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ANALYSIS OF GLYCANS DERIVED FROM GLYCOCONJUGATES BY CAPILLARY ELECTROPHORESIS-MASS SPECTROMETRY

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Abstract

The high structural variation of glycan derived from glycoconjugates, which substantially increases with the molecular size of a protein, contributes to the complexity of glycosylation patterns commonly associated with glycoconjugates. In the case of glycoproteins, such variation originates from the multiple glycosylation sites of proteins and the number of glycan structures associated with each site (microheterogeneity). The ability to comprehensively characterize highly complex mixture of glycans has been analytically stimulating and challenging. Although the most powerful mass spectrometric (MS) and tandem MS techniques are capable of providing a wealth of structural information, they are still not able to readily identify isomeric glycan structures without high order tandem MS (MS^n). The analysis of isomeric glycan structures has been attained using several separation methods, including high-pH anion exchange chromatography (HPAEC), hydrophilic interaction chromatography (HILIC) and gas chromatography (GC). However, capillary electrophoresis (CE) and microfluidics capillary electrophoresis (MCE) offer high separation efficiency and resolutions, allowing the separation of closely related glycan structures. Therefore, interfacing CE and MCE to MS is a powerful analytical approach, allowing potentially comprehensive and sensitive analysis of complex glycan samples. This review describes and discusses the utility of different CE and MCE approaches in the structural characterization of glycoproteins and the feasibility of interfacing these approaches to mass spectrometry.

Keywords

Glycans; Glycoproteins; Capillary Electrophoresis; Microfluidics Capillary Electrophoresis; CE-MS; MCE-MS

Introduction

Glycosylation is considered as the most common and structurally diverse posttranslational modification of proteins. The common complexity of glycosylation patterns and the frequent difficulties associated with resolving such fine structural differences in large biopolymers is attributed to the multiple glycosylation sites of proteins and their associated microheterogeneity. This high complexity and structural variation associated with a glycoprotein (known as protein glycoforms) ultimately define the function and activity of a glycoprotein [1, 2].

Protein folding, stability, and localization, among other key biochemical processes, are defined by the glycosylation of proteins [3]. Moreover, cellular communication, such as cell-cell, cell-matrix, protein-protein, and sugar-sugar interactions, is controlled through specific interactions between a glycan and its target protein(s) [2, 4–6]. Alterations in the

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glycosylation of proteins, either through various site occupancy changes on the polypeptide chain, or in the variation of the oligosaccharide structures occupying a particular site on a protein, modulates the biological activity of proteins (glycoproteins). Additionally, aberrant glycosylations of glycoconjugates have been implicated in many mammalian diseases, such as hereditary disorders, immune deficiencies, cardiovascular disease, and cancer [7, 8]. Recently, devising approaches that allow monitoring the subtle, yet biologically significant, glycosylation changes have been the focus of many biomedical research initiatives. The primary goal of these initiatives is the development of analytical tools that allow effective analysis of glycans which subsequently aid in the diagnosis and prognosis of these diseases, as well as understanding these diseases at the molecular level needed for effective development of cures [8].

Currently, the characterization of biomolecules, including glycans, is routinely attained using mass spectrometry (MS) [9–13]. However, MS and tandem MS techniques, which provide a plethora of structural information, are not suited alone to characterize isomeric glycan structures without high order tandem MS (MS^n). The latter is not feasible for glycans existing at their biological concentrations. Therefore, the analysis of glycan pools containing isomeric structures is only practically feasible using both MS and separation methods. Since glycans are relatively polar molecules, hydrophilic-interaction chromatography (HILIC) has been recognized as a powerful method for the separation of such structures [14, 15]. The retention of different oligosaccharides on silica- or amide-based stationary phases appears sufficient for the identification of core glycan structures present in a typical glycan mixture. However, HILIC glycan separations are typically time-consuming and partially distinguish isomers. This disadvantage has been recently alleviated by the use of HILIC-based micro- and nanofluidic platforms [16–18]. Better resolution is attained using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) [19]. However, HPAEC-PAD analyses also suffer from relatively long analysis time (> 60 min.), poor reproducibility, and low sensitivity [20]. Although the development of high pressure LC systems has permitted the separation of very complex samples in relatively very short time, the feasibility of this approach for the analysis of glycan mixture has not been fully exploited.

Both capillary electrophoresis (CE) and microfluidics capillary electrophoresis (MCE) offer high separation selectivity and efficiencies, allowing the effective resolution of glycan isomers [21–23]. The separation of different glycans has been attained by various capillary electromigration techniques, including capillary zone electrophoresis (CZE) [24, 25], capillary gel electrophoresis (CGE) [26], micellar electrokinetic chromatography (MEKC) [27], and capillary electrochromatography (CEC) [28–31]. Nevertheless, no isomeric separation has been demonstrated by MEKC. CEC, which is based on the movement of a mobile-phase driven by an electroosmotic flow (EOF) through a column containing a stationary phase, permitted the separation of several structurally similar glycans and certain structural isomers have been resolved by CEC [29–31]. However, CGE [26, 32] and CZE [24, 33] have offered superior separation efficiency and resolution of isomeric glycans.

The merging of capillary electromigration in its capillary or microfluidic formats with the most informative and one of the most sensitive detection technique (MS), is only expected to provide a large wealth of information which can only allow better understanding of the biological attributes of biomolecules. This review mainly focuses on discussing the different means by which CE and MCE techniques are interfaced to MS and the utility of these techniques in the structural characterization of glycoproteins.

Release of Glycans from Glycoconjugates

The release of N-Glycans from lipid or protein back-bones is achieved either chemically or enzymatically. However, the excessive sample treatments and preparation steps required in the case of chemical release through hydrazinolysis [34, 35] or alkaline β -elimination [36] have severely limited the use of this approach, since the multiple steps associated with these approaches results in increased sample losses.

Although several commercially available enzymes can be used to release N-glycans from glycoproteins, peptide N-glycosidase F (PNGase F) remains to be the most widely used enzyme [37]. This is an endoglycosidase enzyme which releases the intact glycans as glycosylamines which are readily converted to reducing glycans in the commonly used phosphate buffer. This enzymatic release of glycans also results in the conversion of asparagine to aspartic acid at the N-glycosylation site of the protein. This chemical change and shift in the molecular weight of the deglycosylated peptide is often used to determine the site of glycosylation when mass spectrometers offering high mass accuracy and resolution are employed. PNGase F has a very wide specificity, cleaving all N-glycans except those having possessing fucose residues that are $\alpha(1-3)$ linked to the reducing-terminal GlcNAc. Glycoproteins with such linkages are commonly found in plants. These N-glycans with reducing end fucose attached to GlcNAc through $\alpha(1-3)$ linkages can be enzymatically cleaved using peptide N-glycosidase A[37]. Other endoglycosidases can be more specific as discussed in a review by O'Neill [37].

