

# Advancing Solutions to the Carbohydrate Sequencing Challenge

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## Supporting Information

**ABSTRACT:** Carbohydrates possess a variety of distinct features with stereochemistry playing a particularly important role in distinguishing their structure and function. Monosaccharide building blocks are defined by a high density of chiral centers. Additionally, the anomericity and regiochemistry of the glycosidic linkages carry important biological information. Any carbohydrate-sequencing method needs to be precise in determining all aspects of this stereodiversity. Recently, several advances have been made in developing fast and precise analytical techniques that have the potential to address the stereochemical complexity of carbohydrates. This perspective seeks to provide an overview of some of these emerging techniques, focusing on those that are based on NMR and MS-hybridized technologies including ion mobility spectrometry and IR spectroscopy.

## ■ INTRODUCTION

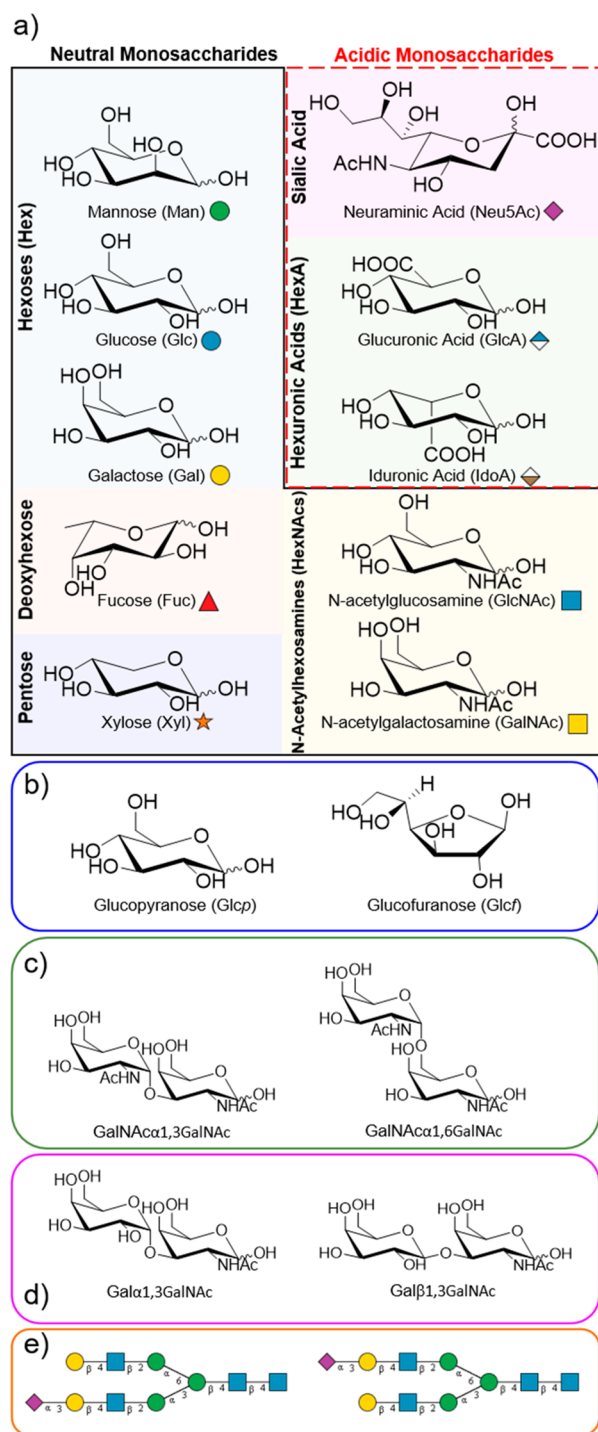
Intricately fine-tuned glycans decorate all cells and have a plethora of functions ranging including mediating cell–cell adhesion, cell motility, protein folding, and immune response.<sup>1,2</sup> Their functions are mediated either by recognition with a glycan-binding protein (GBP) or through the inherent chemical properties of the saccharide including altering the properties of any conjugate to which they are bound.<sup>1</sup> Natural glycans are typically heterogeneous, with the abundances of these different glycoforms varying over time, from individual to individual, by nutritional state, and from the cell environment, along with many other factors that are still poorly understood.<sup>3,4</sup> These glycans are exploited during pathogenesis, and aberrant glycan structures are associated with a number of disease states.<sup>5</sup> Glycoforms may also change during the course of disease progression, so identifying these changes enables early treatment and treatment stratification.<sup>6</sup> Given the

established connection between their structure and their function, full characterization of unknown carbohydrates ideally with minimal sample derivatization/labeling, is critical and represents one of the major roadblocks within the progress of the glycosciences.<sup>7</sup> Furthermore, the development of glycan and glycoconjugate therapeutics will necessitate accurate, precise, sensitive, and high-throughput analytics to unequivocally characterize the carbohydrate structure.

Carbohydrates possess unparalleled levels of structural complexity compared to other classes of biological molecules and are found free, adhered to proteins and lipids, and as polysaccharides. This complexity normally arises from the number of chemically similar (and often isomeric) monosaccharide building blocks, the position and orientation of glycosidic linkages, and branching (Figure 1). The biosynthesis of carbohydrates is not template-driven; although the complex biosynthetic pathways of glycoconjugates have been extensively studied, in particular for human glycans that are all derived from 10 monomeric building blocks, and many structures contain conserved cores. As a result, many analytical approaches rely on these studies to infer chemical structures. Where the pathways are less predictable or unknown altogether, such as in plant metabolites or bacterial glycans, analysis is still very slow. Many analytical approaches require highly purified standards (sometimes in amounts greater than biologically available) that are difficult to obtain by isolation or synthesis, necessitate derivatization to facilitate separation or detection, and often employ cocktails of glycosidases, which are not available for every linkage. As a result, our understanding of endogenous carbohydrate structure and function still lags behind that of other biomolecules, despite carbohydrates representing the most abundant biological class.

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**Figure 1.** (a) Chemical structures of the 10 classical human monosaccharides and their respective Symbol Nomenclature for Glycans (SNFG) nomenclature.<sup>8</sup> Additional isomerism resulting from ring size (b), regioisomerism (c), anomeric configuration of the glycosidic bond (d), and positional isomerism (e).

