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Review

Miniaturized separation techniques in glycomic investigations☆

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Abstract

High-sensitivity glycomic analyses are becoming of a great interest in modern biomedical and clinical research, as well as in the development of recombinant protein products. The evolution of separation techniques for glycomic analysis at high sensitivity is highlighted in this review. These methodologies include capillary liquid chromatography, capillary electrophoresis (CE) and capillary electrochromatography (CEC). The potential of such methodologies in glycomic analysis is demonstrated for model glycoproteins as well as total glycomes derived from biological samples.

Keywords: Glycomics; Oligosaccharides; Capillary liquid chromatography; Capillary electrophoresis; Capillary electrochromatography; Hydrophilic nano-LC; Graphitized carbon packings; Permethylation

Contents

1. Introduction

As a part of the "systems biology" efforts [1–6] aiming at a nearly complete understanding of the complexity of living organisms, glycomics and glycoproteomics deserve particular attention. This is due to the fact that many important processes in mammalian cells are mediated through various actions of glycoproteins and proteoglycans [7]. Consequently, targeted studies of glycosylation processes in the highly organized eukaryotic cells are likely to explain developmental differences between such cells and the less advanced systems which do not feature this sophisticated form of posttranslational modification or use only its simpler structures. Since differences in glycoprotein structures and their levels seem associated with numerous

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diseases and disorders (both hereditary and environmentally-induced), detailed studies of glycosylation are also potentially pertinent to understanding of disease etiologies, a design of better diagnostic procedures and a follow-up of therapeutic steps. Finally, no less important are the current efforts of pharmaceutical and biotech industries to produce new therapeutics which are glycoconjugate-based [8,9].

Just as with the other efforts contributing to systems biology (transcriptomics, proteomics, metabolomics, etc.), the fields of glycoproteomics and functional glycomics are strongly dependent on the current and future advances in analytical methodologies and instrumentation. Perhaps even more so because of the greater sophistication of glycoconjugate molecules when compared to the linear biopolymers such as DNA or polypeptides. Glycoconjugates can feature different forms of linkage, branching into antennas and other types of isomerism. The functional sophistication of glycoproteins seems paralleled by the seemingly complex structural attributes such as unique sugar structures, the structural hierarchies of different glycans at the site of glycosylation and, to some degree, the local "peptide landscape" at that site.

In addressing the analytical needs for a complete structural investigation of a complex glycoprotein, a combination of several techniques is often required. Clearly, a majority of such determinations has been a multimethodological task [10] in which an isolated glycoprotein is typically digested by a protease enzyme and further analyzed by tandem mass spectrometry (MS/MS) to yield a complete protein sequence, while more refined analytical strategies are being applied to the mixtures of resulting glycopeptides in high-sensitivity site-of-glycosylation determinations [11–15]. Such procedures are at the heart of glycoproteomics, which shares many analytical approaches with the mainstream proteomics (e.g., "top down" and "bottom up", or "shotgun" approaches) [16–21]. Quite different are the glycomic studies, which necessitate a quantitative removal of glycans from the polypeptide backbone prior to their separate analysis.

Glycomic investigations, aiming at a total structural analysis of glycoproteins' released glycans, rely also heavily on the use of both mass spectrometry (MS) and analytical separation methodologies. To address structural problems of glycoproteins at different degrees of complexity, additional methodologies, such as the use of specific reagents, fluorescence-tagging chemicals, exo- and endoglycosidase enzymes, and lectins may also be applicable. Nevertheless, MS using electrospray ionization (ESI) or matrix-assisted laser desorption-ionization (MALDI) is becoming the most widely applicable tool of glycoanalysis to which other techniques are viewed as ancillary. Since many important biological processes are mediated through sugar–sugar or sugar–protein interactions, the basic premise of functional glycomics is to access structurally and quantitatively these biological determinants (glycans) and relate their presence/quantities to their physiological or pathological roles. This can apparently be performed even in relatively complex mixtures of glycoproteins (e.g., unfractionated cellular extracts or physiological fluids).

Glycomic studies using different MS ionization techniques and/or mass analyzers rely on the displays of glycomic profiles or "glycomic maps" according to the glycans' molecular mass for a particular type of biological material. For example, the mass profiles of a diseased sample can be quantitatively compared with a control (normal) profile, or progression of a disease and, alternatively, the effects of a therapeutic agent could also be discerned from such data. This approach appears particularly attractive with the use of MALDI-MS and its relatively uncomplicated spectra. However, any type of MS suffers form the general lack of resolution when dealing with isomeric structures, so that a complementary use of suitable separation methodologies in carbohydrate analysis becomes desirable.

The analytical separations of carbohydrates have a long history of development, as reviewed in book chapters and review articles [10,22–27]. The recent studies have particularly emphasized the use of nanoscale separation methodologies based on capillary zone electrophoresis (CZE), capillary electrochromatography (CEC) and capillary liquid chromatography (LC), which are all high-resolution techniques that are also eminently suitable for small-scale biological investigations. Besides the isomer resolution, the use of separation techniques can further simplify determination of glycans in complex mixtures and facilitate preconcentration of their trace components. This latter capability is particularly needed in addressing a wide concentration range in which biologically important glycoproteins can occur in tissue extracts and physiological fluids of interest.

The purpose of this brief review is to summarize and critically evaluate the recent trends in separation of oligosaccharides, with a special emphasis on their use in the analysis of isolated glycoproteins and unfractionated biological materials. There is a distinct methodological overlap with the use of similar techniques in the general area of glycoscience, for example, with the analysis of polysaccharides and glycosaminoglycans. This review supplements the previous accounts of this laboratory on glycoanalysis, in general [22–24,26,27], and glycoproteins [10,25], in particular. The separation methodologies of particular interest are the capillary techniques which combine easily with MS, the ultimate tool in structural glycoanalysis. These will be emphasized in comparison to the more traditional HPLC methodologies used in carbohydrate research, such as ion-exchange chromatography using pulsed-amperometric detection [28], fluorometric detection of aminopyridylated sugars [29–31] or spectrophotometric detection of native solutes [32].

The lack of chromophoric moieties in carbohydrate molecules has been among the challenging tasks of detection in carbohydrate analysis for a long time. This problem is now being gradually overcome through a wider use of LC–MS techniques. Nevertheless, the on-going use of precolumn derivatization for the sake of laser-induced fluorescence (LIF) detection and, to a lesser degree, UV absorbance, is still justified for simpler analytical tasks, such as quality control assays for recombinant products, in affinity studies, or confirmatory monosaccharide analyses [15]. As preliminary screening procedures in functional glycomics, high-resolution chromatographic methodologies, combined with a suitably sensitive detection means, could be used with the advantage of simplicity. The general trend

seems to be, however, toward the development of a general glycomic platform using highly informative MS measurements. Solute derivatization can also offer some distinct advantages for MS measurements as well, as shown below.

2. Chromatographic methodologies

In typical chromatographic systems, the hydrophilic nature of native sugar molecules has a more practical merit than their hydrophobicity, which has been largely confined to the interaction studies of hydrophobic organic molecules (e.g., various pharmaceuticals) bound inside the relatively hydrophobic cavity of oligosaccharides [33,34]. Indeed, the uses of reversed-phase LC in carbohydrate separations are rare [10], with the exception of cases where a hydrophobic tag is placed through the reaction at the reducing end of a saccharide [10], or when the sugar solutes are comprehensively methylated (as seen in one of the procedures detailed below) [25]. This leaves hydrophilic interactions, adsorption-type interactions, and ion-exchange under extreme pH-conditions as the only other viable options. With regard to the use of LC–MS with such columns, it is essential to reduce their dimensions to comply with "micro" (1–10 μL/min) or "nano" (< 500 nL/min) flows.

