Perspective

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Unraveling the Glyco-Puzzle: Glycan Structure Identification by Capillary Electrophoresis

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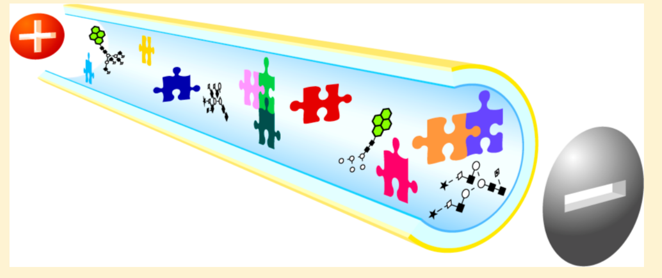
ABSTRACT: State-of-the-art high-resolution separation techniques play an important role in the full structural elucidation of glycans. Capillary electrophoresis (CE) offers a rapid yet simple method for exhaustive carbohydrate profiling. CE is a versatile analytical platform, which can be operated in several separation modes, simply by altering separation conditions during operation. For in-depth glycan structural analysis, CE has also gained significantly from the additional resolution introduced by complementary and orthogonal separation techniques such as ion exchange or hydrophilic interaction chromatography. Commercially available mass spectrometry (MS) interfaces have not only brought this information-rich detection technique within reach, but CE also represents an expedient highly efficient separation inlet for MS, capable of separating isobaric oligosaccharide isomers prior to MS detection and MS/MS fragmentation based identification. This Perspective gives a sophisticated impression of the versatility of capillary electrophoresis for deep structural elucidation of carbohydrates derived from glycoproteins of biomedical interest. Different separation modes for the analysis of both charged and neutral glycans, such as influencing electroosmotic flow, using complexation/interaction based secondary equilibria, and the use of charged and neutral labels are compared. The merits of introducing orthogonal and complementary techniques, such as exoglycosidase digestion arrays, analytical/preparative chromatography and mass spectrometric detection, and extending the dynamic range and resolution of CE are all thoroughly discussed.



The structural elucidation of protein derived glycosylation, which may possess vast structural diversity including positional and/or linkage isomerism, poses a substantial analytical challenge. The discovery of different glycan moieties' involvement in protein confirmation or function, cell−cell signaling, and reflection of cellular or even organism physiological state has attracted major research attention. Because of the analytical complexity associated with glycosylation analysis, a variety of orthogonal analytical techniques is used, often in combined fashion, and generally including separation driven methods combined with optical, chemical, or mass selective detection.[1] Capillary electrophoresis (CE) today is an important tool in unraveling the glyco-puzzle and represents a rapid yet highly resolving analytical platform.

In the current discerning Perspective of the structural elucidation potential of capillary electrophoresis in analyzing complex protein derived carbohydrates of biomedical and biopharmaceutical interest is presented. Auxiliary techniques, such as sequential exoglycosidase digestion, lectin affinity partitioning, analytical/preparative chromatography and mass spectrometry (MS) detection in conjunction with tandem mass spectrometry (MS/MS) fragmentation are discussed based upon their structural identification capabilities when interfaced with CE.

■ TECHNICAL OVERVIEW

The technical simplicity of capillary electrophoresis can be deceptive; CE is a highly adaptive versatile technique, enabling several separation modes simply by altering the separation conditions such as the background electrolyte used. Controlling the electroosmotic flow (EOF) phenomenon, that is the directional bulk flow generated under an electric field by ions attracted to capillary surface charges and its direction (co- or counter-electrophoretic mobility, μ_e), allows altering the migration time window and the experimental run time. EOF toward the detection site facilitates a stable liquid flow and CE current for electrospray ionization (ESI) with hyphenated mass spectrometric detection[2] or can generate a reverse migration order[3,4] when driving against analyte electrophoretic mobilities (with $\mu_{EOF} > \mu_e$). Suppression of the EOF by covalent or dynamic coating of surface charges can remarkably elevate experimental reproducibility. Under arheic (without flow) conditions, capillary zone electrophoresis (CZE) separates analytes based on their charge to hydrodynamic volume ratio differences thus rendering it a powerful tool for carbohydrate

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isomer identification. The selectivity of CE for the separation of similar oligosaccharides can be improved by introducing secondary equilibria, such as borate complexation,[5] micellar surfactants,[6−9] chromatographic (pseudo-) stationary phases,[10] or by polymeric additives.[11]

Protein derived carbohydrates from physiological samples are often only available in limited quantities, lack chromophores or fluorophores, and frequently contain differentially charged species. Therefore, derivatization with charged fluorogenic agents is a popular means to increase detector sensitivity and enable the simultaneous separation of neutral and charged analytes using electric field mediated methods. While optical on-column detection techniques such as laser induced fluorescence (LIF) enable high sensitivity and selectivity they do not however, provide additional glycan structural information. The choice of a labeling agent is strongly dependent on the application and needs to be carefully selected according to the background electrolyte (BGE), EOF, sample characteristics, and also the detection method. Enzymatically released *N*-linked glycans by peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase (PNGase F) maintain the free amino group from the side chain of the parent asparagine and the resulting glycosylamine can be derivatized with amine reactive dyes under basic conditions.[12,13] More commonly, the liberated glycosylamines are converted to reducing sugars (aldehyde form) at acidic pH and reacted with primary amino group containing dyes via reductive amination.[14−16] However, low pH and high temperatures can promote analyte hydrolysis and the potential loss of labile glycan constituents as well as selective labeling need to be diligently precluded by optimizing the derivatization reaction.[17,18] A favorable derivatization agent not only needs to meet reaction chemistry, size, and pH stable charge state requirements but also has to be compatible with the available detection platforms (e.g., laser excitation wavelength). On-column complexation,[19] EOF, or charged residues (e.g., sialic acids) can also provide sufficient separation mobility. Underivatized glycans are disadvantageous for sensitive optical detection but are commonly applied in mass spectrometric analysis.[20] A free reducing end enhances MS fragmentation options but may also increase analyte complexity due to anomericity.[21] Conjugated glycans potentially reach higher ionization yields.[15,22]