The analysis of protein O-glycosylation is still substantially less sensitive than that of N-glycosylation mainly because of the lack of a nonspecific enzyme capable of efficiently releasing O-glycans. O-Glycans are commonly released through different chemical approaches involving several steps, resulting in substantial sample loss, thus subsequently limiting the sensitivity of O-glycan analysis.

Hydrazinolysis [34, 35] and alkaline β -elimination [36] are two chemical approaches commonly used to release O-glycans of proteins. Although hydrazinolysis yields reducing glycans, it constitutes a tedious procedure with many needed precautions [35]. An improved version, involving the use of 70% (w/v) aqueous ethylamine was recently reported [38, 39], but the overall reaction yields were low, as the O-glycans suffered significant peeling reaction and/or other forms of degradation.

β -Elimination approach is widely viewed as the most reliable and universal method for the release of O-glycans. However, this chemical approach is not microscale compatible, as the minute quantities of released glycans are easily lost during the cleaning procedure needed to reduce/eliminate the excessive amounts of salts used in this method. Also, the presence of a strong reducing agent, which converts glycans to their respective alditols, is necessary here to minimize the “peeling reactions” caused by the alkaline medium [40]. The conversion to alditols prevents reductive amination which may often be needed for the attachment of a fluorophore/chromophore to enhance CE, LC or MS sensitivities of MS investigations [41–44]. Sugar alditols are not useful when a polyvalent coupling to a lipid or protein is needed for a subsequent immunoassay [45]. A modified β -elimination procedure so-called ammonia-based β -elimination provides a viable alternative to the hitherto used hydrazinolysis and the Carlson β -elimination methods [46]. This procedure yields free reducing end N- and O-glycans. Its simplicity, the lack of peeling reactions and deacetylation byproducts supplement its effectiveness at microscale levels.

Unlike N-glycans, no endoglycosidases are capable of effective release of O-glycans, with the partial exception of endo- α -N-acetylgalactosaminidase, permitting the release of unsubstituted Core-1 O-glycans [47–49]. This highly specific enzyme has very limited use,

as it does not cover the other core structures. At this time, chemical release methods provide the only universal means for O-linked glycans.

A sensitive alternative to enzymatic digestion was recently described by Mechref and co-worker, involving the use of pronase E in conjunction with permethylation[50]. The cleavage is based on pronase digestion of glycoproteins prior to permethylation. The high pH associated with permethylation allows β -elimination to proceed methylation of hydroxyl and amino groups. This new approach appeared to be at least 50-times more sensitive than all the other chemical approaches. However, this approach will only produce permethylated O-glycans which can only be analyzed by MS and LC-MS [50].

Capillary Electrophoresis and Microfluidics Capillary Electrophoresis of Glycans

Basic Considerations

While glycan pools released from purified glycoproteins, or glycoproteins in biological samples can be readily analyzed by MALDI-MS, fluorescently-tagged mixtures separated by CE and detected by LIF are often viewed as somewhat complementary to MALDI-MS. This is due, as mentioned above, to the fact that different forms of CE are often capable of resolving isomers, which are otherwise indistinguishable by MS. Several types of capillary electromigration techniques have been routinely utilized for glycomic analysis, in either the CZE mode, employing either free-buffer media or gels. However, high resolution and fast analysis inherently offered by these techniques can only be appreciated when used in conjunction with sufficiently sensitive detectors. Detection of glycans at high sensitivity is immensely hindered by their lack of an inherent chromophore or fluorophore. While direct or indirect detection modes have been described for the analysis of underivatized glycans, such analyses lack the necessary sensitivity to analyze glycan mixtures derived from biological samples [51]. Electrochemical techniques have proven more useful for the detection of underivatized glycans than spectroscopic detection. Amperometric and pulsed-amperometric detection are two approaches that have been demonstrated for the detection of carbohydrates, yet they lack reproducibility and the necessary selectivity in the presence of interfering compounds. Fluorophores are commonly attached to the reducing end of the glycan molecules through reductive amination to permit their detection through laser-induced fluorescence (LIF) which is recommended if MS is not available [13]. Since not all N-glycans possess electrical charges, the derivatization procedure introduces ionic groups needed for electromigration of neutral glycans. However, some neutral derivatizing reagents have also been utilized in conjunction with the MEKC mode or in CZE mode using borate buffer, yet the resolution and efficiency associated with such methods have not been satisfactory.

In comparison to the miniaturized LC-based techniques, CZE suffers from a limited capability to inject large aliquots of biological samples. While sample stacking and the use of other means of preconcentration can somewhat reduce this problem, CZE performs best at very small solute concentrations and volumes, and, correspondingly, small column diameters. The detection challenges of CZE, and to a various degree also the other capillary electromigration techniques, can be offset through the use of the highly sensitive LIF and MS techniques.

Capillary Electrophoresis- Laser-Induced Fluorescence (CE-LIF): a Complementary Technique

In 1991, the first glycan analysis by CE-LIF was demonstrated, using 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA) fluorescence-labeling reagent, for amino sugars [33], and glycoprotein-derived N-glycans [25, 52, 53]. This fluorescence-labeling reagent permitted the analysis of N-glycans derived from fetuin at subattomole detection limits. Since then, other derivatizing reagents were utilized in conjunction with CE-LIF for the sensitive detection of glycans.

Several fluorophores, including 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS), 7-aminonaphthalene-1,3-disulfonic acid (ANDS), and 2-aminonaphthalene-1-sulfonic acid (ANS), were chemically attached to glycans through reductive amination chemistry (Figure 1) to enhance the sensitivity of CE-LIF analysis of glycans [54–56]. ANTS allowed faster analyses at high resolution because of its greater charge. Accordingly, this reagent was successfully employed for characterization of glycans derived from various glycoproteins, including human immunoglobulin G [57], ovalbumin [58, 59], fetuin [32, 58], recombinant HIV gp-120 [60], and α_1 -acid glycoprotein [60].