2.1. Nano-LC with hydrophilic columns

Hydrophilic-interaction chromatography (HILIC), a term first coined by Alpert [35] in 1990, has become generally popular in carbohydrate separations. Although LC of carbohydrates (in conventional-scale columns) on unmodified silica was reported in the literature [36], it seems more effective to use the silica materials modified through chemical bonding of suitable polar functional groups such as amine [37–40] or amide [41–44].

Most commonly, the utility of the HILIC approach in the analysis of oligosaccharides has involved fluorescence labeling prior to LC separation [10] with analytical columns used at high flow rates. To comply with the needs of microscale separations and LC/MS, the use of a capillary amide-based column has recently been demonstrated [45], employing a flow rate of 300 nL/min. With the use of nano-LC format and an ion-trap mass spectrometer, oligosaccharide mixtures could be analyzed at low-femtomole sensitivities. The analytical approach was validated through the investigations of N-glycans derived from keyhole limpet hemocyanine and horseradish peroxidase, as illustrated in Fig. 1 [45]. Apparently, the mobile phase used in this investigation was compatible with the MS operation. As a general limitation, amide columns suffer from a significant decrease in separation efficiencies over time and fairly limited lifetimes.

2.2. Nano-LC with graphitized carbon packings

As an alternative to hydrophilic interaction chromatography with amine- and amide-bonded phases, graphitized carbon columns (GCCs) have recently been gaining popularity because of their greater capacity combined with high efficiency, and easy use. GCCs became recently available as microcolumns (supplied through SGE, Ringwood, Australia). Carbohydrate retention is largely based on adsorption, but some hydrophobic interactions are also expected. The unique selectivity of GCCs and their nearly unmatched ability to resolve isomers and other closely related compounds are the result of using the adsorption principle that is feasible with this homogeneous adsorbent without adverse effects of irreversible adsorption.

The retention of organic compounds on GCCs is generally greater than what is observed with reversed-phase materials. Consequently, a greater percentage of organic solvents in the mobile phase is needed to elute carbohydrate solutes. A major advantage of GCCs appears to be their ruggedness, permitting a

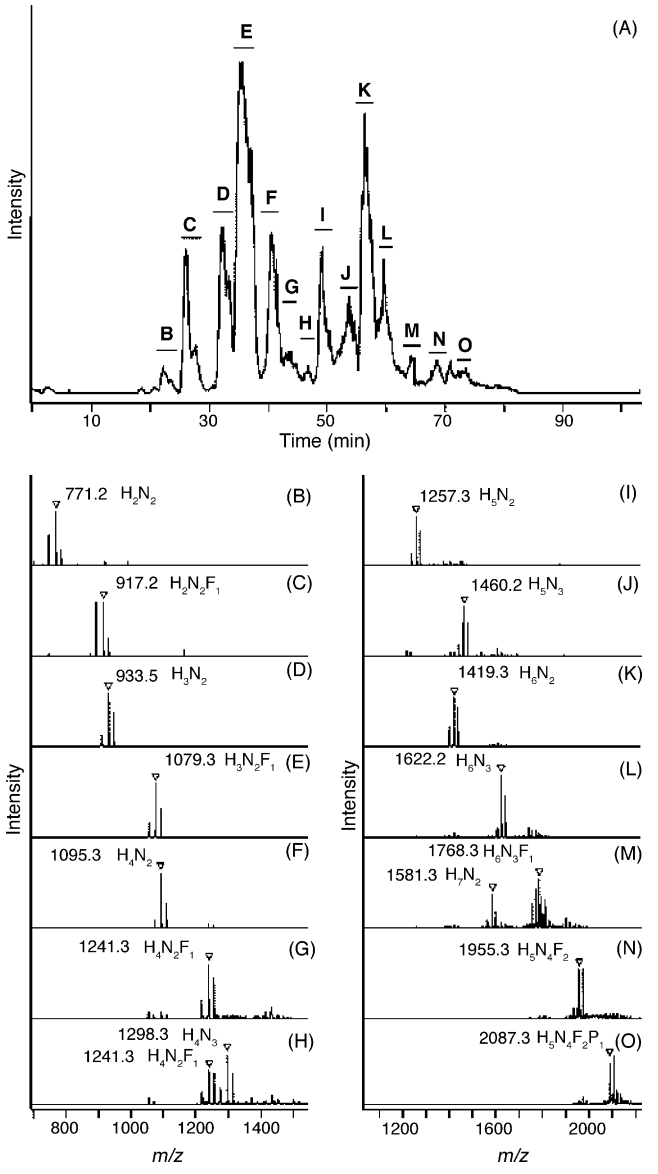

Fig. 1. Nano-LC-ESI-MS of oligosaccharides released from KLH by PNGase F treatment: (A) base peak chromatogram (mass range *m/z* 700–2800); (B–O) mass spectra obtained for the time windows indicated by horizontal bars in (A). Sodium adducts are presented with *m/z* and deduced monosaccharide composition. H, hexose; N, *N*-acetylhexosamine; F, fucose; P, pentose; (▽), sodium adduct. No *m/z* values are given for proton and potassium adducts. Reproduced from [45], with permission.

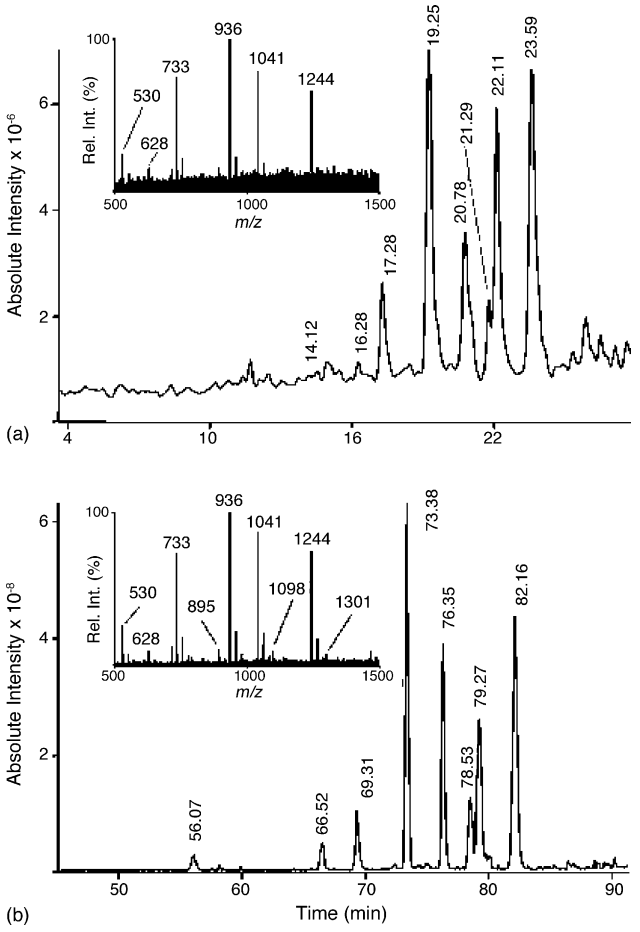

Fig. 2. Comparison of negative ion (a) capillary LC/MS vs. (b) nano-LC/MS analysis of neutral *O*-linked oligosaccharides (5.5 ng) using graphitized carbon chromatography (base peak chromatograms). Combined MS^1 mass spectra of the region where oligosaccharides were eluted are shown as inserts. Reproduced from [54], with permission.

long, repeated use without adverse effects of a decreased performance or reproducibility. The columns are very stable across a wide range of buffer pH and different solvents. In a microcolumn format, CGGs were employed during the LC/MS analyses of the glycans originated from egg jelly surrounding *Xenopus laevis* eggs [46–48], human bronchial epithelial cell cultures [49], membrane proteins from premature aging Huchinson–Gilford progeria syndrome fibroblasts [50], human tear-fluid [51], rat brain Thy-1 [52], and human blood plasma [53].