The transition to online mass detection techniques with electrospray ionization brings about the necessity to alter CE separation conditions, including replacement of outlet buffer reservoir and associated electrical circuit closing as well as introducing volatile background electrolytes, to achieve MS compatibility. Key for successful hyphenation of CE and MS are interfaces that produce a stable spray with low flow (preferably <20 nL/min) and allow nearly independent optimization of the separation and ionization sections. Although, a certain degree of compromise needs to be accepted: popular sheath flow interfaces use a sheath liquid at the capillary outlet, which can be optimized for stable ionization but at the cost of sensitivity due to sample dilution. Direct or sheathless connections generally incorporate low flow rates, high sensitivity, and reduced ion suppression, but a common BGE for reasonable separation performance and ionization yield needs to be found. Liquid junction interfaces introduce a liquid lined gap between the separation capillary and ionization source that allows decoupling optimization of CE separation and ESI parameters at low sample dilution effects. In-depth information about CE−MS technology, including glycan analysis using off-line interfacing and further ionization techniques such as matrix-assisted laser desorption/ionization (MALDI), can be obtained from eminent recent review articles.[20,23−25]

Miniaturization of CE into microchannel devices offers another attractive ESI-MS front end due to high sensitivity analysis of nanoliter amounts of sample.[26] Irrespective of the chosen detection method, microchannel glycan separations generally offer ultra fast separation times on the seconds scale but usually at the cost of resolution; particularly of glycans carrying additional charged constituents, which are often removed prior to analysis.[27−29] For fast screening, e.g., of mAb glycans during clone selection or rapid profiling of clinical samples in disposable chips, where throughput is decisive, a lower separation efficiency can be acceptable. Moreover, recent introduction of longer separation channels has converged CE and microchannel CE efficiencies but such designs yet need to be commercialized.[30−32]

A dramatic gain in throughput is also possible by simply running multiple separation columns in parallel. Multiplexed systems are commercially available and incorporate from 4 up to 96 capillaries. High experimental precision is maintained by coinjection of internal standards for alignment. Multicapillary systems are becoming increasingly established in clinical trials and biomarker discovery studies, where hundreds of samples are screened.[33,34] However, commonly built-in electrokinetic injection systems require careful sample handling, such as maintaining consistent salt or free dye content.[35] Avoiding selective analyte injection is of utmost importance when peak area based quantitation and associated statistical analyses are conducted.

■ GLYCAN STRUCTURE ELUCIDATION

CE offers a variety of separation modes, which incorporate different selectivity and associated glycan structure identification potential. Counter-electroosmotic flow enables increasing charge based migration grouping with respective larger species migrating prior to smaller ones of the same net charge.[36] Selectivity enhancement of structurally related glycan pools can be achieved by online interaction with ionic buffer additives, exhibiting different complexation affinity based upon monosaccharide composition.[37] Zone electrophoresis in aqueous or low viscosity polymer matrix with suppressed EOF provides exceptionally high resolution of isomeric species due to the separation principle of charge-to-hydrodynamic volume differences.[38]

Oligosaccharide Standards. Co-injection of purified glycan standards with an unknown sample mixture poses a simple and effective way for structural assignment. Although, basic knowledge about the nature of the analyte pool present is useful, because glycans with different monosaccharide or linkage patterns can exhibit identical migration behavior. Limited commercial availability of structures, their production via purification with techniques offering restricted separation efficiency (e.g., isomers), and taxonomy mismatch with the sample further restrict practical implementation of standards.

Anticipation of molecular size from analyte migration time can be achieved by the ancillary separation of gradually sized oligomeric sugar standards. Optimal reference standards incorporate equally distributed oligosaccharide pools exhibiting a linear relationship between size and migration time. Thus, linear homooligosaccharide ladders with degrees of polymerization (DP) 1, 2, ..., *n* of glucose$(\alpha 1 \rightarrow 4 \text{ or } 6)_n$, glucose$(\beta 1 \rightarrow$