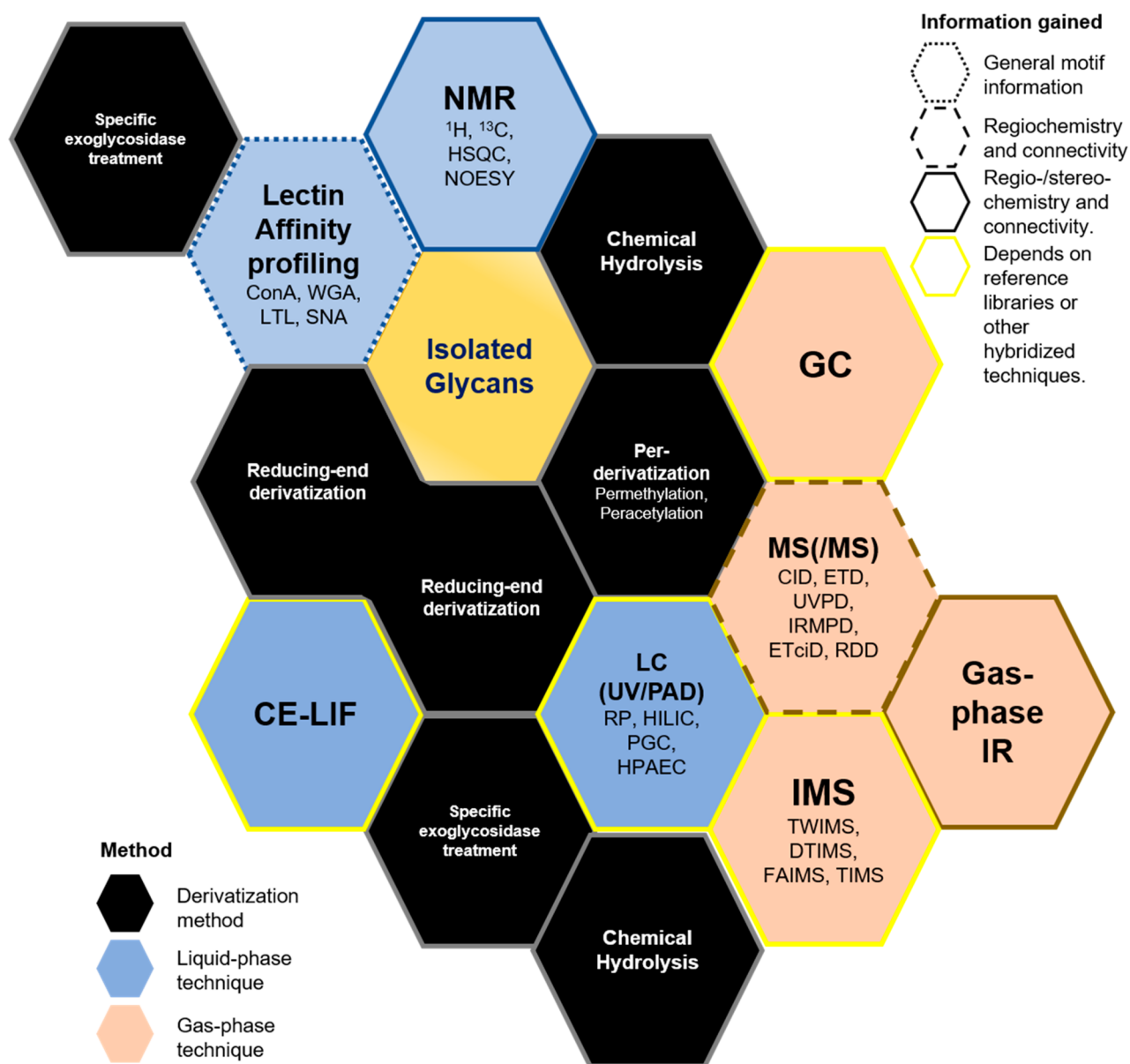
Development of technologies to facilitate glycan research has been highlighted as a key area within the scientific community with identification/sequencing being a crucial contributing facet within this program,<sup>9</sup> given that reliable sequencing techniques are still very slow or not available. This perspective focuses on the challenges associated with glycan analysis and highlights recent promising technologies, in particular, the development of more sensitive NMR methods and the

increasing amount of stereochemical information obtained from MS-based gas-phase techniques.

## ■ A PROBLEM OF SIZE, LINKAGES, NUMBERS, AND "SHAPES"

Carbohydrates exist in a wide variety of sizes, from simple monosaccharides to large insoluble polysaccharides. The biological information encoded in these glycoconjugates relies significantly on stereoisomerism: common monosaccharide building blocks in human glycans such as glucose, galactose, and mannose differ from each other by inversion of just a single stereocenter (Figure 1a), yet each isomer has distinctly different biological roles and properties. This chemical similarity between the building blocks makes their separation and characterization challenging, especially for techniques like mass spectrometry (MS) that rely on mass differences. Oligosaccharides may also be altered enzymatically with functionality such as sulfation, acetylation, and phosphorylation, thereby further complicating analyses. In addition to the individual building blocks, isomerism of glycosidic linkages also needs to be defined. Monosaccharides can be linked by glycosidic bonds at multiple positions (including branching from the same residue) allowing for the formation of linkage regioisomers. The inherent chirality of the anomeric carbon adds  $\alpha$ - and  $\beta$ -stereoisomerism. Finally, the monosaccharides can exist as 6-membered rings, pyranose (*p*), or 5-membered rings, furanose (*f*) (Figure 1). Bacteria also contain a much more diverse range of monosaccharide building blocks compared to the 10 human ones, many of which remain uncharacterized.

Seemingly, carbohydrates are perfectly evolved biomolecules to contain the maximum amount of structural information within the least amount of atomic space; it is perhaps unsurprising that they mediate such a great number of diverse biological processes. The incredibly large numbers of possible permutations,<sup>10</sup> range of monosaccharide building blocks, and size range of the glycans make finding a single analytical approach capable of unambiguously discerning all stereochemical information highly challenging. This is reflected within the glycobiology/glycochemistry community where multiple analytical techniques are normally combined. Fortunately for researchers, biological pathways limit the number of species actually observed to a cohort of common structural motifs. This knowledge has been exploited in the analysis of complex mixtures of animal *N*-glycans, carbohydrates that are commonly found to be linked to asparagine residues in proteins within a defined consensus sequence N-X-S/T, whose biosynthetic pathways and resulting structures are relatively well known. Also, these assemblies all expand from a core Man<sub>3</sub> pentasaccharide structure. These *N*-glycans can now be profiled even in a high-throughput manner.<sup>6,11–15</sup> Although very important for modern biotechnology, *N*-glycan and to some extent *O*-glycan analysis remains a highly specialized field which is not applicable to many other glycoconjugates, the structures of which remain difficult to elucidate. For example, there has been recent growth in bacterial glycomics since their discovery. However, much of the field is limited, as the biological glycosylation pathways are not fully understood and they contain a much larger pool of diverse monosaccharide building blocks compared to human systems.<sup>16</sup> As a result, many glycan structures are unknown or only partially known. Complete structure elucidation is extremely challenging with current analytical approaches;



**Figure 2.** Overview of the analytical techniques and some common sample preparation steps to analyze isolated glycans (and glycoconjugates). Adjoining hexagon tiles indicate that these techniques may be combined, although for clarity, not every single combination is included (such as direct MS/MS or direct LC of isolated glycans). Black hexagons are sample preparation steps. Blue hexagons are solution-phase and orange hexagons are gas-phase analytical techniques. Hexagons with dotted outlines typically provide general glycan motif information with no precise atomistic information; dashed outlines typically provide regiochemistry and connectivity but not stereochemistry; solid outlines can provide the full complement of connectivity, regiochemistry, and stereochemistry. Analytical techniques with a solid yellow outline may be used as a separation technique in combination with other approaches and/or used as a diagnostic tool where the separation time under a standardized set of conditions and instrumentation can be compared to databases of known standards. Therefore, structural information they provide depends on the availability and quality of known standards. Interpretation can also be facilitated by knowledge of biosynthetic pathways of the glycan system being studied.

therefore, most effort in the area focuses on characterizing the enzymatic pathways as a means to characterize structures.<sup>16</sup> Although, this assumes pathways are highly conserved which may not be the case.