Although ANTS has been found effective in glycomic analysis, the instability and high cost of the required He-Cd laser prompted the need to explore alternative fluorophores, requiring a more convenient light source such as the argon-ion laser. Today, 1-aminopyrene-3,6,8-trisulfonate (APTS) is the most commonly used fluorescence reagents for CE-LIF analysis of glycans [61–63]. Chen and Evangelista [61] were the first to use this reagent for the analysis of N-glycans derived from glycoproteins. Glycans were enzymatically released from their glycoproteins and derivatized with APTS under mild reductive amination conditions to preserve sialic acid and fucose residues prior to their CE-LIF analysis. Using similar methodologies, other investigators reported the analyses of N-glycans derived from various glycoproteins, including ribonuclease B [26, 32, 61, 62, 64], fetuin [61, 62], recombinant human erythropoietin [61], kallikrein [61], and a chimeric recombinant monoclonal antibody [24].

Guttman used the same fluorophore for N-glycan sequencing by GCE and exoglycosidase digestions [21]. He employed a carefully designed exoglycosidase matrix with a subsequent comparison of the positions of the separated exoglycosidase digest fragments to maltooligosaccharides of a known size. Accordingly, the appropriate linkage information could be deduced from the positions of separated peaks and combined with the shifts resulting from treatment with a specific exoglycosidase. Although this approach allowed structural identification of N-glycans, the procedure is time consuming and data interpretation is difficult in the case of a complex mixture of glycans derived from biological samples such as tissue or blood.

Ma and Nashabeh [24] were the first to describe the use of CZE to monitor variations in the glycosylation of rituximab, a chimeric recombinant monoclonal antibody, during production. The N-glycans derived from rituximab are neutral, complex biantennary oligosaccharides with zero, one and two terminal galactose residues (G0, G1, and G2, respectively). The method was the same as the one described by Guttman [21], yet CE was employed instead of CGE. All observed glycans were fully resolved, including the positional isomers of G1 (Figure 2a). The two G1 positional isomers were identified by comparing CE profiles obtained from sequential enzymatic digestions (Figure 2b). This method is simple, accurate, precise, and allow somewhat high throughput, and robustness. However, sample preparation is rather lengthy.

Mechref and co-workers have also demonstrated baseline separation of closely related glycan structural isomers using APTS in conjunction with CZE-LIF [65]. The CE-LIF trace (Figure 3a) of the fluorescently labeled standards (core-fucosylated biantennary, asialylated, and mono- and disialylated glycans) clearly illustrates the ability to separate several structural isomers. Monosialylated structures with terminal sialic acid residues attached to either the 1–6 or 1–3 antennas were partially resolved (Figure 3a). As shown in Figure 3b, monofucosylated biantennary glycan structures lacking one terminal galactose residue which were derived from a monoclonal antibody are baseline resolved, further supporting the ability of CZE to resolve closely related structural isomers.

The clinical applicability of CE-LIF for the profiling of serum N-glycans was demonstrated in 2001 by Callewaert and coworkers by using capillary electrophoresis gel-based DNA sequencer [66]. This method was utilized to assess liver cirrhosis [67, 68]. The method allowed the quantification of 14 peaks that were detectable in all samples. Although 5 peaks demonstrated a decrease or increase in abundance that could be correlated with disease progression, quantitative assessment of liver cirrhosis was achievable using the ratio of only two glycan structures identified in the desialylated N-glycans derived from blood serum samples as a triantennary and fucosylated GlcNAc bisecting glycan structures. Although this CE-based N-glycan profiling method distinguished cirrhotic from noncirrhotic chronic liver disease patients, with 79% sensitivity and 86% specificity, it was rather complicated and hard to use.

The same group recently modified and simplified the same approach and labeled it GlycoFibro test [68]. It involved the use of 96-well plate-based serum N-glycomics sample preparation in conjunction with incubation in a polymerase chain reaction (PCR) thermocycler. This configuration yielded APTS-labeled N-glycans which were subsequently analyzed by the capillary electrophoresis-based DNA sequencer. This approach was employed to analyze 376 consecutive chronic hepatitis C virus patients for which liver biopsies were available. Quantification was based on the ratio between a bisecting GlcNAc-modified agalactose glycan and a triantennary glycan. The ratio of these two glycans correlated with the histological fibrosis stage data generated through FibroTest ($p = 0.4–0.5$) [68]. The developed method appears to be effective in assessing liver fibrosis in chronic hepatitis patients; therefore, it might be considered as an alternative to liver biopsy which suffers from several disadvantages, including sampling error and up to 20% interlaboratory variance [68].

Although CE-LIF has offered high sensitivity and resolution for the analysis of N-glycans derived from both purified glycoproteins and biological samples as indicated above, the need to have standards or to use a battery of exoglycosidase to identify glycan structures have somewhat limited its use. This factor has also been the driving force behind the development of CE analysis for N-glycans using buffers that are compatible with mass spectrometry without losing resolution and separation efficiencies.

Interfacing Capillary Electrophoresis and Capillary Electrochromatography to Mass Spectrometry

Enhanced MS sensitivity and CE-MS reproducibility over the past several years have prompted the popularity of employing CE-MS for the analysis of biomolecules. This interfacing merges the better of the two techniques, the high sensitivity of mass spectrometers and the high separation efficiency of CE. Interfacing CE to MS technique also benefits from the high mass accuracy and tandem MS to generate a wealth of information that allows a structural characterization. In the case of glycan analysis, MS and tandem MS

allow the positive identification of isobaric structures which are resolved by CE and subsequently detected and characterized by MS and tandem MS.

Interfacing of CE and CEC to MS built on the approaches that were primarily developed for the hyphenation of liquid chromatography and MS. In the late-1980s, coupling of CE to MS was reported for a continuous-flow fast atom bombardment mass spectrometer (cfFAB-MS) [69, 70], electrospray mass spectrometer (ESI-MS)[71, 72], and ionspray mass spectrometer (ISP-MS)[73]. Interfaces available for coupling CE to MS have been comprehensively reviewed.[74–78]. Coupling CE to MALDI-MS was initially reported by Chang and Yeung[79], and shortly after by Karger and co-workers [80]. CEC coupling to MALDI-MS was later reported by Tegeler *et al.* [81].