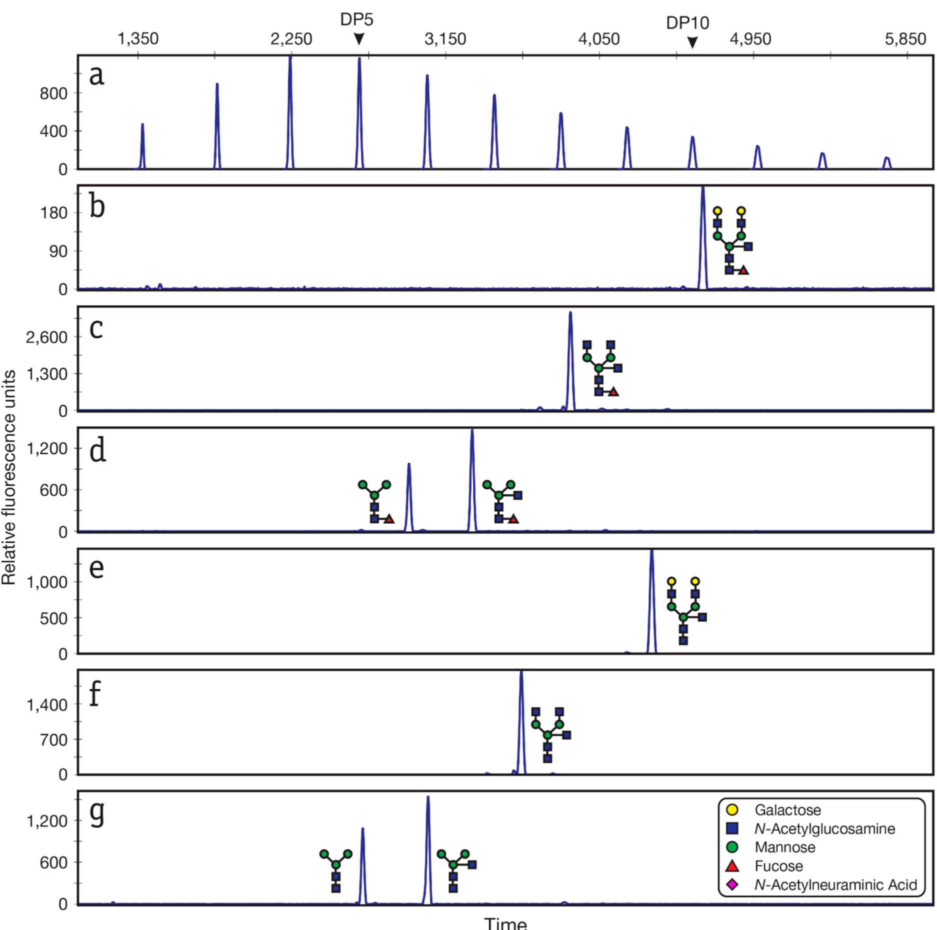


Figure 1. Exoglycosidase sequencing of a purified biantennary core fucosylated complex glycan standard with a bisecting *N*-acetylglucosamine. (a) Separation of an 8-aminopyrene-1,3,6-trisulphonic acid (APTS)-labeled oligomaltose hydrolysate reference standard. The number of glucose units (DP, degree of polymerization) in these structures is indicated. (b) Nondigested standard. Standard digested with (c) β-galactosidase, (d) β-*N*-acetylhexosaminidase, and (e) α-fucosidase. Standard digested simultaneously with (f) β-galactosidase and α-fucosidase, (g) β-galactosidase, β-*N*-acetylhexosaminidase, and α-fucosidase. Symbols used for glycans are those suggested by the Consortium for Functional Glycomics. Reprinted with permission from ref 43. Copyright 2006 Nature Publishing Group.

4)$_n$, and *N*-acetylglucosamine($\beta 1 \rightarrow 4$)$_n$, produced by hydrolyzing starch, cellulose, and chitin-type polysaccharide chains, respectively, are commonly applied standards. Hydrolysates of branched structures, e.g., high-mannose type glycans,[39] are generally not recommended due to the occurrence of positional isomers and the limited DP range. Molecular size standards can either be coinjected with the sample, e.g., oligomer fragments of DNA base pairs in multicapillary sequencer studies,[40] or analyzed in a separate experimental run, where alignment standards are usually introduced in both sample and standard runs.[41,42] Figure 1a,b shows the separation of a glucose-oligomer ladder and a purified glycan standard, respectively.

The respective DP or sugar units (SU) of a sample peak can be obtained by interpolation between adjacent oligosaccharide ladder peaks or polynomial fitting of multiple standard peaks.[44] The conversion from migration time to a size-based scale of SU also promotes interexperiment, -instrument, and -institution precision and comparability, by compensating for potential buffer composition, separation temperature, or column history induced experimental deviations. The accumulation of SU values of glycans with known (e.g., purified standards) or decoded structural identity can evolve into the establishment of a database, capable of decisively supporting structural elucidation or denovo sequencing of unknown glycan pools. Usage of such databases requires consistent experimental conditions to those under which the data was generated, including the appropriate ladder standard. Glucose units (GU), i.e., SU based upon glucose oligomers, are widely used normalization standards in glycan analysis. Maltooligosaccharides ($\alpha 1 \rightarrow 4$ linked oligo-glucoses) are the prevalently used standards in CE, whereas $\alpha 1 \rightarrow 6$ linked isomaltooligsaccharides (also referred to as dextran) are predominantly applied in glycan analysis by hydrophilic interaction liquid chromatography (HILIC). Sole discrepancy in glycosidic linkage type or anomericity of oligosaccharides can impact their hydrodynamic volumes and result in differential migration, thus render SU based structural assignments inaccurate if mismatching standards are used.[44]

Enzymatic Digests. The specific cleavage of monosaccharide constituents from nonreducing termini via exoglycosidase enzymatic digestion represents another powerful means for glycan structure characterization. Depending on enzyme specificity, monosaccharide type, sequence, or even linkage and anomericity can be obtained when digest induced structural/shape changes are accompanied by CE monitoring. Sophisticated digest cascades can also lead to the identification of positional isomers.[45,46] Exoglycosidase digest arrays can either be conducted in parallel, where the sample is evenly split

into one aliquot per enzyme mixture, or in sequential fashion using analysis-digest iterations of the same sample. Sequential processing requires lower amounts of sample and enzymes and takes advantage of the minimal injection volumes needed for CE analysis. The use of nonvolatile buffer components or addition of alignment standards to the sample prior to injection, as for example used in multicapillary sequencer experiments, can impede further digestion steps. Moreover, exoglycosidase digests can cause changes in the sample ionic strength or pH, introduced by salts of the digestion buffer, and promote diffusion induced peak distortion as well as potentially altered electrokinetic injection behavior.[47] Volatile digestion buffer systems are therefore generally recommended to alleviate this issue. Most commercial enzymes are, however, supplied in nonvolatile media and consequently will cause the described problems in CE, if concentrated sample volumes or multiple enzymes are used.

Exoglycosidase digestion of glycans with known structure (e.g., purified standards) can cause a predictable loss of constituents corresponding to enzyme specificity. After reanalyzing the digested product by CE, glycan structural differences can be recorded by the differences in migration time and relative peak area compared to the substrate. Such shifts in migration time, or respective sugar units, can be used to build a knowledge base of monosaccharide residues and associated "contributions".[48] Traces c and d and e and g in Figure 1 exemplify two different digest cascade possibilities of the purified glycan standard analyzed in trace b. Structural identity of hydrolyzed products, respective SU, as well as digest induced shifts can readily be deduced. Additionally, positions of unknowns, e.g., a monogalactosylated biantennary species, can be anticipated half way between the bi- and the agalactosylated peaks as, respectively, depicted in Figure 1b,c.

In the case of dealing with unknown glycan mixtures, increasing structural identity confidence can be gained by tracing respective peaks through several digestion steps or by virtual "reattachment" of constituents in a bottom-up manner, especially when combined with sugar unit shift analysis and CE-based glycan structure reference databases. Relative peak areas before and after digestion nevertheless need to be rationally compared when analyzing complex oligosaccharide pools. Glycans composed of different monosaccharide units can exhibit identical migration, due to, e.g., similar charge to hydrodynamic volume properties but differentially or even unaffected enzymatic digest reactivity. This can be either due to lacking the epitope that matches enzyme substrate specificity or inaccessibility potentially induced by steric hindrance. For example the removal of bisecting *N*-acetylglucosamine (GlcNAc) residues upon hexosaminidase treatment can be hampered as depicted in Figure 1d,g.