## ■ STRUCTURE ELUCIDATION: ANALYTICAL APPROACHES

Often combinations of a diverse variety of methodologies and analytical techniques have been used to elucidate structural information on glycans and their conjugates. These include (micro)arrays, liquid chromatography (LC), capillary electrophoresis (CE), nuclear magnetic resonance (NMR), and MS.

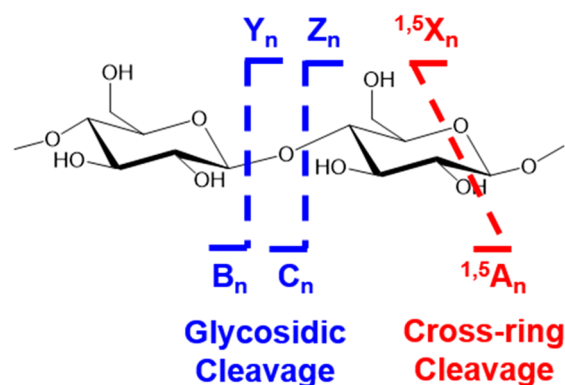
These approaches usually offer different levels of structural information and often incomplete pictures of the glycans being studied. Figure 2 presents an overview of sample-preparation methods and analytical methods that are often combined in different workflows. Classically, glycans are tagged at their reducing terminus with a fluorescent tag and analyzed by (U/H)PLC. The retention times can be standardized to a reference ladder (glucose units, GU) and these GU compared to a standard library of known glycans. This provides non-direct structural information. MS and MS/MS may be combined with LC to provide an alternative detection technique enabling compositions to be discerned directly,

with MS/MS potentially also providing connectivity information. Specific glycosidases can be employed to selectively trim the ends of glycan branches providing indirect structural information. Glycan samples can be perderivatized to improve retention, deduce regiochemistry from MS/MS spectra and improve ionization, among other benefits. Either way, MS(/MS) is typically blind to stereochemical information. Alternatively, complex glycans can be completely hydrolyzed to their monosaccharide components (also potentially combined with prior perderivatization to facilitate regiochemical assignments), and retention of these units can be compared to monosaccharide libraries. This provides information on the composition of monosaccharides present, but will lose much connectivity information. Alternatively, glycans can be directly analyzed by NMR, which can provide a full complement of structural information although this frequently requires greater than biological amounts of samples, is time consuming, and can still miss information especially with no prior knowledge of the sample structure. Overall, there is no consensus approach to identify a glycan, and most approaches are dictated by the glycan “family” (e.g., *N/O*-glycans, polysaccharides, glycolipids, etc.). As a result, multiple strategies are often used in the same glycomics experiment, improving confidence in structural assignments. Some of the more prominent techniques, with a special focus on more recent hybridized-MS approaches, are discussed in the subsections below, and the structural information that can be obtained is discussed in greater detail. It should be noted that the use of LC, and array-based technologies and lectins will not be discussed in great detail here, as they have been recently extensively reviewed in their own right.<sup>17–22</sup> (U/HPLC have been at the forefront of analytical techniques used to separate, isolate and, when standards are available, characterize carbohydrates. Typically, reducing-end tagged glycans retention times are converted to “glucose units” (GU) in reference to the dextran standard, reducing day-to-day or system variations.<sup>23,24</sup> These GU can then be compared to reference libraries. Monitoring shifts in GU upon action of specific exoglycosidases provides additional indirect information.<sup>11</sup> Even though LC does not provide direct structural information, these methods have been incredibly important for the glycomics community, especially *N*-glycans.<sup>6,23–25</sup> Certainly, any strategy that seeks *de novo* identification of the complex sequences within carbohydrates will undoubtedly need pre-separation of complex biological material prior to reproducible analyses.

Classically, arraying extensive libraries of glycans and screening them with fluorescently tagged lectins whose binding motifs have been characterized can generate vast amounts of structural information rapidly. These experiments can suggest the presence of certain motifs within the arrayed glycans that can be collaborated in conjunction with exoglycosidases treatment (i.e., is binding lost to a certain motif after a glycan has been treated with specific exoglycosidase(s)). These approaches do not always provide direct fine structural information, and promiscuity in lectin binding can lead to possible false assignments. Also, exoglycosidases do not exist for every natural glycosidic linkage. They can also be challenging for identifying unusual glycans that may be encountered in bacterial or plant analysis. Similarly, enrichment of targeted glycans with lectin affinity methods downstream of other technologies provides clues on motifs

present, which can increase the confidence in identification upstream.

**Mass Spectrometry.** Over the past few decades, advancements in MS instrumentation including detectors, dissociation techniques, and mass analyzers have seen application of MS within the glycomics community increase significantly. Low amounts (fmol) of glycans can be analyzed rapidly, sometimes with extraordinary levels of resolving power. Combined with knowledge of biological pathways, MS profiles of glycans facilitate rapid characterization of the structural “families” (possible isomers still complicate elucidation by MS alone), potentially in a quantitative manner. Tandem mass spectrometry (MS/MS) is particularly useful to dissociate glycans into smaller fragments that can reveal structural motifs directly. Glycans dissociate either across the glycosidic bond (forming B-, Z-, C-, and Y-ions) or across the monosaccharide ring (A- and X-ions) (Figure 3).<sup>26</sup> Glycosidic dissociation pathways



**Figure 3.** Domon–Costello nomenclature for common fragments generated by gas-phase dissociation techniques.<sup>26</sup>

typically reveal broad sequence and composition information, whereas cross-ring dissociation can reveal regiochemical information.<sup>27</sup> Usually, neither type of product ion is diagnostic for anomeric differences or leads to stereochemical assignment of the monosaccharide building block. Commercial instrumentation exists in a variety of different “layouts” of ionization techniques, dissociation methods, and mass analyzers. As a result, instrumentation that manipulates glycan ions can be chosen that best suits an experimental pathway required.

Oligosaccharides typically vary from being neutral to highly acidic and therefore are studied as both positively and negatively charged species. The nature of the adduct formed upon ionization can be influenced by doping with salts (sodium, lithium, nitrate, etc.). Any attached conjugates or derivatization also influences the preferred adduct (e.g., glycopeptides readily protonate). Consequently, there is significant variation in ionization efficiency between oligosaccharides that can make quantification challenging for MS, although addressable. For example, permethylation can be used to sequester these differentially ionizable groups, thereby minimizing this variation and improving ionization efficiency and therefore sensitivity.<sup>28</sup> Permethylation also facilitates identification of branching patterns and stabilizes labile sialic acids that often dissociate during matrix-assisted laser desorption/ionization (MALDI) because of ion heating. An alternative inventive strategy for sialic acid stabilization was

found by selective chemical reaction of the  $\alpha$ 2,3 and  $\alpha$ 2,6 regioisomers carboxylate generating a  $m/z$  shift.<sup>29</sup>