ESI Interfaces for Capillary Electrophoresis

Interfacing of electromigration techniques and MS through ESI requires overcoming several limitations, including (i) proper grounding of the CE electrical circuit and (ii) flow-rate compatibility. CE usual flow-rates are 1 to 100 nL/min, while that for cf-FAB, electrospray, ionspray and MALDI-MS interfaces is 1–200 μ L/min flow-rate. Therefore the addition of sheath-liquid is needed to overcome this flow rate incompatibility. However, this addition prompts analyte dilution and the introduction of additional “chemical noise”. Micro- and nano-electrospray systems have provided a solution to the flow rate incompatibility, thus allowing higher efficiency and ion sampling rates at extremely low flow-rates compatible with CE.

Poor sensitivity associated with the reduced ionization efficiency of the analyte or arcing between the ESI sprayer needle and the electrode of the CE result from Improper grounding of the CE electrical circuit associated with ESI interface. However, this problem is easily addressed by using a 40–100 M Ω resistor to ground both CE column outlet and the ESI sprayer needle. There are three ESI interfaces developed for pairing CE or CEC and MS, namely (i) sheathless, (ii) (coaxial) sheath flow, and (iii) liquid-junction.

Sheathless-flow Interface

In 1987, Smith and co-workers were the first to describe the use of stainless-steel capillary sheath for electrical contact[71]. The same later on described the incorporation of silver on the stainless-steel capillary sheath [72]; however, the lack of stability of this interface originating from the flaking of the silver and gold coatings of the capillary limited the utility of this arrangement. Mazereeuw *et al.* were the first to describe the direct application of the high voltage to the inlet of a tapered separation capillary outlet to form a microspray tip [82]. This arrangement eliminated the need for the electrode or conductive coating of the spray capillary, and the sheath liquid. On the other hand, Zare and coworkers [83] inserted a thin gold wire about 2 mm into the capillary separation outlet to establish the electrical potential needed for ESI. In this arrangement, the authors claimed the ability to sustain a stable electrospray current by the CE electroosmotic flow; however, this aspect of the interface design was questioned by others [82]. Although other arrangements based on the sheathless-flow concept were reported, the instability and high day-to-day variations associated with this design have limited the use of this type of interface.

Sheath-flow Interface

Smith and coworkers were the first to describe a coaxial delivery of sheath liquid to make-up for the discrepancy between CE flow-rate and ESI required flow-rate [84]. Similar designs were employed to couple CE to cfFAB-MS [70], and ISP-MS [85]. Generally, this type of interface requires a mixing of a sheath liquid with CE outlet flow at the tip of the separation capillary to produce the needed flow-rate to sustain the ESI. Three concentric

capillaries are used in this design, where the innermost capillary is the CE separation capillary. The sheath-liquid (make-up flow) is provided by a second stainless-steel capillary through which the ESI potential is applied, while a nebulizing gas is supplied through the third (outer) capillary to sustain uniform ESI and suppresses the effects of corona discharge. The make-up liquid introduces dilution and additional “chemical noise”, thus rendering this interface insensitive. Moreover, capillary position and flow-rate as well as composition of sheath liquid, need to be optimized to sustain reproducible and efficient ESI.

Liquid Junction Interface

A third design for CE-MS interface involved partial disconnection of the CE separation column and the ESI emitter, thus allowing for a choice of appropriate make-up solvent and flow-rate for optimized ESI performance. This decoupling of the CE and ESI emitter allows individual optimization for each of the two components. Henion and coworkers [73] were the first to use this interface to couple CE to MS. In their reported arrangement, a stainless-steel tee was used to provide a 10–25 μm gap between the ends of the CE separation column and the electrospray needle. The limited use of this interface is attributed to (1) the required high degree of precision in the alignment and the spacing of the CE and sprayer capillaries, (2) the poor mixing of the CE electrolyte and the make-up liquid, and (3) the increased band-broadening associated with this arrangement. However, these issues were rectified by a controlled flow-rate delivery of the make-up fluid and the implementation of a self-alignment system [86].

Other improvements of the original arrangement include the use of a porous junction design which is based on making a small section of the CE capillary porous, thus providing the needed electrical connection to sustain ESI. Initially, glass joint- μESI immersed in a reservoir of 1% acetic acid [87], and a microdialysis tubing [88, 89] were described. In the case of the latter, the limited mass transfer of the microdialysis tubing substantially reduced/eliminated the loss of sensitivity caused by the make-up liquid introduced in the sheath flow or liquid junction interface. The liquid flow in this arrangement is due to the column electroosmosis, the pressure resulting from the capillary effluent and the flow induced by ESI. A liquid junction interface, using a removable electrospray tip enclosed in a sub-atmospheric chamber and placed in front of the sampling orifice of the mass spectrometer, was described by Karger and co-worker [90]. This arrangement allowed a good control of the flow from the liquid reservoir that feeds the liquid junction by the chamber pressure. The use of a liquid junction through a porous segment around the entire circumference of the capillary outlet was also described as a means to sustain an electrical connection [91]. However, these designs were laborious and made the capillary very fragile. The creation of porous tip at the capillary outlet appears to overcome some of these limitations [92, 93]. The simplified version of this design involves the creation of the porous section by removing 1–1.5 in. of the polyimide coating of the capillary and etching using 49% aqueous solution of hydrofluoric acid until it is porous (30 min) [92]. This etching process also simultaneously reduces the outer diameter of the CE separation capillary outlet, thus permitting efficient and reproducible ionization. The porous capillary outlet tip is then inserted into the ESI needle (metal sheath) prior to filling the needle with the background electrolyte to achieve the electrical connection to the capillary outlet. The etching process reduces the wall thickness of the etched section, including the tip of the capillary, to 5–10 μm , which for a 20–30 μm i.d. capillary results in stable electrospray at *ca.* 1.5 kV. This porous junction design is simple, reproducible, automatable, and does not require any mechanical tools. The interface design does not add any dead volume, since it leaves the capillary inner wall intact. Moreover, this design is recently adopted by Beckman-Coulter, Inc. (Brea, CA) for its new CESI 8000 commercial system.

CE was recently interfaced to MS using a pressurized liquid junction nanoflow electrospray interface and surface-coated capillaries [94]. In this configuration, the separation capillary and the spray tip were positioned in an electrode vessel containing the appropriate spray liquid. Inside the liquid junction, an electrode spray potential was applied, thus sustaining a stable electrospray at nl/min flow rates without an external pump. This interface provided high durability of the spray tip and allowed independent optimization of the CE separation and ESI conditions [94].