The hydrolysis of sialic acids, which introduces additional molecular charges, by sialidase treatment prior to analysis, is a rather regularly applied practice in CE based glycan investigations.[29,33,34,49−52] However, associating sialic acid removal with technical limitations of electromigration based methods is a misapprehension. For example, CZE with suppressed EOF offered superior separation efficiency of additionally charged thus faster migrating glycans, also when compared with HILIC methods, due to decreased analyte diffusion.[53] The removal of sialic acids generally results in decisive reduction of CE profile complexity, by merging previously distributed corresponding species with a differential degree of sialylation as well as sialic acid linkage and positional isomers. This reduction of complexity coincides with the loss of information and potentially correlated physiological features, when investigating glycans of biomedical interest.[54,55] Although, the combination of previously distributed low abundant species upon sialidase digestion might also allow for the investigation of other biologically important glycan features, formerly undetected due to dynamic range issues. When electrokinetic injection is used, equalizing analyte charge states will also support diminishing potentially biased injection.

Different endoglycosidases, used for the release of asparagine-linked sugar moieties from glycoproteins, can be applied for specific glycan type-based partitioning. While PNGase F liberates all classes of *N*-linked glycans from mammalian glycoproteins, Endo-β-*N*-acetylglucosaminidase H (Endo H) specifically releases high-mannose and hybrid type oligosaccharides.[56] Endo H cleaves substrate glycans between the two GlcNAc residues in the core region and readily enables the identification of the specific glycan types by CE.[57] Determination of the intersection and difference sets with the respective PNGase F released pool enables type-based classification,[58,59] when the alterations by the missing GlcNAc residue and associated SU shifts are accounted for.

Exoglycosidase digests are usually carried out in low reaction volumes (~10 μL) with overnight incubation to achieve exhaustive enzymatic processing. On-column enzymatic digestions represent a practical, rapid, low sample, and enzyme consuming alternative with incubation times down to only a few minutes or even during the separation process itself by passing the injected sample through an enzyme plug.[60] Direct introduction of exoglycosidases into narrow bore separation channels can increase enzymatic reaction speed due to decreased diffusion limitations, but such endeavors are sometimes incompatible with certain enzymes[60] and often accompanied by some loss in separation efficiency.[51,61,62]

Lectin Affinity. As opposed to consecutively cutting glycan constituents, carbohydrate binding proteins, incorporated into the separation column, can result in specific peak retardation or disappearance, based upon their respective structural affinity. Lectin affinity CE enables the structural classification of glycans by type (e.g., high-mannose[51,62,63]), antennary branching (e.g., triantennary[64,65]), monosaccharide features (e.g., fucose,[60,65,66] bisecting GlcNAc,[64] galactose,[60] sialic acid[65]), or even by their glycosidic linkage type (e.g., α2−3 or 2−6 linked sialic acids[60,67]) via specific binding reactions. Similar to on-column enzymatic digestions, lectins can either be added to the BGE[63−67] or introduced as a distinct zone,[51,60,62] but generally no incubation time is needed. Although, not all lectin−carbohydrate interactions are well understood, binding associations are generally low and peak distortion can limit area based comparison of experiments with/without lectins, affinity CE represents another fast and valuable structure identification tool.

Additional Separation Dimensions. Additional orthogonal separation dimensions offering different selectivity can drastically increase the resolution. Especially when investigating the identity of heterogeneous carbohydrate pools, the reduction of sample complexity by preparative separation and subsequent analysis of the collected fractions by CE is often indispensable. Analytes can either be partitioned into groups of multiple species according to sample properties such as charge state, or, often by using several preparative techniques, into distinct glycan species. The preparation of such purified carbohydrate standards enables the identification strategies outlined under

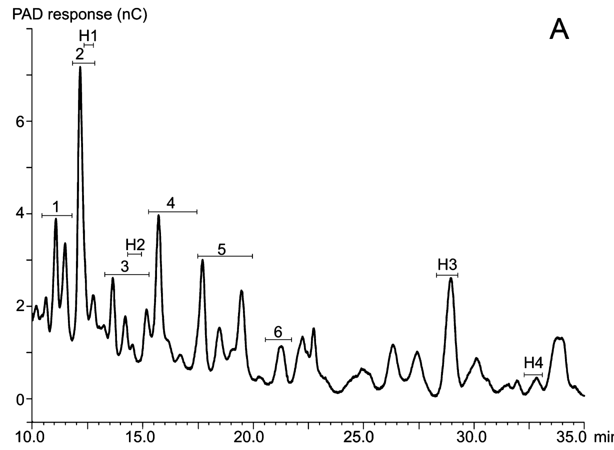
Oligosaccharide Standards and Enzymatic Digests, when combined with exoglycosidase digestion. For highly complex samples consisting of a multitude of species, this approach can be extremely labor-intensive or even impossible due to restricted sample availability and resolution limitations of preparative techniques in separating closely related structures. Difficulties in structural identification arising from comigration of species with similar charge to mass (or equivalent hydrodynamic volume) ratios in CE can be minimized by molecular charge or size based fractionation. Orthogonal separation mechanisms, such as analyte polarity based surface interaction, can also distinguish previously comigrating species, due to differential separation selectivity.

Chromatographic techniques with various stationary phases and corresponding distinct selectivities accompanied by higher associated injection volume requirements are ideally suited for preparative fractionation prior to CE analysis. Compatibility with subsequent CE analysis is reached by desalting or the application of volatile liquid phases. Similar to glycan analysis by CE, sensitive detection in liquid chromatographic (LC) techniques often requires the introduction of fluorescent dyes. However, on the basis of the different selectivity of chromatographic phases, difficulties can arise from certain properties of labeling agents (e.g., charge, polarity) which were optimized for glycan separation by CE. The combined application of preparative LC and analytical CE techniques with sensitive detection thus generally requires a compromise.

Anion-exchange chromatography (AEC) fractionation is one of the popular means to separate glycan pools by their degree of sialylation or other charge including modification (e.g., phosphorylation). Each charged fraction may contain several glycan structures, differing, e.g., in branching degree, composition of monosaccharides, and their position or linkage but all exhibiting the respective equal net negative charge. In CZE under arheic conditions, for example, comigration of larger structures holding higher charge states with smaller analytes, can aggravate structural identification. Additional charges introduced by sialic acids can be removed via sialidase digestion and the resulting changes in relative peak areas monitored, as outlined under Enzymatic Digests. In cases where the ratio of charged vs neutral species is particularly unbalanced in favor of the latter, e.g., sialo- vs asialo-glycans on human IgG, and only trace amounts of certain charged species are observed, exoglycosidase digestion induced increase of the corresponding neutral species can be marginal. Partitioning into charged fractions resolves overlaps between differentially charged species and enables the monitoring of peak shifts to unoccupied regions upon charge removal and direct comparison to the associated neutral fraction.[53]

Figure 2 shows the high pH high performance AEC (HPAEC) fractionation and consecutive CE analyses of human serum *N*-glycans released via endo H. While sialidase digest of the total pool in trace BS could result in inconclusive changes of the corresponding neutral species, individual analysis of preparatively captured fractions and their sialidase digestions revealed the identity of the underlying monosialylated hybrid type species, as presented in Figure 2B.[57]

Highly charged labeling agents, such as the commonly used 8-aminopyrene-1,3,6-trisulphonic acid (APTS), can critically increase the total negative charge of analytes, thus potentially causing excessive retention on AEC phases. Moreover, the fundamental separation principle in AEC is based upon differences in charge distribution across the hydrated molecule.