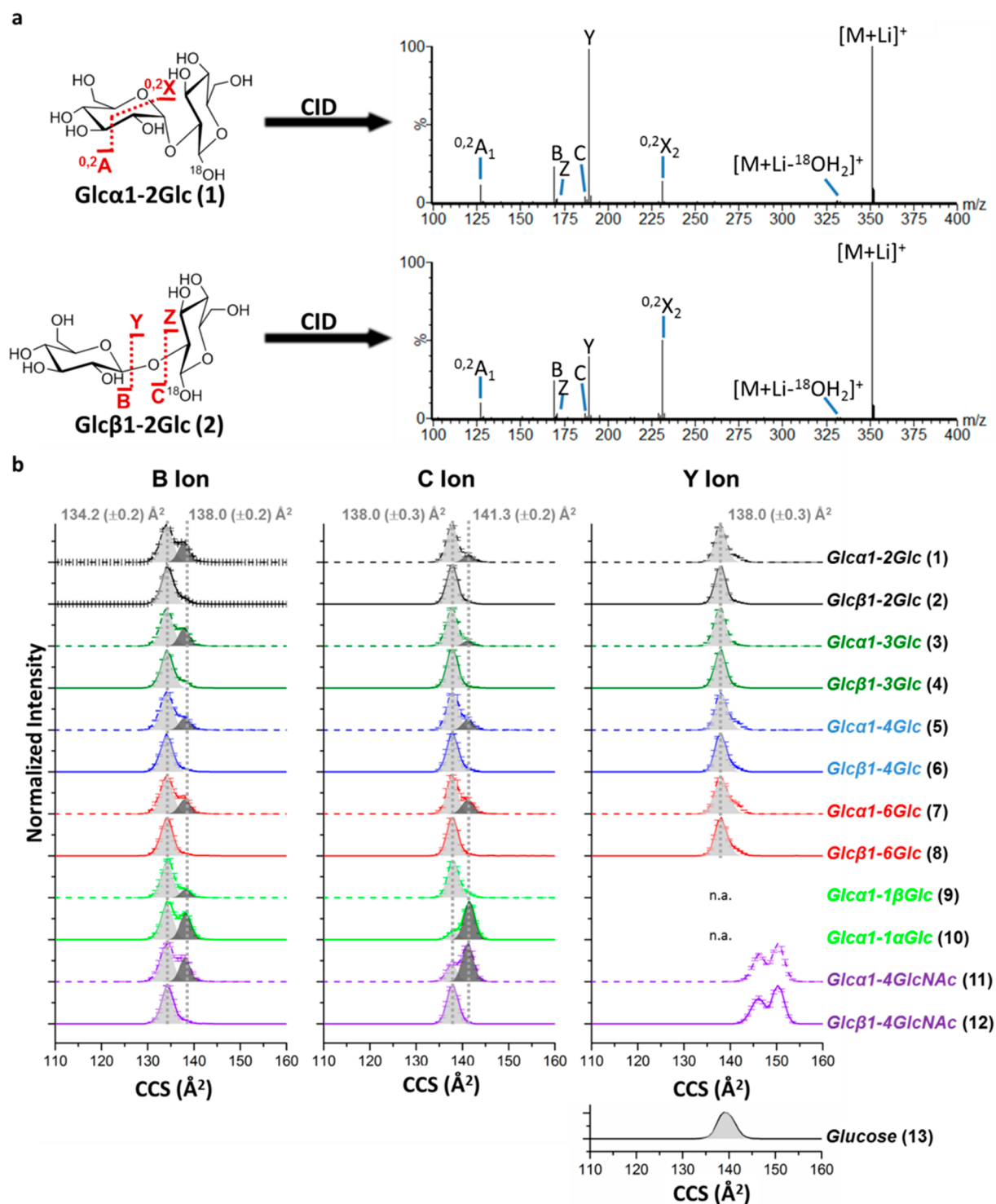
Electrospray ionization (ESI) can readily be coupled to chromatographic separation as they both deal with solution-phase samples; the time scales of MS and LC complement one another, being of the order of microseconds and seconds, respectively. LC facilitates separation (and purification) of the heterogeneous glycan mixtures prior to MS. This reduces signal suppression of analytes by salts, other additives, and importantly other analytes. Spectra then consist of fewer ions, making analysis simpler and consequently reducing the likelihood of incorrect sequencing. An especially prominent example for glycans is the separation of isomeric forms that are indistinguishable by MS alone.<sup>30</sup> Without LC separation, any MS/MS spectra will be chimeric and will likely lead to misidentification or will miss important information. As a result, coupling LC and MS is highly beneficial for glycan analysis. A great advantage of using gas phase-based methods for structural analysis is the opportunity to dissociate large complex structures to more tangible fragments that are amenable to analysis by MS. Numerous dissociation techniques have been employed in an attempt to maximize the amount of structural information that can be garnered. Collision-induced dissociation (CID) is by far the most prominent technique,<sup>25,31–37</sup> but electron-based dissociation techniques (electron capture dissociation, ECD; electron detachment dissociation, EDD; electron transfer dissociation, ETD; etc.),<sup>38–42</sup> infrared multiple photon dissociation (IRMPD),<sup>43</sup> ultraviolet photodissociation (UVPD),<sup>44</sup> and high-energy CID (HCD)<sup>45</sup> have also been examined as well as combinatorial strategies like radical-directed dissociation (RDD, combines UVPD and CID),<sup>46</sup> ETciD, and EThcD.<sup>47</sup> The choice of technique greatly affects the fragmentation pathways and thus information gained. As a generalization, CID and IRMPD often lead to fragmentation of the more labile bonds, which are normally glycosidic linkages, whereas the high-energy CID, electron-based dissociation, and UVPD lead to greater cross-ring fragmentation (Figure 3).<sup>43,46</sup> Combinatorial fragmentation approaches such as ETciD, EThcD, and RDD typically display advantages between the two complementary techniques.<sup>46</sup> In reality, the dissociation pathways and resulting fragment ions are influenced by numerous factors, many associated with the analyte ion in question, namely the chemistry of the glycan and its conjugate, neutralist charge state,<sup>38,39</sup> any adduct,<sup>43,48</sup> and any derivatization. Glycosidic linkages of sialic acids that decorate the termini of many glycans, readily break by CID (and in-source dissociation) generating little useful information. Similarly, losses of other modifications such as sulfation and phosphorylation tend to be the most favorable pathways and make identifying the residues and positions they modify challenging. Glycans can be chemically derivatized to stabilize some of these groups.<sup>49</sup> When dealing with glycopeptides, CID favorably breaks apart the glycan first, whereas the peptide portion remains primarily intact. As a result, the site of glycosylation within the peptide sequence can be difficult to identify. In comparison, electron-based dissociation methods preferentially fragment the peptide backbone over the neutral glycan.<sup>50</sup> Combining these strategies generates complementary information, increasing the confidence and level of structure assignment. Mass spectra are sensitive to differences in precursor structure such that diagnostic product ions can be generated even from isomeric precursors.<sup>25,51–55</sup> Ashwood et

al. recently developed a workflow that resulted in product ions diagnostic for sialylation linkage ( $\alpha$ 2,3 versus  $\alpha$ 2,6) and arm position for native reduced *N*- and *O*-glycans.<sup>54</sup> Additionally, the ratio of certain fragment ions will likely differ between isomers, facilitating differentiation.<sup>56,57</sup> In fact, Pett et al. recently discovered that the intensities of specific product ions generated by HCD of glycopeptides differed between  $\alpha$ 2,3 versus  $\alpha$ 2,6-linked sialic acids.<sup>57</sup> Because of the number of factors that dictate how carbohydrates fragment, the pathways and thus spectra are difficult to predict *a priori* and, due to the numerous different fragment ions generated (many of which are isomeric or isobaric), interpretation of glycan MS/MS spectra are often difficult. Also, precise knowledge of the structures formed after dissociation are not well understood although several groups have postulated structures.<sup>37,48,53,58</sup> Computational tools capable of interpreting tandem mass spectra lag behind those developed, e.g., for proteomics, and are still being developed and improved.<sup>59,60</sup>

