
	1827.678*	0.2	0.3
	1890.758	2.2	0.2
	1665.625	0.6	0.2
	1437.514	1.8	0.2
	1396.486	0.7	0.2
	2077.751	4.5	0.1
	2052.718	1.9	0.1
	2928.016*	1.9	0.1
	1316.486*	2.3	<0.1

Table 2

The 10 most abundant glycans identified with ^{1}H-NMR. Symbols as in Table 1. Reproduced from [102] with permission.

Glycan structure[b]	Identified by		Quantification		Compound Number
	MS [Da]	Accuracy [ppm]	NMR (L,C,S)[c]	MS [%][d]	NMR [%/nmol][e]	
	1624.604	3.9	L,C,S	20.4	13.9/5.2	1
	1624.604	3.9	L,C,S	11.4	14.6/5.5	2
	1462.552	4.9	L,C,S	19.6	22.9/8.6	3
	1786.656	3.1	L,S	7.5	3.0/1.1	4
	2255.800	2.8	L,C,S	7.7	5.8/2.2	5
	2110.762	2.8	L,C,S	13.9	15.9/5.9	6
	1234.436	1.9	L,C,S	2.9	7.9/3.0	7
	2093.746	2.5	L,S	1.4	4.8/1.8	8

| Glycan structure[b] | Identified by | | | Quantification | | Compound Number |
	MS [Da]	Accuracy [ppm]	NMR (L,C,S)[c]	MS [%][d]	NMR [%/nmol][e]	
	2400.832	0.4	L,S	0.6	10.0/3.7	9
	2093.746	2.5	L,S	0.1	1.2/0.4	10