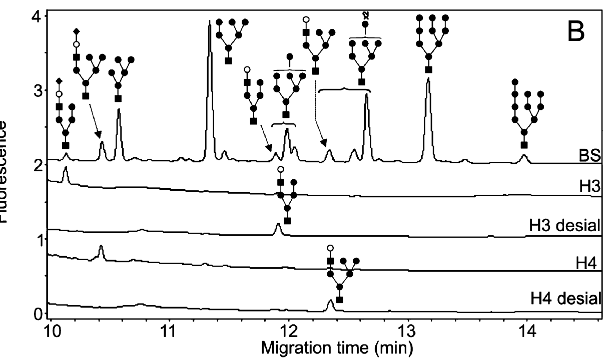
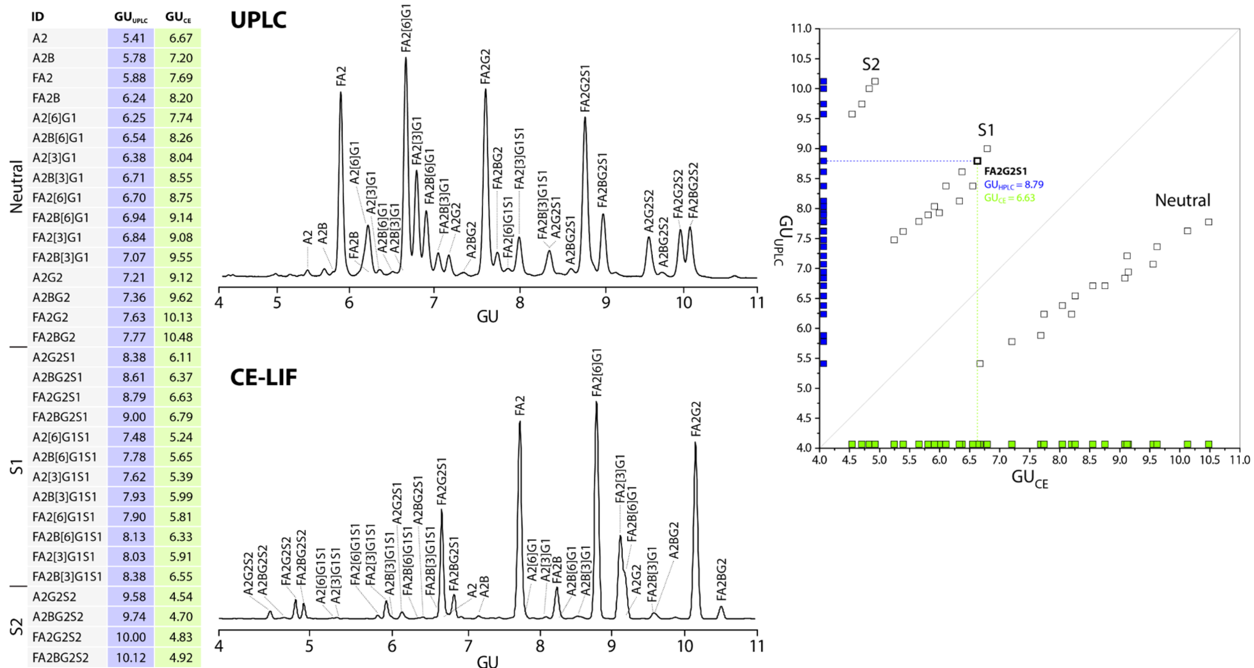


Figure 2. (A) HPAEC-PAD profile of human serum *N*-glycans released via endo H. Areas subjected to fraction collection are indicated. (B) CE-laser induced fluorescence detection (LIF) trace of APTS labeled total glycan pool from blood serum (BS), collected fractions H3, H4 and their respective sialidase digests (H3 desial, H4 desial). Glycan symbols as in Figure 1. Adapted with permission from ref 57. Copyright 2011 John Wiley & Sons, Inc.

The subdivision into distant non- and reducing terminal charges further complicates the application of highly charged dyes for AEC-based fractionation. Using APTS as an ionic dye for CE separation and sensitive detection is still possible if fractionation is carried out using underivatized glycans and only subsequent dye conjugation. This was demonstrated by either blind fraction collection adhering to pre-established retention time windows from the separation of 2-aminobenzamide labeled glycans[53] or pulsed amperometric detection (PAD) following HPAEC separation.[57] On the basis of its separation principle, AEC allowed for additional size-based separation within each charged fraction, when operated with fluorescently labeled glycans and sensitive optical detection.[68] Supplemental size-based fraction collection of underivatized glycans was also achieved by UV detection[69,70] or by HPAEC-PAD, which enabled sensitive elution monitoring at maintaining adequate resolution, as exemplarily shown in Figure 2A. HPAEC-based separation generally requires a subsequent buffer exchange step due to nonvolatile strongly alkaline mobile phases, the described associated ionic strength mismatch issues in CE separation, and high pH-induced sample epimerizat[illegible].[71]

Hydrophilic interaction liquid chromatograph[illegible]a high resolution fractionation alternative to AEC, also offering orthogonal selectivity. Depending on the chromatographic column, retention is a function of hydrophilicity and associated glycan size or a combination with ionic interactions,[72] when