Caution must be taken when interpreting MS/MS of underivatized carbohydrates, as fucose and sialic acid residues have been observed to “rearrange” to different positions during CID/HCD,<sup>61</sup> significantly increasing the likelihood of false identification. However, these rearrangement reactions do not occur for per-derivatized glycans and seem to be reduced by studying metal adducts compared to protonated species. Recently, evidence has been presented that these rearrangements may occur during MS analysis in the absence of significant activation, so sample preparation and instrumentation conditions need to be carefully selected to minimize these unwanted rearrangements.<sup>62</sup> As the variety of glycans analyzed increases, undoubtedly more such positional rearrangements will be found for other monosaccharides to complicate analytical work flows.

A major restriction of MS is typically “blindness” to isomerism, although recently *in situ* additives have been discovered that can be used to discern any aldo- or ketohexose or pentose—including its enantiomeric form—using only MS.<sup>63,64</sup> As discussed, MS/MS can be sensitive to isomeric differences especially positional and regioisomerism, although it is less sensitive to stereochemical variations. MS/MS thus usually only reveals snippets of structural information. As a result, alternative analytical techniques that produce complementary information are still being investigated. Nonetheless, these “snippets” of MS/MS-derived structural information may still be highly valuable, especially to restrict potential candidates and thus facilitate identification when used in combination with other analytical techniques such as NMR spectroscopy.

**Ion-Mobility and Mass Spectrometry (IM-MS).** Over the past decade, investigations into the applications of ion-mobility spectrometry (IMS) for glycan analysis have increased.<sup>65,66</sup> Much of this derives from the commercialization of instrumentation that increased its availability compared to early experiments that used “home-built” IMS instrumentation. Most IMS techniques separate ions based on their mobility through an inert drift gas under the influence of a weak electric field. This mobility can be converted to a rotationally averaged collision cross section (CCS,  $\Omega$ ) that is an intrinsic property of that ion under the experimental conditions used. Importantly, these CCS values may differ based on the conformation of the glycan analyte, possibly enabling separation of isomers. As a result, IMS is frequently hybridized with MS (IMS time scale milliseconds versus



**Figure 4.** Comparison of tandem mass spectra associated with disaccharide epimers *Glcα1-2Glc* 1 and *Glcβ1-2Glc* 2 with ion-mobility (IM) arrival time distributions (ATDs) of selected glycosidic product ions. (a) Collision-induced dissociation (CID) of isomeric diglucosides generate similar tandem mass spectra especially for the  $\alpha/\beta$ -anomers. Disaccharides were selectively <sup>18</sup>O-labeled at C1 of the reducing glycan by dissolution in <sup>18</sup>O-water, allowing mass discrimination of the C/Y- and B/Z-product ion pairs. (b) Ion mobility mass spectrometry generates ATDs associated with B- and C-product ions generated by CID. Significantly, the  $\alpha$ -glucose terminating precursor display more than one species compared to their  $\beta$ -glucose counterparts. It is important to note that Domon–Costello nomenclature<sup>26</sup> for glycosidic product ions does not extend to non-reducing carbohydrates; hence, B- and C-ions for 9 and 10 correspond to  $m/z$  169 and 187, respectively, despite it being highly likely the C-ion is formed by a Y-ion type mechanism. Displayed ATDs are the sum of three normalized and calibrated replicates. Intensity error bars are shown for all spectra whereas CCS error bars are only displayed for the B-ions of 1–2 for clarity, although the standard deviation is  $<0.3 \text{ \AA}^2$  for all shown ATDs. n.a. = not applicable. Reproduced with permission from ref 53. Copyright 2017 American Chemical Society.

microseconds for MS), readily providing orthogonal information in the same experiment.

Additionally, separation of isomeric glycans prior to MS/MS facilitates identification of these glycoforms that may otherwise be missed or incorrectly assigned.<sup>31,35</sup> Experimental CCS values can be compared to database values<sup>67,68</sup> or theoretical computational derived CCSs providing an insight into potential gas-phase conformations.<sup>53,69</sup> IMS can therefore be used to separate isomeric species and/or to provide structural information. Several different IMS techniques exist such as drift tube IMS (DTIMS), traveling-wave IMS (TWIMS), and field-asymmetric IMS (FAIMS). The details and benefits of these techniques are discussed elsewhere.<sup>70</sup> A multitude of papers have explored separation of isomeric glycans and their conjugates by IMS including structures that differ by regiochemistry, glycosidic bond stereochemistry and monomer stereochemistry.<sup>53,69,71–73</sup> Because the resolving power of IMS is fairly poor compared to other separation techniques, much effort has been made into methodologies to improve separation, especially since isomeric glycans may only differ by the stereochemistry at a single position, which may not significantly alter the overall conformation. Given that IMS separates ions by their rotationally averaged CCS-to-charge ratio, the charge state,<sup>74,75</sup> polarity, identity of the adduct,<sup>76–78</sup> ion-pair coupling,<sup>79</sup> and derivatization<sup>80</sup> have all been studied. However, there is likely no universal optimal approach to improve IMS separation. Therefore, it is beneficial to systematically test the optimal set of conditions to separate the glycans/glycoconjugates of interest. Improving IMS resolving power by increasing the drift length, including using a cyclic drift cell, has been shown previously, however, comes at the cost of sensitivity, which will be hugely detrimental to IMS's ability to deal with biological matrices.<sup>81</sup> Nevertheless, these technologies are still being explored. In this context, it is interesting to note a promising recent approach termed “Structures for Lossless Ion Manipulations” (SLIM)-IMS that has been developed to have long serpentine path lengths (13 m) which have markedly improved resolution without previously observed large losses in sensitivity.<sup>82</sup> More recently, SLIM serpentine ultralong path with extended routing (SUPER) IM-MS with a 112.5 m path length allowed the trapping of an increased amount of ions (2–3 orders of magnitude) compared to conventional approaches and the much easier separation of a variety of di- and trisaccharides and a variety of isomeric human milk oligosaccharides.<sup>83</sup> Recently, Ujma et al. also reported a new instrument where ions could be injected into a cyclic IMS separator and separated over multiple cycles. Additionally, ions could be ejected back into a “pre-store” prior to the cyclic IMS separator, collisionally dissociated, and re-injected back into the cyclic IMS cell, enabling tandem IMS (IMS/IMS) experiments. Using this instrument, they showed excellent separation of anomers and ring-open forms of a series of three pentasaccharides, including separations of mixtures of these oligosaccharides.<sup>84</sup>