Although flow rates at μL/min could be successfully used with the graphitized carbon columns for the analysis of glycans, only the major structures were effectively detected under such conditions. The situation improved substantially with the use of nanocolumns at much lower flow rates. As seen in Fig. 2, even the low-abundance N- and O-glycans were observed at high-sensitivity [54]. The use of negative-ion MS facilitated a simultaneous detection of femtomole quantities of both neutral and acidic oligosaccharides, with the observation of several structural isomers. Additionally, this procedure was found effective in analyzing N- and O-glycans isolated from mucosal surfaces and ovarian cancer cells [54].

2.3. Anion-exchange chromatography-mass spectrometry

Anion-exchange chromatography of glycans with pulsed-amperometric detection has become one of the standard methodologies in glycobiology because of its simplicity and quantitative reliability in the picomole range. While mass spectrometers are capable of detecting glycans at a level that is many orders of magnitude lower than amperometric detectors, interfacing this chromatographic technique to MS is not convenient due to the incompatibility of the mobile phases with MS. Several attempts [55–59] were made to address this problem, but neither approach was completely successful.

3. Electromigration approaches in glycomics

3.1. Capillary electrophoresis

While glycan profiles released from different glycoproteins (e.g., recombinant glycoprotein products) or even unfractionated glycoprotein mixtures can be displayed through MALDI-MS, fluorescently-tagged mixtures separated by capillary electrophoresis (CE) and detected by laser-induced fluorescence are often viewed as somewhat complementary to MALDI-MS, since the CE approach is often capable of resolving certain isomers which are otherwise indistinguishable by MS. During the last decade, several types of capillary electromigration techniques have been significantly utilized for glycomic analysis, including capillary zone electrophoresis employing either free-buffer media or gels, and micellar electrokinetic chromatography (MEKC). A high resolution and fast analysis offered by these techniques can only be appreciated when used in conjunction with sufficiently sensitive detectors. While still utilized in certain applications [10], the sensitivity of UV absorbance is marginal for carbohydrates, and fluorescence-tagging procedures for laser-induced fluorescence detection are generally recommended if mass spectrometry is not available. Since not all N-glycans are electrically charged, the derivatization procedure is also expected to introduce ionic groups needed for electromigration. Some neutral derivatizing agents, however, have also been utilized in conjunction with MEKC mode or with a borate buffer that permits complexation with oligosaccharides, thus imparting the electrical charge. Some intriguing aspects of CE-LIF, MEKC-LIF and CE-MS in glycomics are discussed below.

3.1.1. Capillary zone electrophoresis

In comparison to the miniaturized LC-based techniques, CZE suffers from a limited capability to utilize large aliquots of biological samples at the capillary inlet. 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 peak volumes and, correspondingly, small column diameters [60–62]. The detection challenges of CZE, and to a various degree also the other capillary electromigration techniques, can increasingly be met through the inherently sensitive LIF and rapidly evolving high-sensitivity MS techniques.

LIF necessitates the use of fluorogenic or fluorescence-tagging reagents, which are optimally "tuned" to reliable laser technologies; typically, argon-ion lasers are preferred over helium/cadmium laser. In 1991, our laboratory first demonstrated carbohydrate analysis by CE/LIF combination, using 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA) as a derivatizing agent, for amino sugars [63] and glycoprotein-derived N-glycans [63–65]. With the detection limits reaching subattomole levels, the oligosaccharide constituents of bovine fetuin (CBQCA-labeled N-glycans) were resolved into four major (expected) peaks and some minor components. Since then, additional efforts have been made to exploit the potential of various derivatizing reagents for high-sensitivity glycomic analysis through CE.

The effect of the charge groups in 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) on the electrophoretic separation of glycans was investigated [66–68]. As expected, a greater charge caused faster analyses and higher resolution, making ANTS one of the most effective derivatizing agents among the aminonaphthalene derivatives for the CE analysis of N-glycans derived from glycoproteins. Accordingly, this reagent was successfully employed for characterization of glycans derived from various glycoproteins, including human immunoglobulin G [69], ovalbumin [70,71], fetuin [70,72], recombinant HIV gp-120 [72], and α_1-acid glycoprotein [72].

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 utilizing a more convenient light source such as the argon-ion laser. Accordingly, the most commonly used fluorophore for CE-LIF today is 1-aminopyrene-3,6,8-trisulfonic acid (APTS) [73]. Using this reagent, Chen and Evangelista [74] devised a complete method for the analysis of N-glycans derived from glycoproteins. It is based on a combination of specific chemical and enzymatic conversions coupled with CE/LIF. First, N-glycans are released enzymatically from glycoproteins and derivatized with APTS under mild reductive amination conditions to preserve sialic acid and fucose residues. The method was successful in profiling the heavily sialylated N-glycans. Using similar methodologies, additional authors reported analyses of N-glycans derived from various glycoproteins, including ribonuclease B [73–79], fetuin [74,75], recombinant human erythropoietin [74], kallikrein [74] and a chimeric recombinant monoclonal antibody (mAb) [80].

Guttman devised a method for multistructure sequencing with N-glycans by gel CE and exoglycosidase digestions [76,81]. It involves a carefully designed exoglycosidase matrix with a subsequent comparison of the positions of the separated exoglycosidase digest fragments to maltooligosaccharides of a known size, in terms of relative migration shifts. 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.

Ma and Nashabeh [80] extended the use of CZE of APTS-labeled glycans to monitor certain variations in the glycosylation of rituximba, a chimeric recombinant monoclonal antibody, during production. The N-glycans derived from rituximba are neutral, complex biantennary oligosaccharides with zero, one and two terminal galactose residues (G0, G1, and G2, respectively). The method was based on releasing N-glycans from the glycoprotein via PNGase F, then derivatizing them with APTS prior to a CE mapping. All observed glycans were fully resolved, including the positional isomers of G1. The two G1 positional isomers were identified by comparing CE profiles obtained from sequential enzymatic digestions. N-Glycans were first released enzymatically by treating the glycoprotein with PNGase F; as an example, CE profile of APTS-derivatized N-glycans is illustrated in Fig. 3a. The N-glycans were further incubated with β-*N*-acetylglucosaminidase to remove the terminal GlcNAc residues on the G0 and G1 isomers, as seen in Fig. 3b. 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 (see Fig. 3b). Finally, 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. The method has several advantages over other schemes, including simplicity, accuracy, precision, high throughput, and robustness.

More recently, the use of APTS in conjunction with CZE-LIF permitted a baseline separation of closely related structural isomers [15]. The CE-LIF trace (Fig. 4 upper trace) of the fluorescently labeled standards (core-fucosylated biantennary, asialylated, and mono- and disialylated glycans) clearly illus-

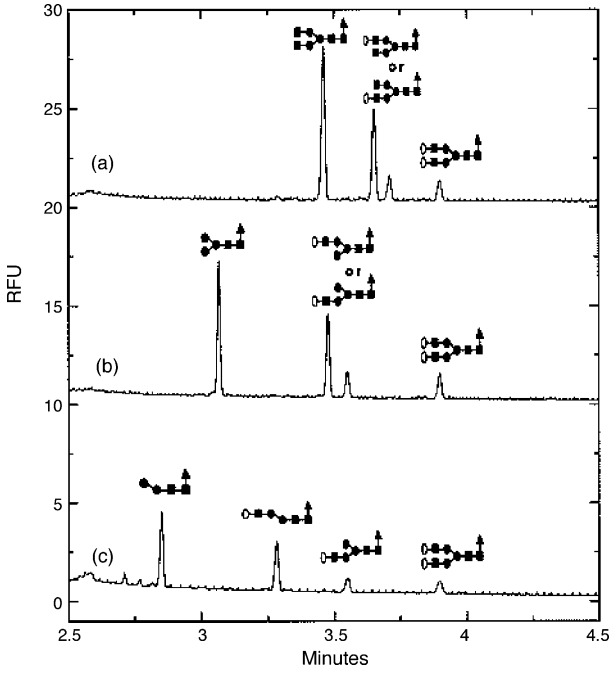

Fig. 3. Electropherograms of the glycans obtained from the rituximba after sequential enzymatic digestion steps: (a) PNGase F; (b) β-*N*-acetylhexosaminidase; (c) α1-2,3 mannosidase. Samples were derivatized before CE analysis. Reproduced from [80], with permission.