Group	ID	GU_{UPLC}	GU_{CE}
Neutral	A2	5.41	6.67
	A2B	5.78	7.20
	FA2	5.88	7.69
	FA2B	6.24	8.20
	A2[6]G1	6.25	7.74
	A2B[6]G1	6.54	8.26
	A2[3]G1	6.38	8.04
	A2B[3]G1	6.71	8.55
	FA2[6]G1	6.70	8.75
	FA2B[6]G1	6.94	9.14
	FA2[3]G1	6.84	9.08
	FA2B[3]G1	7.07	9.55
	A2G2	7.21	9.12
	A2BG2	7.36	9.62
	FA2G2	7.63	10.13
	FA2BG2	7.77	10.48
S1	A2G2S1	8.38	6.11
	A2BG2S1	8.61	6.37
	FA2G2S1	8.79	6.63
	FA2BG2S1	9.00	6.79
	A2[6]G1S1	7.48	5.24
	A2B[6]G1S1	7.78	5.65
	A2[3]G1S1	7.62	5.39
	A2B[3]G1S1	7.93	5.99
	FA2[6]G1S1	7.90	5.81
	FA2B[6]G1S1	8.13	6.33
	FA2[3]G1S1	8.03	5.91
	FA2B[3]G1S1	8.38	6.55
S2	A2G2S2	9.58	4.54
	A2BG2S2	9.74	4.70
	FA2G2S2	10.00	4.83
	FA2BG2S2	10.12	4.92

Figure 3. Comprehensive annotation of the UPLC−fluorescence and CE−LIF profiles of the N-glycans released from human serum polyclonal IgG using a combination of exoglycosidase digestion and weak anion exchange fractionation. Insets show a table of the experimentally determined GU value for each glycan structure and a comparative 2D plot. N-Glycan nomenclature from ref 91. Reprinted from ref 53. Copyright 2011 American Chemical Society.

using amide- or amine-based stationary phases, respectively. HILIC-based separation enables the collection of fractions with high individual structure purity as opposed to common analyte property based grouping in AEC; however, the higher resolution of the HILIC fractionation results in an increased number of fractions and associated larger scale downstream analysis. Combination of HILIC fractionation and exoglycosidase digestion of purified analytes is also commonly applied for accurate glycan structural elucidation.[73−75]

Similar to AEC, the application of polar and ionic labeling agents, such as APTS (log P −1.21) for CE analysis, was preceded by either intact or removed charged glycan constituents, using underivatized[35] or APTS labeled oligosaccharides,[43,74] respectively. The motivations for these distinct strategies, however, seem to originate from mass spectrometric detection and CE instrument compatibility considerations. Other fluorescent labeling agents, such as 2-aminobenzoic acid, (log P 0.78) featuring lower charge and polarity, were successfully applied for both HILIC-based fractionation and CE analysis using high sensitivity fluorescence detection.[73,76]

Applications of reverse phase (RP) and size-exclusion chromatography (SEC) glycan fractionation are limited due to lower associated applicability and selectivity, respectively, when compared to HILIC and generally low efficiency for relatively small sugars (0.1−5 kDa range), respectively. Only few high performance columns serving ranges in the low molecular weight region exist,[77] and exclusive size-based preparative partitioning on ion-exchange columns suffers from low resolution.[78] As opposed to HILIC, retention on RP stationary phases is based on hydrophobicity thus providing only weak interaction for polar glycans. Although, type based separation of high mannose, complex and fucosylated complex glycans can be achieved,[79] and RP-based fractionation could resolve comigration of species from the distinct groups.[80] Also the derivatization with more hydrophobic dyes, such as 2-aminoacridone (log P 2.95), can facilitate RP fractionation potential.[81]

Capillary electrophoresis can also be operated in preparative mode using larger capillary diameters and several interchangeable outlet buffer reservoirs for fraction collection.[82] Low injection plug volumes often require several iterations of the fractionation process to achieve a sufficient amount of analyte per collected fraction.[83] Micropreparative CE has nevertheless been successfully applied for off-line MALDI ionization and hyphenated mass detection, where higher concentration is reached by liquid phase evaporation and concentration within the MALDI matrix.[84,85]

One often perceived limitation of CE is the low sample injection volume. However, this facilitates experiment repetition and also the consecutive execution of different CE separation modes. Hence, the simplification of complex samples via elaborate preparative fractionation can be avoided by the introduction of additional analytical separation modes with different selectivity. Glycans comigrating in one separation dimension can be separated in another with appropriate selectivity and vice versa. Such multidimensional mapping of oligosaccharides originates from the application of different stationary phases for liquid chromatography.[86] Nanoliter injection volumes and the versatility of CE allow the facile operation of several different separation modes, including EOF and on-column complexation phenomena, on the same instrument or even capillary, by simple changing the electrolyte used. Normalized migration times of identified carbohydrate species from each separation dimension span a multidimensional database thus facilitating structural identification of unknowns at increasing confidence.[87,88]

Combination of analytical CE and LC glycan separation methods comes at costs of additional instrumentation and sample preparation steps, e.g., introduced by different fluorescent labels optimized for each technique, but enables