As mentioned previously, CCSs can be derived from IMS measurements and used directly to differentiate structural features. CCSs for specific precursor ions have been stored in databases, such as GlycoMob,<sup>67</sup> and can act as an additional identifying parameter along with MS(/MS), LC retention times, etc.<sup>68</sup> While these databases will be helpful for the identification of glycans formed from well understood pathways for which we have standards, they do not help in the characterization of unknown species. Alternatively

performing IMS on product ions generated by dissociation of oligosaccharide precursors has several advantages: there will be fewer potential isomeric combinations for smaller analyte fragments, and consequently the need for standards is dramatically reduced; fragment-based approaches also benefit from the abundance of common glycan motifs, that arise due to economic use of biosynthetic machineries. In a recent example of a fragment-based approach, the Pagel group demonstrated that *N*-glycans terminating in biologically relevant  $\alpha$ 2,3-Neu5Ac or  $\alpha$  2,6-Neu5Ac regioisomers could be differentiated on the basis of the mobility of a terminating B<sub>3</sub>-trisaccharide fragment.<sup>85</sup> Also, *N*-glycoform structural features could be discerned from the CCS of product ion.<sup>31–33</sup> Both et al. were able to separate and identify terminal hexose and *N*-acetylhexosamine monosaccharide fragments generated by CID of glycopeptides and free-reducing disaccharides on the basis of the drift time of the terminal fragment ion.<sup>73</sup> Crucially, these drift times were independent of the initial glycoconjugate structure. Subsequently, they determined that in fact these terminal B- and C-monosaccharide product ions retained memory of the anomeric configuration of the glycosidic bond from which it was derived, as well as the stereochemistry of the glycan building block (Figure 4).<sup>53</sup>

These studies suggest an attractive carbohydrate sequencing strategy: multistage fragmentation (MS<sup>*n*</sup>) combined with a database of  $\alpha/\beta$ -monosaccharide fragment CCSs could enable a significant amount of carbohydrate structures to be annotated rapidly. At the moment, there are some limitations that need to be addressed to make such an approach fully viable. First, IMS resolving power on commercial instrumentation is currently incapable of separating mixtures of  $\alpha/\beta$ -hexose and *N*-acetylhexosamine product ions, so analysis of branched glycans is more challenging. Second, the dissociation energy can affect the arrival time distribution of product ions (with high energies possibly inducing a secondary reaction pathway), which will complicate analysis and identification. Finally, for larger glycan analytes, it is less likely that dissociation will lead to the formation of mono-/disaccharide terminal fragment ions. Nevertheless, this fragment-based methodology shows promise as a possible “sequencing tool”, particularly as IMS resolution continues to improve. Increasing knowledge of glycan dissociation pathways can undoubtedly be exploited to facilitate approaches like this one.

**Gas-Phase Infrared Spectroscopy and MS.** Application of gas-phase IR action spectroscopy with MS for carbohydrate analysis is a fairly new field of research. Isolated ions are typically irradiated with a high fluence IR beam to induce multiple photon absorption (IRMPD) overcoming the barrier to dissociation. since IR photons energies are typically lower than the dissociation threshold. The efficiency of light absorption depends on the frequency of the incident light as well as the analyte's atomic structure(s). With tunable IR lasers, a spectral range can be scanned and a gas-phase vibrational spectrum of an *m/z* selected ion can be constructed. Alternatively, a weak complex between the target analyte and a so-called “messenger” (e.g., He and H<sub>2</sub> that do not absorb IR, bind weakly without imparting an effect on the analyte, and produce an easily measurable *m/z* shift) can be produced and the efficiency of its dissociation measured at various IR wavelengths.<sup>86,87</sup> Care must be taken when interpreting these spectra in case the messenger(s) differentially interact with the target analyte ion in such a way that it

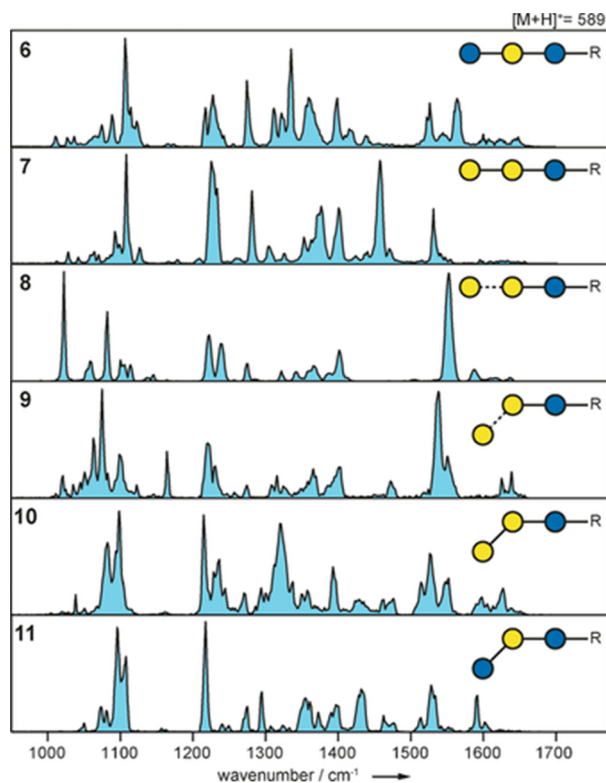
perturbs its structure.<sup>88</sup> Features from experimental vibrational spectra can be compared with theoretical vibrational spectra to derive energy-minimized gas-phase simulated structures and provide insight into potential 3D conformations.