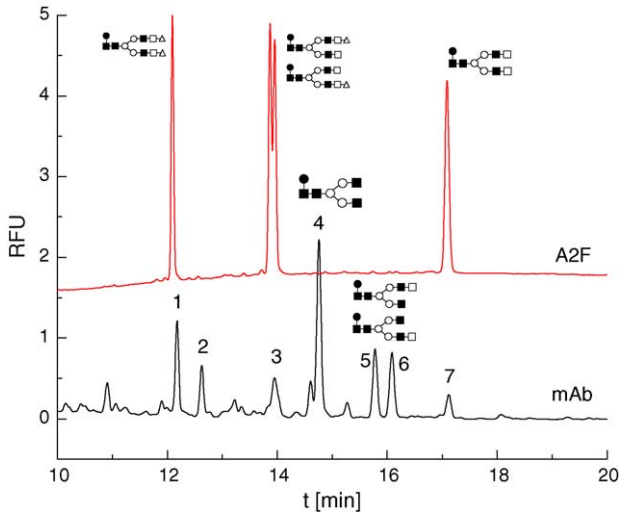

Fig. 4. CE profile of APTS-labeled glycans derived from mAb. The upper trace represents standard core-fucosylated biantennary/disialylated, monosialylated, and asialylated glycans. Conditions: column, polyacrylamide-coated 50/365 mm I.D./O.D.; length, 50.5 cm total, 40.5 cm effective length; temperature, 25 °C; injection pressure, 0.5 psi for 5.0 s; voltage, 15 kV anodic electroosmotic flow; λ_{ex} 488 nm, λ_{em} 520. Reproduced from [15], with permission.

trates the ability of this technique to separate structural isomers. Monosialylated structures, which differ only in the attachment of a terminal sialic acid residue to 1–6 or 1–3 antenna, were partially resolved in this CE-LIF analysis scheme. The CE-LIF analysis of the N-glycans derived from a monoclonal antibody revealed the presence of seven major components in addition to many minor components (Fig. 4 lower trace) [15]. In this figure, the resolving power of CZE is also evident from the ability to baseline-resolve monofucosylated biantennary glycan structures lacking one terminal galactose residue.

Honda et al. [82] introduced an ultramicroscale method for the analysis of carbohydrates using CE-LIF. Their method is based on labeling carbohydrates through their initial conversion to *N*-methylglycamines, before tagging these products with 7-nitro-2,1,3-benzoxadiazole (NBD). The tagged carbohydrates are then analyzed through CE-LIF. Conversion of N-glycans to *N*-methylglycamines was achieved by incubating the sample with dimethylamine–borane complex at 40 °C. Next, *N*-methylglycamines were coupled to NBD, while the coupling reaction was achieved in 5 min at 70 °C. Although the derivatization involves two steps, the procedure is carried out simultaneously in "one pot" arrangement without a loss in derivatization efficiency. This method allowed a high-resolution profiling of N-glycans derived from fetuin through the use of borate buffer.

3.1.2. Micellar electrokinetic chromatography (MEKC)

The separation systems using micelles and other molecular aggregates as a pseudostationary phase [83] have often been found effective in resolving small molecules with minor structural differences. In the area of carbohydrate analysis, this notably includes differently substituted amino sugars as CBQCA derivatives [65] and optical isomers as ANTS derivatives [84]. While hydrophobicity is the general cause of retention in typical micellar systems, a fine-tuning of separation conditions, through a choice of buffer systems or the use of additives (forming mixed micellar entities), has often been found profitable. A proper choice of fluorescent tagging structures can be a further supplement to the success of MEKC analysis. Unfortunately, such separation conditions are seldom acceptable in MS analysis. As detailed below, various forms of MEKC could be utilized in separating glycan mixtures.

2-Aminoacridone is yet another reagent that has been recently utilized in derivatization of N-glycans. While this is a neutral fluorophore, a borate buffer or MEKC must be employed to allow the separation of neutral oligosaccharides. The N-glycans derived from different glycoproteins were profiled by CE using this procedure: ovalbumin [85], fetuin [85,86], ribonuclease B [85,87,88], and IgG antibodies [87,89]. 2-Aminobenzamide is yet another neutral fluorophore to be used in conjunction with MEKC or CZE (in presence of anionic cyclodextrin as a pseudostationary phase) for profiling glycoprotein N-glycans [89].

3.1.3. Capillary zone electrophoresis-mass spectrometry (CZE-MS)

The popularity of CZE-MS interfacing has substantially increased over the past several years due to the notable improvements in sensitivity and reproducibility. This approach takes a full advantage of the extreme sensitivity of a mass spectrometer and unmatched separation efficiency of CZE, as illustrated above. In coupling CZE to a proper MS technique, high mass accuracy and tandem MS operations can be easily achieved. The application of CE-MS and tandem MS to glycoscreening in biomedical field has been highlighted [90]. More recently, CZE interfaced to a Q-Trap mass spectrometer was featured in the study of N-glycans from cellobiohydrolase I, with special interest in phosphorylated residues [91]. A tandem MS operation was mediated through a modified triple quadrupole where the Q3 region can be operated as a conventional quadrupole mass filter or as a linear ion-trap with axial ion ejection [92]. A simultaneous analysis of neutral and charged glycans was achieved through fluorescence labeling with 8-aminopyrene-1,3,6-trisulfonate (APTS) which imparts negative charges to the labeled structures. Generally, APTS labeling of these oligosaccharides permits high-resolution CE, a better ionization in the negative ion mode, a simultaneous detection of neutral and charged oligosaccharides, and generation of predictable tandem MS spectra (Fig. 5). A differentiation of phosphorylated glycan isomers was achieved through this system, while MS/MS data furnished additional structural information [91].

3.2. Capillary electrochromatography

During the last decade, CEC has often been emphasized as a technique combining some desirable features of capillary electromigration principles (e.g., flow-induced radial solute mass transfer) and chromatography (a wide choice of suitable stationary phases). Moreover, it is easier with CEC (compared to CZE or MEKC) to preconcentrate dilute samples at the capillary inlet. Perhaps, most importantly, a recent progress in CEC features prominently monolithic columns, which principally offer