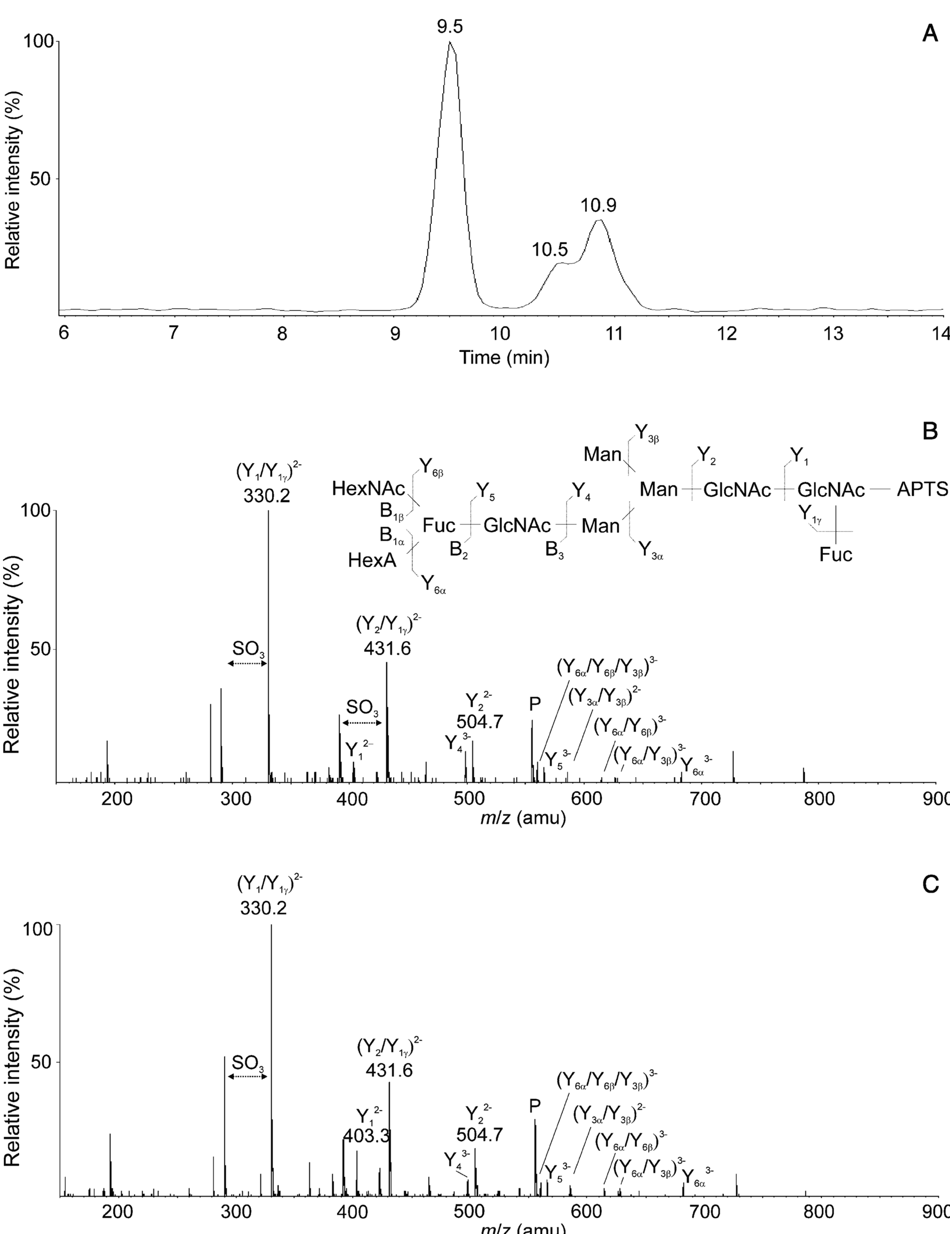


Figure 4. CE−MS/MS [illegible] of the fixed m/z 555.7 in Q1 of a quadrupole ion trap MS (A) and MS/MS fragmentation spectra of the respective peaks at 10.5 (B) and 10.9 min (C). The peak at 9.5 min corresponds to $Man_5GlcNAc_2$ at m/z 557.8. P, precursor ion. Fragmentation nomenclature as in ref 93. Reprinted with permission from ref 102. Copyright 2007 Oxford University Press.

orthogonal glycan separation.[53,75] Although, fluorescent dyes suitable for both platforms have been presented,[73,76,89,90] instrumental limitations such as available detection method, can restrict their application. Disregarding the increased necessary sample handling, the application of two distinct analysis routes allows for monitoring potential experimental discrepancies originating from, e.g., loss of labile glycan constituents or salt content. Also one might not want to change a certain analysis route due to already existing databases that can be used for peak identification in the other dimension.[69]

Figure 3 depicts the comprehensive annotation of the human IgG N-glycan pool separated in both ultrahigh pressure LC (UPLC)-HILIC and CE−LIF. Exhaustive glycan identification was achieved by the combined application of preparative anion exchange fractionation and exoglycosidase digestion steps requiring over 48 h of total analysis time. In a two-dimensional plot, inset in Figure 3, all 32 oligosaccharides present were well separated and revealed clustering based upon their degree of sialylation. Combining high experimental precision, granted by the use of glucose unit values following time based normalization, with the beneficial orthogonality of the separation platforms allowed for exhaustive and confident structural annotation of the IgG N-glycan pool within only 20 min of using the 2D space and made additional fractionation or digestion techniques obsolete.

CE−MS: Molecular Mass and Fragmentation. Mass spectrometry offers an information-rich complementary detection method for glycan analysis, facilitating the measurement of distinct analyte mass and charge properties when used online, independent from standard coinjection or exoglycosidase digest patterns. Glycan composition can be estimated

based upon the addition of monosaccharide constituent masses. However, because of the high structural diversity, numerous combinations can result in equal mass values. Moreover, on the monosaccharide level stereosiomers (e.g., glucose and mannose) exhibit the same masses, thus MS can only detect different classes of sugars such as hexose (glucose, galactose, mannose), N-acetylhexosamine (GlcNAc or GalNAc), deoxyhexose (fucose), or sialic acids. Information about the identity of monosaccharides may be deduced from additional sample information such as glycan class (e.g., N-, O-glycan), taxonomy, and associated biosynthetic pathways. Such information, if available, provides very limited potential of elucidating linkage and positional isobaric isomers. Therefore to increase the overall level of information in each associated experiment, MS detection can be combined with monosaccharide sequencing by exoglycosidase digest induced mass shift monitoring or more commonly the application of tandem mass spectrometry (MS/MS) following collision induced dissociation (CID).[92] In tandem MS or higher order MS^n approaches of pseudomolecular oligosaccharide cations formed during positive ionization, glycans first break at their most labile bonds resulting in a series of glycosidic cleavages. Glycosidic linkage cleavage allows determination of monosaccharide sequence and branching degree, based on B/Y or C/Z-ion series if negative ionization is used,[93] whereas cross-ring cleavages and associated A/X-ions allow deduction of linkage positions.[92] Because of the complexity and potential interpretation ambiguity, it is often reasonable to investigate fragmentation patterns with purified glycan standards similar to those under study. However, informatics platforms such as GlycoWorkBench[94] and GlycoPeakFinder[95] have greatly helped in simplifying the interpretation of oligosaccharide MS/MS spectra.

Problems arise from direct infusion of complex sample mixtures, where selected precursor ion masses potentially contain structural isomers. Fragmentation of multiple species can lead to incorrect spectral interpretation and glycan mass based structural conclusions. Hyphenation of MS with separation based techniques can provide additional structural information or even resolve isomeric species and thus prevent erroneous structural interpretation from the resulting spectral data. Concentration of complex analyte pools into distinct bands using separation techniques results in increased dynamic range and associated sensitivity for MS detection. Furthermore, less analyte will enter the MS together and thereby reduce the suppression of less abundant ions by predominant ones. Ion suppression effects as well as biased quantitation of differentially charged analytes and associated ionization yield can be resolved by upstream CE separation due to the inherently high efficiency of CE based separations.