MALDI Interfaces for Capillary Electrophoresis

Chang and Yeung were the first to describe the coupling of CE to MALDI MS [79]. A UV laser which vaporizes and ionizes the effluents from a CE separation capillary was used in conjunction with calcium chloride solution (0.5 mM) which acted as both a CE electrolyte and a matrix. This arrangement produced pulsed-ions analyzed by a TOF mass spectrometer. Karger and co-workers [80], introduced a vacuum deposition interface for coupling CE to MALDI-TOF MS. Liquid samples consisting of the analyte and matrix were deposited on a moving tape in the evacuated source chamber of a TOF mass spectrometer. The vacuum deposition procedure generated significantly more reproducible signal intensity, eliminating the need for a “sweet spot” searching relative to the conventional dried-droplet method.

Murray and co-workers [95], described an on-line CE-MALDI-TOF MS interface using a rotating ball. This interface is based on a rotating stainless steel ball that transports samples from atmospheric pressure to the high vacuum of the mass spectrometer for desorption and ionization. The ball is rotating at 0.03 to 0.3 revolutions/minute. The sample is deposited directly from the separation capillary on a 19-mm ball on which a mixing of the matrix and the sample takes place using a capillary delivering matrix solution. The sample and matrix mixture is dried prior to passing a polymer gasket into the MALDI-TOF MS ionization chamber. The ball is cleaned after it rotates out of the ionization chamber, using a solvent-saturated felt.

The above mentioned MALDI-MS interfaces were developed mainly for CE-MS of peptides. A sample deposition device for interfacing CEC to MALDI MS for the analysis of glycans was recently described by Tegeler *et al.* [81]. The device consisted of an inlet and outlet buffer reservoirs connected to a matrix reservoir through a connection sleeve. The matrix reservoir is connected to a deposition capillary via another connection sleeve. CEC eluent was transported to the matrix reservoir via a capillary that was connected to the deposition capillary by the connection sleeve inside the matrix reservoir. This connection sleeve also acted as a mixing chamber, allowing the CEC eluent to be mixed with the matrix prior to deposition. The utility of this arrangement was demonstrated for complex glycan mixtures separated by CEC using hydrophilic-phase monolithic columns, with the capillary flow being deposited on a standard MALDI plate along with a suitable matrix solution [81].

Glycomic Analysis by CE-MS

The application of CE-MS and tandem MS to glycoscreening in the biomedical field has been recently described and discussed in several reviews [13, 96–100]. Off-line coupling of CE to MALDI-MS for the structural characterization of APTS-labeled N-glycans derived from glycoproteins was first described by Suzuki *et al.* [64]. The CE-resolved components were collected using an automated high-resolution fraction collector. The authors also described an on-probe sample cleanup with a cation-exchange resin to acquire a negative-ion mass spectra of the APTS-labeled N-glycans derived from glycoproteins using a mixture of 6-hydroxypicolinic and 3-hydroxypicolinic acids (1:1 ratio) as a matrix. Singly-deprotonated ions with a detection limit of *ca.* 30 fmol for APTS-labeled maltoheptose were detected. Highly sialylated structures (tri-, tetra-, pentasialylated and higher) detection

sensitivity was not at par with that of asialylated or mono- and di-sialylated structures. This could be due to the in-source fragmentation of the highly labile sialic acid.

Both CE-ESI-MS [101] and CE in conjunction with MALDI-MS [102] were employed to characterize mannoooligosaccharide caps in mycobacterial lipoarabinomannan (ManLAMs) which are key molecules in the immunopathogenesis of tuberculosis. APTS-labeled mannoooligosaccharide caps were detected as $[M-2H]^{-2}$ molecular ions in the negative ion mode using triethylammonium formate buffer in the case of CE-ESI-MS analysis. Unequivocal structural characterization at the picomole level was attained since some sequence fragment ions were detected in the spectra. Mannoooligosaccharide caps were released by a mild acid hydrolysis from 85 μ g of cellular ManLAMs of *M. bovis*. Preferential ManLAMs cleavages, occurring in the arabinan domain, were achieved as a result of this treatment, thus leading to the formation of arabinose, mannoooligosaccharide caps and the mannan core. This was unequivocally confirmed through CE-ESI-MS in which arabinose, mannose, mannose-arabinose, mannose-mannose-arabinose and mannose-mannose-arabinose-arabinose were detected [101]. The last two structures were previously misassigned using CE-UV and CE-LIF analysis in which the structural assignment was based on matching the migration times to standards [103].

On the other hand, CE-MALDI-MS analysis allowed the analysis of only 5 μ g (300 pmol) of *M. tuberculosis* ManLAMs subjected to a mild acid hydrolysis prior to APTS labeling and CE separation. Off-line MALDI MS analysis was performed on the fractions collected from the CE separation. The matrix consisted of a (9:1) mixture of 2,5-dihydroxybenzoic acid (DHB) and 5-methoxysalicylic acid (MSA), while on-probe sample cleanup using cation-exchange resin was required. Mass characterization of the deprotonated molecular ions $[M-H]^{-}$ and the y-type ion fragments obtained in post-source decay experiments allowed the successful assignment of the mannoooligosaccharide cap structures [102]. These two studies confirmed that Off-line CE-MALDI-MS, in this case, was more sensitive than CE-ESI-MS as suggested by the amount of ManLAMs consumed in each study. This better sensitivity of the off-line CE-MALDI-MS was also confirmed through the ability of this approach to detect the minor compounds observed in CE-LIF analysis which were not identified in the CE-ESI MS study [102].

Off line CE-ESI-quadrupole time-of-flight (QTOF) MS configuration was recently utilized for glycoscreening of O-glycosylated peptides derived from the urine of patients with a hereditary *N*-acetylhexosaminidase deficiency (Schindler's disease) [104]. The sensitive identification of the different glycopeptides present in urine samples was achieved using the data-dependent acquisition capability of the QTOF instrument which allowed on-the-fly switching between the negative-ion mode MS scans and low-energy collision-induced dissociation tandem MS on selected precursor ions. The low detection levels of glycans typically derived from biological matrixes was attained using this approach. Sialylated O-glycosylated peptides, undetectable in complex mixtures by direct ESI-MS, were identified using this off-line CE-ESI-MS configuration.