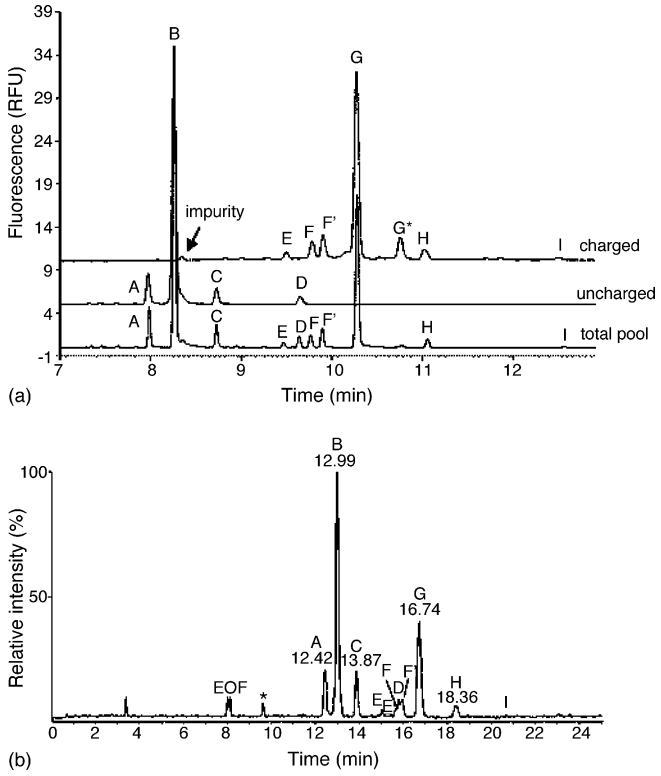

Fig. 5. CE-LIF electropherograms of the APTS-derivatized total CBH I N-glycan pool and of the uncharged and charged glycans (a); CE-MS base peak electropherogram of the total CBH I N-glycan pool (b). The neutral glycans are labeled A–D, the charged ones E–I. Unlabeled neutral glycans are detected in the EOF; unlabeled phosphorylated glycans migrate more slowly and are indicated by an asterisk. Reproduced from [91], with permission.

a wide range of retention selectivity together with separation conditions that appear compatible with MS operation.

The examples of carbohydrate applications of CEC include the separation of aminobenzamide derivatives [93] 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. Among these efforts, CEC monolithic columns were recently shown [94–96] where a high resolving power associated with this approach is capable of dealing with very complex pools of glycans (Fig. 6). This is clearly illustrated for the case of bile salt-stimulated lipase (BSSL) from human breast milk, which is a relatively large glycoprotein consisting of 722 amino acid residues [97] with numerous O-glycosylation sites near the C-terminus. The CEC/ion-trap MS profile of chemically cleaved N- and O-glycans using a hydrophilic monolithic column is depicted in Fig. 6. The high selectivity of these columns assisted a partial or complete resolution of several structural isomers, as suggested by the detection of several *m/z* values at different retention times. These CEC columns were shown to be very effective in conjunction with ESI-MS, using both the ion-trap [94,96] and FT-MS [95] mass analyzers, as well as MALDI-MS using a microdeposition device prior to MS analysis [98]. The potential of the latter technique was exploited in the analysis of N- and O-glycans derived from BSSL. The high microheterogeneity of the glycan structures derived from this glycoprotein is

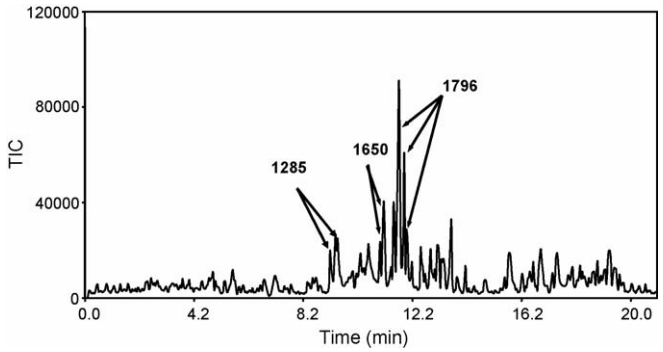

Fig. 6. Mass electrochromatogram of a complex fraction of the O-linked glycans chemically released from human bile salt-stimulated lipase. Conditions: amino column 28 cm, field strength 500V/cm, mobile phase acetonitrile/water/ammonium formate buffer (240 mM, pH 3.0, 55:44:1, v/v/v), injection 1 kV, 10 s. Reproduced from [94], with permission.

evident in Fig. 7, which depicts 3D and 2D CEC/MALDI/TOF-MS recordings. Approximately 50 distinct peaks were displayed in this figure. Many of the depicted peaks differ by a few mass units, while some other peaks are structural isomers [98].

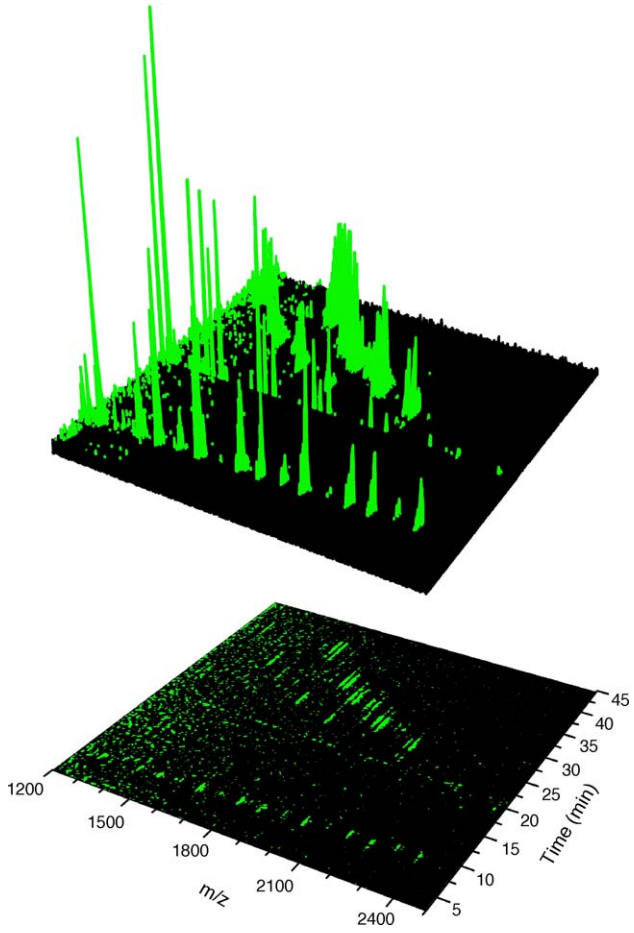

Fig. 7. 3D Electrochromatogram of the mixture of N-linked and O-linked glycans derived from BSSL. Experimental conditions: cyano capillary column, 28 cm; mobile phase, 2.4 mM ammonium formate buffer in a 60/40 mixture of acetonitrile/water; field strength, 535 V/cm; injection, 10 kV, 10 s; matrix, 20 mg/ml DHB in 80/20, methanol/water. Reproduced from [98], with permission.

4. Capillary LC/MALDI-TOF/TOF mass spectrometry: a general platform for functional glycomic investigations

A suitable general platform for functional glycomics must, first of all, consider the best possible structural technique that is capable of distinguishing different forms of isomerism at high-sensitivity. During the recent past, distinctly different approaches and modern MS technologies were applied to satisfy this requirement. The use of sequential fragmentation in tandem MS (for example, MS^n in an ion-trap or quadrupole ion-trap) was found to be effective in distinguishing isobaric structures among methylated branched oligosaccharides [99–105], albeit at the expense of a need for considerable sample quantity and limited compatibility with a prior LC separation (due to incomplete derivatization). The effectiveness of a particular ionization technique, i.e., the use of ESI versus MALDI, must also be considered in view of the relatively inefficient ionization of oligosaccharides when compared to peptides. While MALDI forms exclusively the sodium adduct ions of oligosaccharides in the positive-ion mode, multiply-charged states are formed in ESI that cause a loss in sensitivity as a result of splitting the signal of a single analyte into different detectable entities.