MS interfacing generally comes at the compromise in CE resolution, introduced by a MS friendly BGE, the missing outlet buffer reservoir, differential ionization interface gas pressure, siphoning effects, and band broadening in longer capillaries. On the other hand, the more informative MS detection facilitates the detection of comigrating nonisobaric analytes. High-resolution off-line CE glycan separation with photometric detection can be altered stepwise toward MS compatible conditions. On the basis of relative abundances, respective peaks can be assigned in the CE−MS base peak electropherogram (BPE) and previously gathered structural information matched or even amended.[71,96,97] The more accurate quantitation achieved by optical detection of heterogeneous analyte pools can also be incorporated online proximal to the mass spectrometer.[98−100]

The separation of uncharged glycans in CE is problematic because on-column complexation with borate or metal ions is generally not MS compatible and sole EOF based separation lacks selectivity. Isocratic elution in CEC[21,101] or the introduction of ionic labeling agents enable the parallel separation of both neutral and charged species. Labels are chosen not only based on CE separation optimization but also MS ionization considerations. Neutral or positively charged labeling agents, such as 9-fluorenylmethyl and 3-aminopyrazole, are used for positive MS ionization polarity modes[12,102] and vice versa for the more commonly used APTS, 8-aminonaphtalene-1,3,6-trisulfonic acid and 2-aminobenzoic acid in the negative mode.[90,103,104]

Figure 4A shows the CE−MS BPE of APTS labeled N-glycans from the structural subunit 1 of Rapana venosa hemocyanin at m/z 555.7 isolated in Q1 of a quadrupole ion trap MS.[102] Counter-EOF conditions with the MS at the cathodic side allowed separation based on increasing negative charge and larger species migrating prior to smaller ones within each charged group. The peak at 9.5 min corresponded to the labeled $Man_5GlcNAc_2$ structure, whereas peaks at 10.5 and 10.9 min represent isomers of a different glycan species with an additional negative charge. Similar tandem MS spectra of the peaks at 10.5 and 10.9, depicted in respective parts B and C of Figure 4, endorsed matching the monosaccharide sequence and suggested the presence of positional isomers that were separated due to the high resolving power of CE.

Labeling at the reducing end can provide valuable information on the site-attachment of specific glycan constituents due to fragment ions either including or lacking the mass and charge introduced by the labeling agent. A- and X-type ions and associated linkage position elucidation, however, are generally low when the reducing end of glycans is occupied, because cross-ring fragmentation predominantly occurs at the proximal end.[105]

Unlabeled glycans that carry charged constituents, such as sialic acids or phosphate residues, were well separated in counter-EOF CZE based upon their charge degree and molecular size.[106−109] Such acidic glycans are most sensitively detected by negative ion MS, but positive mode allows quantitation of differentially charged species, i.e., glycan pools containing both positive and negative oligosaccharides, using, e.g., acidic mobile phases in CEC that can protonate sialic acids.[101] Glycans with an unoccupied reducing end produce more informative MS/MS spectra due to higher abundance of A/X-ions, but reducing terminal mutarotation can increase sample complexity.[21] MS/MS fragmentation analysis of glycans holding terminal sialic acids often leads to predominant B_1 ions originating from the loss of such charged glycan constituents and lower amounts of more informative C-type fragments.[110]

Chemical derivatization via amidation, methylation, or permethylation can stabilize sialic acids[111] and thus provide greater structural elucidation potential in MS/MS fragmentation,[112] but coinciding charge neutralization and increased hydrophobicity limit their online CE−MS application. Offline CE fraction collection and spotting on MALDI plates combines CE efficiency with less complex MS spectra originated from mostly singly charged ions.[84,85,113] Low mass loading in CE limits chemical derivatization options of collected fractions. These derivatizations are generally recommended to prevent

glycan degradation during the higher energy laser desorption/ionization.[114]

■ CONCLUDING REMARKS AND PERSPECTIVES

The pursuit of unraveling the glyco-puzzle progresses with the evolution of high-resolution separation techniques. Capillary electrophoresis represents a rapid and high-resolution separation tool, which after sequencing the human genome found one of its prime applications in the analysis of complex carbohydrate mixtures. The ability of separating both linkage and positional isomeric species based on inherent molecular shape differences, such as hydrodynamic volume, has rendered CE indispensable for fine structural analysis. The underlying separation principle can easily be influenced by capillary surface modifications, replacing the background electrolyte, special buffer additives, or ionic labeling agents to meet sample specific optimal separation conditions. Despite the versatility of CE, the introduction of additional separation dimensions should be considered necessary to aid elucidating the vast structural heterogeneity of glycans. Moreover, a parallel analysis route, introducing, e.g., alternative labeling chemistry or orthogonal separation, can prove beneficial for method validation and obviating potential systematic bias. Application of complementary analytical approaches not only increases structural identification accuracy but eventually opens up new possibilities for the establishment of a score-based confidence system based on data integration from the individual dimensions.

Sophisticated exoglycosidase digestion and mass spectrometric detection represent further powerful means to increase structural elucidation confidence. Hyphenation of CE with MS remains technically challenging but facilitates both sensitive and highly informative detection when combined with the resolving power of CE. MS also enables sensitive detection of unlabeled glycans thus excluding potential associated analyte deterioration or selective functionalization. Several informatics-based endeavors are being pursued to assist and (semi-) automate the demanding interpretation of glycan MS and MS/MS fragmentation spectra.

Glycoinformatics tools to aid CE based glycan structure elucidation still remain marginal. Extensive mapping databases that enable structural inference from normalized detection times of fluorescently derivatized glycans currently exist only for HPLC based techniques and complementary databases for CE are necessitated. However, the immense structural diversity of glycans, time-consuming experiments, and the limited availability of glycan standards impede an exhaustive accumulation of such data. As opposed to surface interaction based separation techniques, glycan analysis results by CE could more easily be predicted based on computed geometrical characteristics and a plethora of structure database entries generated in silico. The combination of the different data resources will improve the confidence and quality of glycan structure identification, for instance by CE separation based structure constraints for subsequent MS/MS spectra annotation. Therefore, it is anticipated that developing novel glycoinformatics tools will play an equally important role as high-resolution separation techniques and sensitive detection methods in further resolution of the glyco-puzzle.

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Notes

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