One of the pioneering experiments for IR-MS involved plotting the major product ion intensities generated by IRMPD of several mass-selected lithiated diglucosides that differed by the regiochemistry and anomeric configuration of the glycosidic bond against the wavelength of FELIX<sup>89</sup> (scanned between 7 and 11  $\mu\text{m}$ ). The reproducible 2D plots were unique for each parent, highlighting that the IRMPD was highly sensitive to subtle structural variations in the ionized species (despite their recorded IR spectra being poorly resolved and fairly similar).<sup>90</sup> A similar approach showed that lithiated diglucosides<sup>91</sup> and  $\alpha$ - and  $\beta$ -*O*-methyl-glucopyranoside anomers<sup>92</sup> could also be distinguished using more accessible tunable CO<sub>2</sub> lasers scanning between 9.2 and 10.8  $\mu\text{m}$ . Many of these examples focused on differences between tandem mass spectra rather than IR action spectra. Brown et al. performed IRMPD on all eight unmodified deprotonated D-aldohexoses and again noted differences between tandem mass spectra.<sup>93</sup> However, a strong absorption in the 1700  $\text{cm}^{-1}$  region was observed for all species. This signal was compared with theoretically generated vibrational spectra and corresponded to a carbonyl signal, highlighting these structures existed as their linear rather than cyclic forms in the gas phase. This observation would have been impossible to directly monitor by MS alone.<sup>93</sup> Rabus et al. also found ring-open structures for the C<sub>1</sub>-ion derived from deprotonated lactose. However, they likely form from a ring-opening reaction of the closed-ring structure (whose anomeric configuration was retained) since the barrier for conversion is relatively small ( $\sim 51 \text{ kJ mol}^{-1}$ ) and is entropically favored. They also propose dissociation pathways, structures, and conformations of other fragment ions (Z<sub>1</sub>-ion and cross-rings) by combining MS/MS, IR spectroscopy, and theoretical simulations.<sup>94</sup> In comparison, Gray et al. and Schindler et al. found that C-ions generated by CID of lithiated diglucosides retained the stereochemistry of the parent's glycosidic linkage at the C1 position, irrespective of the regiochemistry of the glycosidic bond or identity of the adjacent reducing residue.<sup>53,95</sup> These experiments highlight the differences in fragmentation chemistry depending on the polarity and identity of the ion adduct where the anomeric configuration of the glycosidic bond is retained under certain circumstances and lost in others. Contreras et al. performed IRMPD on lithiated  $\alpha$ -/ $\beta$ -*O*-methyl-*N*-acetylglucosamine and  $\alpha$ -/ $\beta$ -*O*-methyl-*N*-acetylgalactosamine and found unique features or peak shifting between the stereoisomers/anomers, enabling all to be differentiated. Energy-minimized conformations and corresponding theoretical IR spectra were generated that showed good correlation with experimental measurements, providing a snapshot of potentially viable gas-phase conformations which can be used as evidence to explain observable differences between infrared spectra.<sup>96</sup> Isobaric sulfated and phosphorylated hexosamines,<sup>97</sup> sulfated hexosamines<sup>98</sup> and GAG fragments<sup>99</sup> that differ in the position of sulfation, epimeric *N*-acetylhexosamines,<sup>100</sup> epimeric hexosamines,<sup>101</sup> epimeric hexuronic acids,<sup>102</sup> regioisomeric sialic acids,<sup>103</sup> and regio-/stereoisomeric disaccharides<sup>95</sup> have all been demonstrated to be distinguishable using IR action spectra as unique fingerprints. This is a milestone in our analytical capabilities, as many of these species are inseparable (or poorly separated) by LC, MS(/MS), or conventional IMS.

However, many of these spectra display poor resolution despite only consisting of mono-/disaccharides, possibly masking additional highly diagnostic features. Poor resolution has been proposed to occur due to two factors: the conformational flexibility of gas-phase carbohydrate ions at room temperature, and thermal activation of ions upon sequential absorption of photons during IRMPD.<sup>104</sup> Consequently, cold-ion IR-MS approaches have recently been applied to glycan analysis. Khanal et al. studied isomeric GAG disaccharide fragments (three chondroitin sulfates and two heparan sulfates) on a "home-built" instrument that combined a DTIMS, a cryogenic ion trap, a time-of-flight mass analyzer, and an IR laser. The drift tube could be operated in tandem IMS-IMS mode, where ions with a specific drift time could be selectively gated. Mobility- and *m/z*-selected ions could be injected into a cryogenic ion trap maintained at 13 K. In this trap, carbohydrate ions would be cooled through collisions with H<sub>2</sub> buffer gas and, upon cooling, would form weakly bound H<sub>2</sub>-analyte messenger complexes that could be probed with a tunable IR laser (as discussed previously).<sup>105</sup> The IR action spectra of these cryogenically cooled fragments were extremely well resolved, providing diagnostic fingerprint spectra for all species.  $\Delta\text{UA-(6S)GlcNAc}$  displayed two conformations within the arrival time distribution. Using this instrument setup, both could be isolated prior to IR irradiation, and separate IR spectra could be recorded for the two conformers. These conformers produced unique spectra, highlighting the wealth of information that can be produced from these hybrid MS experiments. This same strategy was recently applied to isomeric pairs of milk oligosaccharides, LNnT/LNT, 2'-FL/3-FL, and LNDFH I/LNDFH II, tagged with a N<sub>2</sub> messenger. Many of these are difficult to separate by LC but were readily distinguished by their vibrational spectra.<sup>106</sup> The Rizzo group also performed IMS prior to cryogenic infrared spectroscopy (using N<sub>2</sub> as the messenger tag) to separate and selectively probe glycan mixtures. Rather than using DTIMS (2 m length) which displays poorer IMS resolving powers, they make use of TWIMS on a cyclic SLIM (13 m path over seven cycles) device which displays much greater resolving power. Under these conditions ions whose CCS differed by roughly 0.5% were resolvable. However, for each cycle they observed an ion loss of up to 3%. Gal $\beta$ 1,4GlcNAc displays a single peak by DTIMS, whereas two are observed using SLIM. It was postulated that these two species are mutarotamer isomers Gal $\beta$ 1,4GlcNAc $\alpha$  and Gal $\beta$ 1,4GlcNAc $\beta$ , primarily as every glycan they studied displayed two features in the SLIM IMS spectra. These could be separately probed and generated unique IR spectra. Mixtures of isomeric disaccharides and tetrasaccharides were separable by their SLIM device that could then be interrogated by IR.<sup>107</sup> Recently they also showed, by combined selective <sup>18</sup>O-labeling of the C1-OH group, cryogenic IR-IR double resonance spectroscopy, and quantum mechanical computations, that for protonated glucosamine three conformers were potentially present: chair <sup>1</sup>C<sub>4</sub>  $\alpha$ -anomer, chair <sup>4</sup>C<sub>1</sub>  $\alpha$ -anomer, and chair <sup>4</sup>C<sub>1</sub>  $\beta$ -anomer. The  $\alpha/\beta$  ratio of these anomers was found to be  $\sim 1.8:1$ , close to the solution-phase measurements of 1.7:1 (NMR and polarimetry). These measurements suggest the barrier to mutarotation is too high in the gas phase.<sup>108</sup> Finally, Mucha et al. employed a different approach avoiding the requirement of a messenger.<sup>109</sup> Their approach required doping of ions into helium droplets consisting of an average size of  $\sim 10^5$  atoms cooling the ions to 0.37 K. Irradiation with a high-energy IR



laser and absorption of multiple resonant photons caused the ion to be ejected from the droplet in a mechanism yet to be defined. The ejection yield, rather than a fragmentation yield, was measured as a function of IR wavelength to generate action IR spectra. This facilitated differentiation of protonated isomeric mono- and disaccharides, modified at the reducing terminal with an aminopentyl linker, providing remarkable resolution and informational content in the IR space (Figure 5). Un-derivatized isomeric biologically relevant milk oligo-



**Figure 5.** Gas-phase IR spectra associated with a series of isomeric synthetic trisaccharides that differ only by regiochemistry, anomeric configuration or monomer composition. R corresponds to an aminopentyl moiety. Structures are depicted using SNFG nomenclature,<sup>8</sup> where solid and dashed lines correspond to  $\beta$ - and  $\alpha$ -configurations, respectively, and the angle of the connector defines regiochemistry (in this example, horizontal lines are linked at the 4-position and the angled the 3-position). Reproduced with permission from ref 109. Copyright 2017 Wiley-VCH Verlag GmbH & Co.

saccharides LNnT and LNT and blood group antigens Le<sup>b</sup> and Le<sup>y</sup> could be readily identified by unique features within their spectra.