For the branched structures and differently linked residues in the oligosaccharides from typical glycoproteins, it is not sufficient to generate secondary fragments due to the cleavages at the sites of glycosidic bonds; sufficient energies must be available to the analyte sugar molecules to form the more informative cross-ring fragments. As the low-energy collision-induced dissociation (CID) in the ion-trap analyzers [94,96,106] or the postsource decay in MALDI/time-of-flight (TOF) MS instruments [107] are generally inadequate to accomplish this fragmentation task, we have chosen a MALDI/TOF/TOF MS with high-energy CID capabilities to furnish extensive fragmentation of model neutral [108–112] and acidic [108,113] glycans. An alternative approach pursued elsewhere for extensive fragmentation of glycans involves Fourier-transform ion cyclotron resonance MS used in conjunction with a sustained off-resonance irradiation (SORI) [114], CID [47,114], IR multiphoton dissociation (IRMPD) [115–117], or electron capture dissociation (ECD) [115,116]. In our MALDI/TOF/TOF experiments, we were able to distinguish a subtle form of isomerism in the mannose-rich glycans [109] from the ratios of diagnostic cross-ring fragments as well as different linkages of sialic acid residues and/or their attachment to a specific antenna within a branched structure [113]. The example illustrated in Fig. 8 demonstrates the ability of this technique to assign sialic acid residues to a particular antenna. *N*-Acetylneuraminyl-lacto-*N*-neo-hexaose from human milk (for structure, see Fig. 8a) is a branched sialylated oligosaccharide which has both 6-linked antenna and 3-linked antenna. The sialic acid residue in this structure is present on the 3-linked antenna. The MS/MS spectrum of *N*-acetylneuraminyl-lacto-*N*-neo-hexaose is shown in Fig. 8b. The most informative fragments in the spectra are $^{1,5}X_{2\beta}$, $^{3,5}X_1$, $^{0,4}A_4$, and $^{2,4}A_4/^{2,4}X_{3\alpha}$. These fragments conclusively allowed the assignment of the sialic acid residue to the 3-linked antenna. Furthermore, the presence of the diagnostic fragments associated with α2-6 linkage, as discussed above, as well as the presence of the C1 fragment (*m*/*z* 416.1), suggest an attachment of the terminal sialic acid residue to be through α2-6 linkage. Much of this structurally/functionally important information had previously been available only through NMR spectrometry, which generally requires highly concentrated samples. Using new bioinformatic approaches [118] to MALDI/TOF/TOF MS fragmentation data, there is a distinct possibility to distinguish the finest structural details on previously separated glycans.

In choosing the MALDI/TOF/TOF approach for structural elucidation of glycans, it was natural to evaluate the merits of per-*O*-methylation as an alternative for analyzing native glycans. While sample derivatization always introduces an additional procedural step, the advantages of permethylation for MS [119,120] are substantial: stabilization of sialylated structures; enhanced sensitivity; and improvements in the MS/MS interpretation capabilities. Serendipitously, the oligosaccharides are made sufficiently hydrophobic through quantitative methylation to allow their separation from complex mixtures by means of capillary reversed-phase LC using "MS-friendly" mobile phases [121]. In addition to offering predictable fragmentation, permethylation also facilitates a simultaneous analysis of neutral and sialylated oligosaccharide solutes.

Oligosaccharide permethylation was previously described as a prelude to MS and tandem MS analysis of oligosaccharides and other glycoconjugates through the use of two different procedures. The first, originally described by Hakomori [122], utilizes the anion dimethyl sulfoxide ($DMSO^-$, commonly referred to as the dimsyl anion) to remove protons from the sample prior to their replacement with a methyl group. However, this methylation procedure tends to be non-quantitative, which is perhaps acceptable for MS studies of an isolated glycan, but not for LC–MS where just one solute may produce multiple chromatographic peaks. The second procedure, which is now more commonly accepted, was introduced in 1984 [119] and modified more recently [120]. It is based on the addition of methyl iodide to carbohydrates, which are dissolved in dimethylsulfoxide solution containing powdered sodium hydroxide and traces of water. The popularity of the modified procedure stems from its speed, experimental simplicity, effectiveness for both O- and N-glycans and "cleaner" products. Unfortunately, when exploring this procedure at microscale, we found difficulties in recovering the small amounts of methylated glycans, which are presumably due to oxidative degradation and peeling reactions induced by the high pH and heat generated during the liquid-liquid extraction step. These difficulties led us to develop an on-line permethylation procedure using a sodium hydroxide-packed capillary as a microreactor [123] that can easily be incorporated into a valve-operated analytical system. This approach has been successfully utilized for the permethylation of glycans from model proteins as well as total glycomes derived from biological samples (Fig. 9) [123].

Any successful glycomic/analytical platform will be dependent on an effective release of glycans from the polypeptide backbone of a glycoprotein or glycopeptides. When considering a permethylation as the next treatment step, a glycan cleavage procedure must be compatible with permethylation condi-

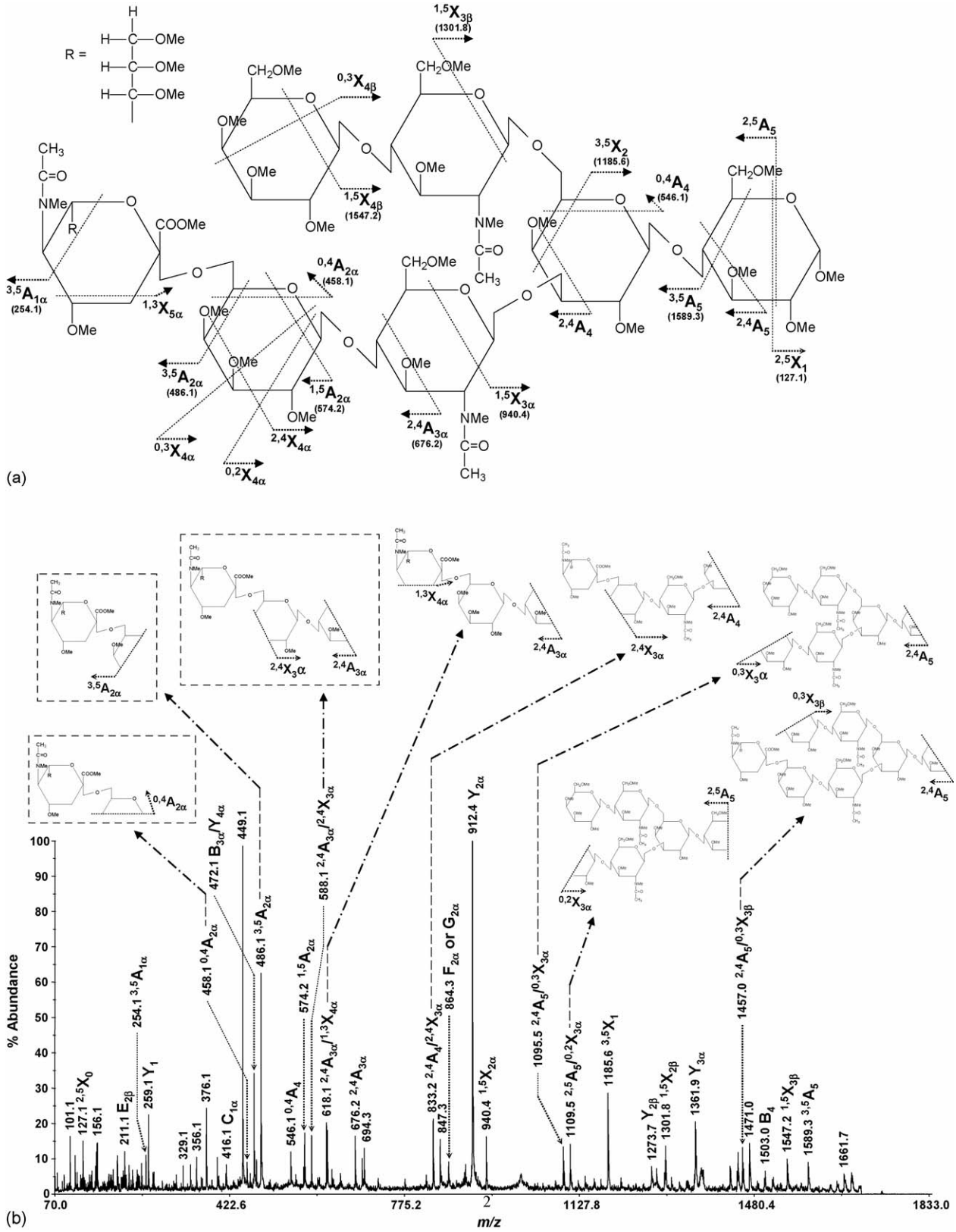

Fig. 8. A tandem MS spectrum of permethylated *N*-acetylneuraminyl-lacto-*N*-neo-hexaose isolated from human milk (precursor ion, *m/z* 1736.8). Structure and observed cross-ring fragments are depicted in (a), while (b) depicts the spectrum with insets illustrating internal fragments. Reproduced from [113], with permission.