Harvey and coworkers described the analysis of negatively charged labeled and native glycans using CE coupled to an ion-trap mass spectrometer through ESI sheath-flow interface design [105]. A stable spray was sustained in a 60–100% methanol solution as a coaxial sheath liquid, while nitrogen was employed as a sheath gas. This configuration was employed to analyze native N-glycans derived from fetuin as well as ANTS-labeled N-glycans derived from ribonuclease B and fetuin. A CE separation attained in this study using 20 mM 6-aminocaproic acid buffer was comparable to the ones usually achieved using 10 mM citric acid, a buffer commonly utilized in the CE-LIF analyses of labeled glycans. The

use of ANTS as a labeling reagent was driven by its high ionization efficiency over a wide pH range and its resistance to fragmentation during tandem MS experiments [105].

Phosphorylated N-glycans derived from cellobiohydrolase I were analyzed using a Q-Trap mass spectrometer coupled to CE [106]. A tandem MS was acquired through a modified triple quadrupole, where the Q3 region was employed as a conventional quadrupole mass filter or as a linear ion trap with axial ion ejection [107]. Simultaneous negative ion detection of both neutral and charged glycans was achieved through APTS labeling of the glycans. Generally, APTS labeling of glycans offers several advantages, including high-resolution in CE, a better ionization in the negative ion mode, a simultaneous detection of neutral and charged oligosaccharides, and generation of predictable tandem MS spectra (Figure 4). The described analyses allowed the distinguishing of phosphorylated glycan isomers, while tandem MS data furnished additional structural information [106].

Labeling of N-glycans derived from glycoproteins attained through the transglycosylation activity of Endo- β -N-acetylglucosaminidase (Endo-M) was recently reported [108]. This endoglycosidase possesses both a hydrolytic activity toward the glycosidic bond in the *N,N'*-diacetylchitobiose moiety of the N-glycans of glycopeptides, and a transglycosylation activity to transfer both the complex-type and high-mannose type oligosaccharides of the N-linked sugar chains from glycopeptides to suitable acceptors having an *N*-acetylglucosamine residue. A suitable fluorescent acceptor such as NDA-Asn-GlcNAc was used in conjunction with this enzyme to fluorescently label N-glycans derived from glycoproteins prior to CE-TOF-MS analysis. This approach was initially validated using N-glycans derived from ovalbumin. Pronase digestion of glycoproteins, which required prior to treatment with Endo-M, made this derivatization approach lengthy. However, the derivatization approach alone reaches completion in 30 min [108].

Gennaro and Salas-Solano were the first to describe the on-line CE-LIF-MS configuration which permitted the direct characterization of N-linked glycans from therapeutic antibodies [109]. Shown in Figure 5 is the schematic illustration of this online CE-LIF MS configuration. The inherent mass accuracy of the MS permitted the identification of major and minor glycan. This study demonstrated for the first time the ability to attain CE-MS separation efficiency somewhat comparable to that commonly observed in CE-LIF analysis [109]. Shown in Figure 6 are 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 are seen clearly in both the on-line LIF and MS electropherograms. The MS electropherograms (b and c) suffer from a shift in migration time and some loss in separation efficiency, which is due to dead volumes originating from the addition of the MS detector.

Glycomic Analysis by CEC-MS

Generally, CEC is referred to as a technique pairing the desirable features of both capillary electromigration (flow-induced radial solute mass transfer) and chromatography (a wide choice of suitable stationary phases). Moreover, CEC allows an easy preconcentration of dilute samples at the capillary inlet. Although a recent progress in CEC features prominently monolithic columns, which principally offer a wide range of retention selectivity together with the separation conditions that appear compatible with MS operation, stationary phases designed specifically for the separation of glycans have not been in abundance or their performance has not been very reproducible.

Examples of carbohydrate applications of CEC include the separation of aminobenzamide derivatives [110], using a hydrophobic monolithic stationary phase. Other approaches to separating native glycans using “MS-friendly” mobile phases lead to the use of columns featuring hydrophilic interactions. CEC monolithic columns suited for the separation of very complex pools of glycans have been demonstrated [29–31]. However, the production of these CEC monolithic columns have been limited to one research group suggesting the difficulty associated with the assembly of such columns.

An application of these monolithic CEC columns based on hydrophilic interactions involved coupling to FT-MS for the analysis of closely related glycan structures [31]. Shown in Figure 7a is a mixture of O-glycans chemically released from mucin and analyzed by CEC-ESI-FT-MS. This type of mass spectrometer offers an average mass measurement accuracy of 3.9 ppm with external calibration. The mass resolution and accuracy of FT-MS paired with the CEC separation allowed the separation and characterization of two glycan structures that differ from each other by 1 m/z unit. These were an acidic glycan with m/z 756 (at 10.5 min in Figure 7b) and a neutral glycan with m/z 757 (at 12 min in Figure 7b). These structures are not distinguishable by MALDI-TOF-MS. CEC analysis allowed the separation of these two glycans, while the high mass accuracy of FT-MS allowed their accurate mass determination (756.2683 m/z and 757.2851 m/z , Figure 7c and 7d, respectively). Tandem MS provided additional structural information, thus enabling their unequivocal structural assignment. Upon collision-induced dissociation (CID), the acidic glycan with m/z value of 756.2683 and GlcNAc(NeuGc)GalNAc-ol composition easily lost its acidic residue at the nonreducing end to form the fragment at m/z value of 449 (Figure 7e). Sialic acid residues are very labile in CID; therefore, they are readily lost. Conversely, the neutral glycan with m/z value of 757.2851 produced two product ions with m/z values of 449 and 611. These fragments originate from the loss of a fucose residue and fucose and hexose residues from the nonreducing end of the structures (Figure 7f), thus suggesting FucHexHexNAcGalNAc-ol to be the composition of this glycan.

Employing the CEC-MALDI-MS interface described above [81], the analysis of N- and O-glycans derived from bile salt-stimulated lipase (BSSL) from human breast milk, which is a relatively large glycoprotein consisting of 722 amino acid residues with numerous O-glycosylation sites near the C-terminus [111], was achieved. The high microheterogeneity of the glycan structures derived from this glycoprotein is depicted in Figure 8, in which 3-D and 2-D CEC-MALDI-MS electropherograms are illustrated. Over 50 distinct peaks are depicted in this figure. Many of the depicted peaks differ by a few mass units, while some peaks appear to possess the same m/z values, yet are separated in time, hence they are structural isomers.

Microfluidics Capillary Electrophoresis

The fact that glycoproteins are commonly encountered at sub-microgram levels prompts the need to miniaturize the previously described separation methodologies. Sample loss to the surfaces of devices being used can occur prior to MS measurements which commonly permit accurate measurements of small amounts. A sample loss during sample preparation (ultrafiltration, dialysis, lyophilization, etc.) can easily become a bottleneck of the entire analysis. Working at such a reduced scale also introduces other problems such as contamination (dust, solvent, reagent impurities, etc.). Accordingly, minimizing sample handling and transfer steps during analysis are critical. In high-sensitivity work, reducing column diameters, solvent flow-rates and the overall surface area that a glycan sample may encounter during analysis is very significant. The ability to integrate multiple steps such as sample preparation, purification, separation and detection to a small analytical unit, such as

a microchip is invaluable to minimizing sample losses and, consequently enhancing analysis sensitivity.

Thus far, chip-based approaches employed in glycomic analysis involve devices which incorporate microchannels, allowing a sample injection, preconcentration and separation. Both electrophoretic and chromatographic modes of separation in microchips have been performed. The former is commonly referred to as microchip capillary electrophoresis (MCE), which commonly uses different detection principles such as refractometry [112], electrochemical detection [113–116], UV [117], and LIF [118–120].

An interesting application of MCE involving a total serum protein N-glycome profiling was recently reported [67, 121]. This method was an extension of the method described above [66]. Alumina-silicate glass chips with a double-T injector were fabricated. A uniform introduction of all molecular types without a bias commonly associated with an electrokinetic injection in CE was achieved through the use of this type of injector. The profiling of the major N-glycans in human serum was attained in 12 min with a resolution comparable to what has been achieved through gel-based DNA sequencers using MCE with 11.5 cm effective length filled with 4% linear polyacrylamide permitted. Profiling of two serum samples from a noncirrhotic chronic hepatitis patient and a cirrhotic patient demonstrated the potential of ME in clinical studies (Figure 9). The change in the ratio of two glycan structures (namely, complex triantennary and fucosylated bisecting biantennary) was determined by the same group to be diagnostic for liver cirrhosis in a previous study using gel-based DNA sequencer (Figure 9B) [67]. MCE generated profiles (Figure 9A) supported these findings. The two peaks associated with glycan structures were well resolved in MCE, while the ratio between the peaks representative of the two structures was comparable to the gel-based DNA sequencer results. Accordingly, this study demonstrated the practical aspects of MCE analysis, allowing rapid, reproducible and quantitative comparison between the different liver cohorts.

Although the same group reported recently the development of a new method that is substantially shorter than the aforementioned methods, the separation efficiency demonstrated by the new method was substantially lower [122]. Moreover, the new method involved only 10 min incubation with PNGase F, which is substantially shorter than what is commonly used. This short incubation time was not justified or supported by any data. The new method was supposed to be an improvement of the other methods which were based on comparing the ratio of two peaks corresponding to fucosylated GlcNAc bisecting and triantennary glycan structures; however, these two peaks are not base-line resolved, thus making their quantification partially non practical.

Smejkal *et al.* employed a commercially available microfluidic chip electrophoresis system, which is primarily designed for DNA analysis [123]. This system successfully separated APTS-labeled N-glycans derived from human plasma glycoproteins enriched by boronic acid-lectin affinity chromatography. Although the separation was attained in 40 s in a microfluidic channel of 14 mm length, the resolution of such separation was substantially lower than that observed in conventional CE.

Jacobson and co-workers recently reported the efficient electrophoretic separations of N-glycans on MCE with analysis times less than 3 min [124]. A spiral channel design with an effective separation length of 22 cm was employed (Figure 10a). MCE performance was comparable to that of CE, thus confirming the absence of any negative effects arising from the noncircular cross section of the microchannel. The microfluidic device was employed to analyze APTS-labeled glycans derived from model glycoproteins and blood serum. The separation efficiency provided by MCE was adequate to resolve N-glycan positional isomers

derived from ribonuclease B and linkage isomers derived from asialofetuin. The separation efficiencies attained for the complex APTS-labeled N-glycans derived from blood serum (Figure 10b) was highly comparable to those observed in the case of APTS-labeled N-glycans derived from model glycoproteins. The same group recently enhanced the separation efficiency of APTS-labeled glycan using serpentine separation channels and asymmetrically tapered turn [125]. The tapered turns were designed to minimize band broadening originating from the “racetrack” effect. This serpentine design permitted an increase in the length of the separation channels beyond what was attainable using spiral channel design described above without sacrificing separation efficiency.

CONCLUSIONS

Although the CE spatial resolution in relatively short separation times has been demonstrated here, wide glycomic application of this technique has been limited by the mismatch between the amount detected and sample concentration. While LIF is permitting highly sensitivity CE analysis of glycans, this detection approach does not offer definitive identification when complex sample is being analyzed. Glycomic analyses are currently taking advantage of what MS offers in terms of sensitivity and identification through tandem MS and high order tandem (MS^n). It is expected that a further enhancement in MS sensitivity is still feasible, permitting a greater use of CE in future glycomic studies.

Since the first papers on CE-MS coupling were published during the late 1980s, substantial advances have been made in the performance of MS. Some ingenious technologies for the coupling of CE with different types of MS instruments now exist due to the intensive research of different groups pursuing the goal of separating isomeric oligosaccharide mixtures. However, the CE methods developed for different analytical tasks in glycobiology often utilize the buffer systems that are not compatible with MS measurements. Another important issue is the sample capacity of different CE modes, favoring CEC over CE as a sensible compromise between the resolution of closely related structures and the adequate MS and MS/MS recording conditions (as demonstrated in Figure 7). With MALDI and the miniaturized ESI available as alternative ionization techniques for carbohydrate MS measurements, it is also important to take into account suitability of their different derivatization procedures and protocols in a CE-MS combination.

Although the efficiencies of MCE separations have been shown to be very comparable to those of CE, yet at much shorter analysis time (sec. vs. min), MCE applications in glycomic analysis of complex samples is still lacking behind. Unfortunately, this is often driven by the misconception that complex samples will plug the microfluidic channels, yet MCE channel dimensions are very comparable to those of CE. There is an acceptance of the ability of MCE to accommodate the separation of complex systems. This is evident by the limited increase in MCE publications.