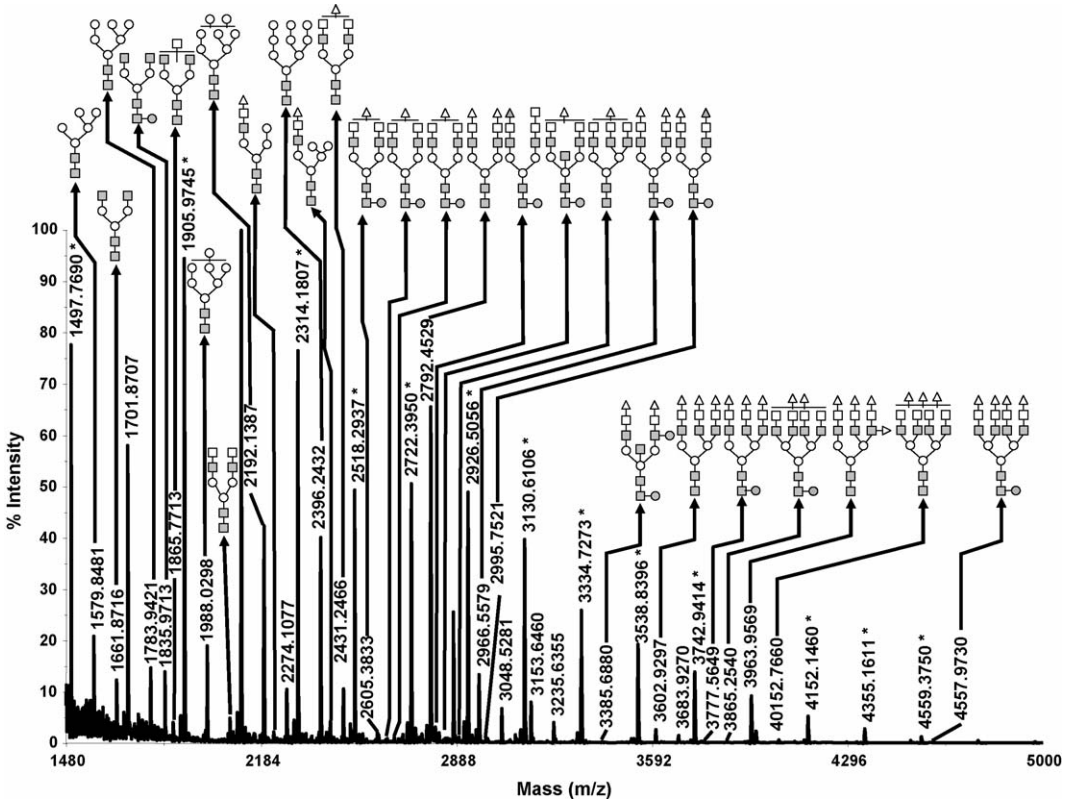

Fig. 9. MALDI/TOF MS glycomic profile of permethylated N-glycans derived from 20 mg of rat liver tissue by using 500 μm I.D. fused silica capillary. Symbols: (■), *N*-acetylglucosamine; (○), mannose; (□), galactose; (●), fucose; (△,) *N*-acetylneuraminic acid; (▲), *N*-glycolylneuraminic acid. Signal marked with asterisks are originating from glucose ladders that are commonly present in tissue samples. Reproduced from [123], with permission.

tions. In our current approach, we prefer the recently developed microscale β-elimination [124] or glycanase-released glycans which are derivatized through a reductively stabilized Schiff base prior to permethylation. In fact, this step may be preferable for short-chain oligosaccharides, which are often obscured by the interferences associated with noise originating from the MALDI matrix clusters.

Serendipitously, the hydrophobicity of permethylated and terminus-modified glycans renders them amenable to a reversed-phase LC separation, which is popular, convenient and easy-to-interface to ESI- or MALDI-MS. Recently, in conjunction with the on-line permethylation, a system was developed, incorporating derivatization, C18 trapping of permethylated glycans, and C18 nano-LC separation of the glycans derived from model glycoproteins and biological samples. The on-line permethylation and LC/MALDI-MS analysis of glycans derived from a mixture of glycoproteins are depicted in Fig. 10. A reversed-phase separation allowed resolving structural isomers, the presence of which could be confirmed by MALDI/TOF/TOF-MS.

5. Chip-based approaches

Miniaturization of the previously described separation methodologies represents a general trend in modern glycoanalysis. This is being driven by the fact that glycoproteins are commonly encountered at the sub-microgram levels. While measuring such small amounts is currently feasible with the modern instrumental techniques, proteins can easily be adsorbed on the surface of glassware before such measurements. A 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. Miniaturizing separation, in terms of reduced column diameters, solvent flow-rates and the overall surface area that a glycan sample may encounter during analysis, is thus becoming significant in high-sensitivity work. 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 analytical 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 electrophoresis (ME), which commonly uses different detection principles such as refractometry [125], electrochemical detection [126–129], UV [130], and LIF [131–133]. Conversely, a microfluidic chip with an integrated face-seal rotary valve, media-filled preconcentration and separation columns, and nanoelectrospray tip for MS has been described by [134], as based on chromatographic principles.

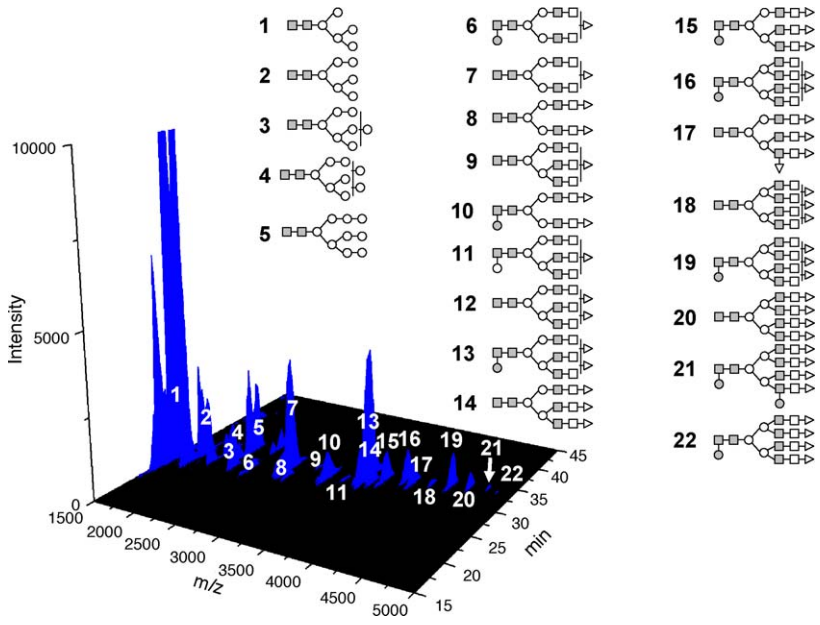

Fig. 10. LC/MALDI/TOF-TOF MS of on-line permethylated glycans derived from a mixture of glycoproteins.