Abbreviations

ANTS	8-Aminonaphthalene-1,3,6-trisulfonic acid
ANDS	7-aminonaphthalene-1,3-disulfonic acid
ANS	2-aminonaphthalene-1-sulfonic acid
APTS	1-aminopyrene-3,6,8-trisulfonate
BEG	background electrolyte
CE	capillary electrophoresis

CEC	capillary electrochromatography
CGE	capillary gel electrophoresis
CZE	capillary zone electrophoresis
CBQCA	3-4-carboxybenzoyl)-2-quinolinecarboxaldehyde
CID	collision induced dissociation
cfFAB-MS	continuous-flow fast atom bombardment mass spectrometer
EKC	electrokinetic chromatography
EOF	electroosmotic flow
ESI	electrospray ionization
HPAEC	high-pH anion exchange chromatography
HPLC	high-performance liquid chromatography
HILIC	hydrophilic interaction chromatography
GC	gas chromatography
ISP-MS	ionspray mass spectrometer
LIF	laser-induced fluorescence
MS	mass spectrometry
MALDI	matrix-assisted laser desorption ionization
MCE	microfluidics capillary electrophoresis
PCR	polymerase chain reaction

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Figure 1.
Reductive amination derivatization approach.

Figure 2.

Electropherograms of glycans obtained from a chimeric recombinant antibody (rituximab) after sequential enzymatic digestion steps: (a) PNGase F; (b) β -N-acetylhexosaminidase; (c) α -1,2,3-mannosidase. Samples were derivatized before CE analysis. The N-glycans were incubated with β -N-acetylglucosaminidase to remove the terminal GlcNAc residues on the G0 and G1 isomers, as seen in (panel b). The migration time of G2 remained unchanged, since it does not possess terminal GlcNAc. On the other hand, G0 exhibited a larger change in migration time than G1 isomers because of the removal of two GlcNAc residues from G0, relative to one from G1 (panel b). The use of α (1-2,3)-mannosidase, which specifically cleaves terminal α (1-3) linkage, in conjunction with a change in the CE profile, indicated the predominance of G1 with a galactose residue on the mannose α (1-6) arm (panel c). Reproduced from ref.[24] with permission.

Figure 3.

CE profile of APTS-labeled glycans derived from a monoclonal antibody. The upper trace represents standard core-fucosylated biantennary/disialylated, monosialylated, and asialylated glycans. Conditions: column, polyacrylamide-coated 50/365 μm ID/OD; length, 50.5 cm total, 40.5 cm effective length; temperature, 257C; injection pressure, 0.5 psi for 5.0 s; voltage, 15 kV anodic electroosmotic flow; λ_{ex} 488 nm, λ_{em} 520. Reproduced from ref. [65] with permission.

Figure 4.

CE-LIF electropherograms of the APTS-derivatized total CBH I *N*-glycan pool and of the uncharged and charged glycans (a). CE-MS base peak electropherogram of the total CBH I *N*-glycan pool (b). The neutral glycans are labeled A–D, the charged ones E–I. Unlabeled neutral glycans are detected in the EOF; unlabeled phosphorylated glycans migrate more slowly and are indicated by an asterisk. Reproduced from [107], with permission.

Figure 5. Schematic of the CE-MS system with on-line LIF detection. The system comprised of a CE system with a CE-MS capillary cartridge allowing an external detection. Fitting a PVA coated capillary with an ellipsoid from Picometrics (Toulouse, France) and using a separate Picometrics Zeta-LIF Discovery detection system consisting of a 488 nm laser unit and an LIF detection cell connected to the detector via a fiber-optic cable permitted online LIF detection. The detection cell is fitted with an adjustable arm, approximately 20 cm from the ESI tip. A MicroTOF mass spectrometer equipped with an orthogonal grounded ESI source was used. A sheath liquid consisting of a 50:50 isopropanol:water (0.2% ammonia) was added coaxially to the separation capillary at 4 μL/min flow rate. Reproduced from ref.[109] with permission.

Figure 6. Expanded-scale electropherograms of APTS labeled N-glycans derived from 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. Reproduced from ref. [109] with permission.

Figure 7.

(a) 2-D contour plot of a mixture of O-glycans cleaved from mucin; (b) zoomed 2-D contour plot, (c and d) spectra of glycans with m/z values of 756 and 757 and their corresponding tandem mass spectra (e and f). An average resolution of $\sim 30,000$ is demonstrated.

Experimental conditions: cyano column, 26 cm; mobile phase, 2.4 mM ammonium formate buffer (240 mM, pH 3) and 0.2 mM sodium acetate in the mixture of acetonitrile/water (71:29, v/v); field strength, 600 V/cm; injection, 12 kV, 30 s. Reproduced from [31] with permission.

Figure 8.

3D Electrochromatogram of the mixture of N-linked and O-linked glycans derived from human bile salt-stimulated lipase. Experimental conditions: cyano capillary column, 28 cm; mobile phase, 2.4 mM ammonium formate buffer in a 60/40 mixture of acetonitrile/water; field strength, 535 V/cm; injection, 10 kV, 10 s; matrix, 20 mg/ml DHB in 80/20 methanol/water. Reproduced from [81] with permission.

Figure 9. (A) MCE profiling of serum samples from a noncirrhotic chronic hepatitis patient (upper trace) and cirrhotic patient (lower trace). (B) Profiling of the same samples using the ABI377 gel-based DNA-sequencer. Symbols: $\square$, N-acetylglucosamine; $\circ$, mannose; $\square$, galactose; $\bullet$, fucose. Reproduced from ref. [67] with permission.

Figure 10a

Figure 10b

Figure 10.

(A) Schematic of the microfluidic device with the spiral channel design used for glycan analysis. The effective separation length was 22 cm from the cross intersection to the detection point indicated by the arrow. The reservoirs and corresponding channels are labeled with their functions. The microchip substrate was 5 cm by 5 cm. The narrow and wide channels were 60 and 240 μm wide at the top, respectively. (B) Electropherogram of N-glycans released from a blood serum sample from a stage IV breast cancer patient separated on the microfluidic device. The separation length was 22 cm, and the separation field strength was 750 V/cm. The separation efficiencies for components C1, C2, and C3 are listed in Table 2. The symbols are as in Figure 9. Reproduced from ref. [67] with permission.