Recently, an interesting application of ME involving a total serum protein N-glycome profiling was reported [135]. The chips were fabricated in alumina–silicate glass device with a double-T injector. This type of injector insures a uniform introduction of all molecular types without a bias commonly associated with an electrokinetic injection in CE. ME with 11.5 cm effective length, filled with 4% linear polyacrylamide, permitted the profiling of the major N-glycans in human serum in 12 min with a resolution comparable to what has been achieved through gel-based DNA sequencers. Profiling of two serum samples from a noncirrhotic chronic hepatitis patient and a cirrhotic patient demonstrated the potential of ME in clinical studies (Fig. 11). In a previous study using gel-based DNA sequencer [136], the same authors founds that the change in the ratio of two glycan structures (namely, complex triantennary and fucosylated bisecting biantennary) is diagnostic for liver cirrhosis (Fig. 11B). These results were also observed in the ME-generated profiles (Fig. 11A). The two peaks associated with glycan structures were well resolved in ME, while the ratio between the peak representative of the two structures was comparable to the gel-based DNA sequencer results. Using ME for this kind of analysis is more advantageous, since the other approach is considerably more laborious and time-consuming.

The separations of oligosaccharides with an integrated microchip (50 mm (*L*), 75 μm (*W*) and 50 μm (*D*) packed with graphite material) were recently described by Lebrilla and coworkers [137]. Effective separations of oligosaccharides was achieved at 300 nL/min flow rate. The chip was interfaced to an orthogonal TOF mass analyzer, offering mass accuracy of less than 5 ppm at a wide dynamic concentration range. This setup was found effective in the analysis of model oligosaccharides as well as complex mixtures, including O-glycans derived from mucin and different oligosaccahrides from human milk. The high mass accuracy allowed the assignment of 97 different structures, many of which were structural isomers.

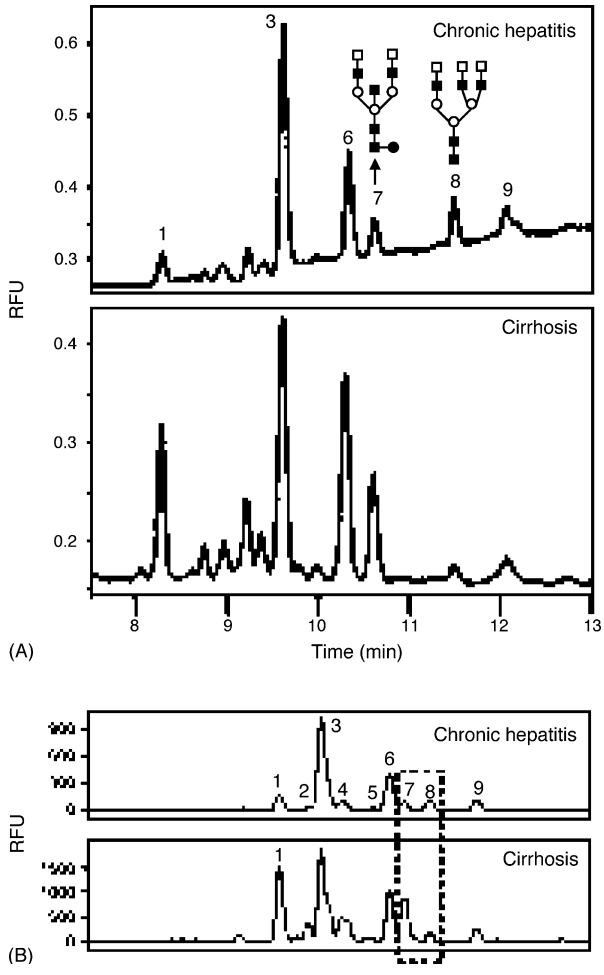

Fig. 11. (A) ME profiling of serum samples from a noncirrhotic chronic hepatitis patient (upper trace) and cirrhotic patient (lower trace); (B) profiling of the same samples using the ABI377 gel-based DNA-sequencer. Symbols: (■), *N*-acetylglucosamine; (○), mannose; (□), galactose; (●), fucose. Reproduced from [136], with permission.

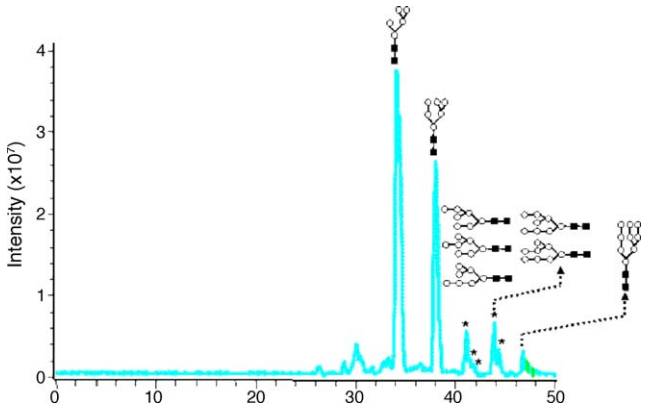

Fig. 12. Base peak chromatogram of solid-phase permethylated glycans derived from ribonuclease B. Structural isomers are designated with asterisks. Symbols: (□), *N*-acetylglucosamine; (◯), and mannose.

More recently, we have utilized the integrated microchip of similar dimensions packed with a reversed-phase C18 medium in conjunction with our solid-phase glycan permethylation [123] for the analysis of glycoprotein-derived glycans. A base-peak separation of solid-phase permethylated glycans derived from ribonuclease B is illustrated in Fig. 12, where different structural isomers associated with N-glycans were resolved from each other. An ion-trap mass spectrometer was utilized in this study. The potential of employing the chip in the general platform for functional glycomic investigations described above is currently being investigated.

6. Conclusions

Quantitative measurements in functional glycomics are viewed as essential to a better understanding of cellular and molecular interactions associated with different physiological and pathological processes in mammalian systems. Such measurements often demand high-sensitivity combined with a specific structural identification of biological determinants or biomarkers of a disease condition. These are currently best secured through the uses of biomolecular MS, which, in turn, performs best if combined with capillary separation techniques.

The main purpose of this brief review has been to evaluate critically the current status of different capillary separation methodologies as a useful adjunct to MS (using additional detection techniques) and when used in a direct combination. In terms of overall resolving power, the electromigration techniques such as CZE, MEKC and CEC, ensure most directly the resolution of close oligosaccharide isomers in comparison with chromatographic procedures. Laser-induced fluorescence is the most preferred detection procedure in high-sensitivity profiling of glycan mixtures by capillary electrophoresis. Coupling these techniques to MS still remains a nontrivial task, although several promising attempts have been made. The inherent limitations of preconcentrating solutes at the capillary inlet challenge the sensitivity of most MS detectors. The separation buffers must carefully be chosen for CZE and CEC combination with MS, while MEKC is not compatible with this type of detection.

In capillary LC separations of oligosaccharides, important advances were recently demonstrated through the design of hydrophilic packings and carbon-based materials. The latter packings have shown reproducible separations of structurally related glycans and suitability for MS investigations in a direct coupling.

A new platform for complete glycomic analyses of complex oligosaccharide mixtures involves an on-line quantitative permethylation preceding capillary reversed-phase LC/MALDI-tandem MS. Permethylated glycans are very suitable for LC/MS at high-sensitivity using either MALDI or ESI mode. Numerous isomers can be adequately resolved through LC after permethylation.

The studies on microfluidic analytical systems initiated for the benefits of genomic, proteomic and cellular microanalysis are likely to find their parallels in glycoproteomics and functional glycomics. There are recent indications of considerable promise in this direction.

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