Current IR-MS approaches typically do not fit into the timeline of online LC, since it takes a long time to scan through the IR region and enough ions must be accumulated at each scan point to record reliable representative action IR spectra. However, the first example of online HPLC-MS IRMPD was recently published for mono-/disaccharide anomers, where they significantly reduced the flow rate (from 400 to 100  $\mu$ L/min) to facilitate acquisition of IRMPD spectra. Acquisition times were also reduced from  $\sim$ 10 to  $\sim$ 6 min.<sup>110</sup>

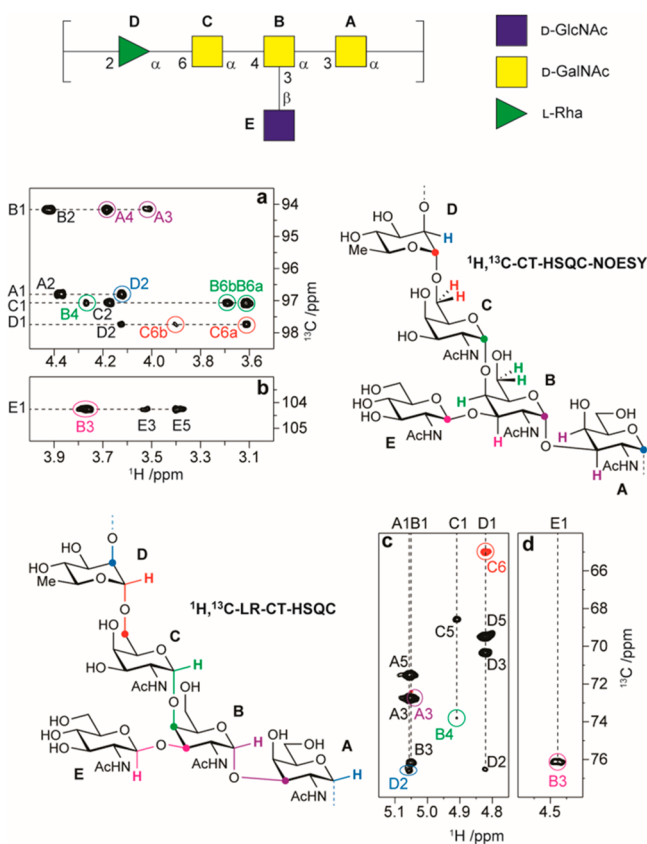
**NMR Spectroscopy.** Like any small organic molecule, nucleic acid, or protein, carbohydrates are readily detected by solution-state  $^1\text{H}$  NMR spectroscopy due to the very high

natural abundance (99.985%) and high frequency of the nucleus. NMR spectroscopy has the advantage over MS that each NMR active nucleus in a molecule gives rise to a resonance, with a characteristic chemical shift being dependent on the structure of the molecule. Elucidation of the complete primary structure of a glycan molecule can be carried out by NMR spectroscopy, in particular, if other NMR active nuclei such as  $^{13}\text{C}$ ,  $^{15}\text{N}$ , and/or  $^{31}\text{P}$  are utilized, since there exists a plethora of multidimensional techniques to unravel their connectivity. For determination of the absolute configuration of sugar residues, as well as identification of the sugar *per se*, 2D NMR of diastereomeric compounds obtained by derivatization with optically active 2-butanol, also as mixtures of different sugars, is readily performed.<sup>111</sup>

The general limitation of NMR spectroscopy is that it is insensitive compared to MS, and in many cases milligram amounts of material are used for NMR-based structural analysis. However, sub-milligram quantities of oligosaccharides of unknown structure have been elucidated where their  $^1\text{H}$  and  $^{13}\text{C}$  resonances were assigned using standard equipment with cryogenically cooled NMR probes.<sup>112</sup> Glycans are also present in what is referred to as natural products, e.g., components of saponins which carry one or more sugars in their structure and an aglycone entity. In natural product chemistry the amount of material may be highly limited and specialized NMR microprobes (5  $\mu$ L of solvent) are commonly used. It should thus be possible to analyze nanomole amounts, with present-day dedicated cryogenically cooled NMR probes.<sup>113</sup> The capabilities of NMR spectroscopy in addition to the application of cryogenically cooled NMR probes, will be improved by developing linear prediction techniques, non-uniform sampling, and  $^1\text{H}$ -decoupling of  $^1\text{H}$ -detected spectra, i.e., pure shift techniques.

Even if the sensitivity becomes sufficient to detect  $^1\text{H}$  NMR resonances, glycan structural elucidation may still be hampered due to lack of  $^{13}\text{C}$  and  $^{15}\text{N}$  spectral information, since the natural abundance of these nuclei is  $\sim$ 1% and  $<$ 1%, respectively;  $^{31}\text{P}$ , on the other hand, has a natural abundance of 100%, so when present, does not lead to any major sensitivity limitation. The remedy for the low natural abundance for  $^{13}\text{C}$  and  $^{15}\text{N}$  is to use isotopically enriched samples, in particular, uniform  $^{13}\text{C}$ ,  $^{15}\text{N}$  enrichment for which optimized NMR techniques have been developed and demonstrated for resonance assignment of sugars and sequence establishment between residues for both oligo- and polysaccharides (Figure 6).<sup>114</sup> Notably, it is possible to carry out 3D  $^1\text{H}$ ,  $^{13}\text{C}$ ,  $^{15}\text{N}$  NMR experiments where the carbon nuclei are  $^{13}\text{C}$ -enriched in the polysaccharide material but the  $^{15}\text{N}$  nuclei are still at natural abundance of only 0.37%.<sup>114</sup>

In contrast to nucleic acids and proteins, the spectral dispersion of carbohydrates is still limited, with most  $^1\text{H}$  NMR signals resonating within  $\sim$ 1 ppm and only a few resonances being observed outside of the bulk spectral region, viz., on the one hand those from anomeric protons and on the other those from deoxy functions such as methyl groups of 6-deoxy sugars. The resonance assignment process may thus be cumbersome, but will likely be aided by NMR chemical shift prediction tools such as GODDESS<sup>115</sup> or CASPER.<sup>116</sup> Using the latter, structural elucidation of an oligo- or polysaccharide can be performed solely by NMR without any resonance assignments provided by the user; i.e., only unassigned NMR spectra are supplied. The capability of predicting NMR chemical shifts accurately and rapidly is also highly beneficial when NMR



**Figure 6.** Sequence determination of polysaccharides by NMR. The structure of the repeating unit of the O-antigen polysaccharide of *E. coli* O142 is shown in CFG (Consortium for Functional Glycomics) notation on the top of the figure. Selected regions of the HC-CT-HSQC-NOESY ( $2T = 44$  ms,  $\tau_m = 100$  ms) (a,b) and long-range HC-CT-HSQC ( $2T = 22$  ms and optimized for  $J_{CH} = 20$  Hz) (c,d) spectra of the  $^{13}\text{C}$ -enriched O-antigen polysaccharide of *E. coli* O142, showing intra- and inter-residue correlations from anomeric atoms (the latter are highlighted by colored ovals). The same color nomenclature is used to illustrate the respective inter-residue correlations in the structures located on the right or left side of the respective spectra. Reproduced with permission from ref 114. Copyright 2014 Springer Science+Business Media.

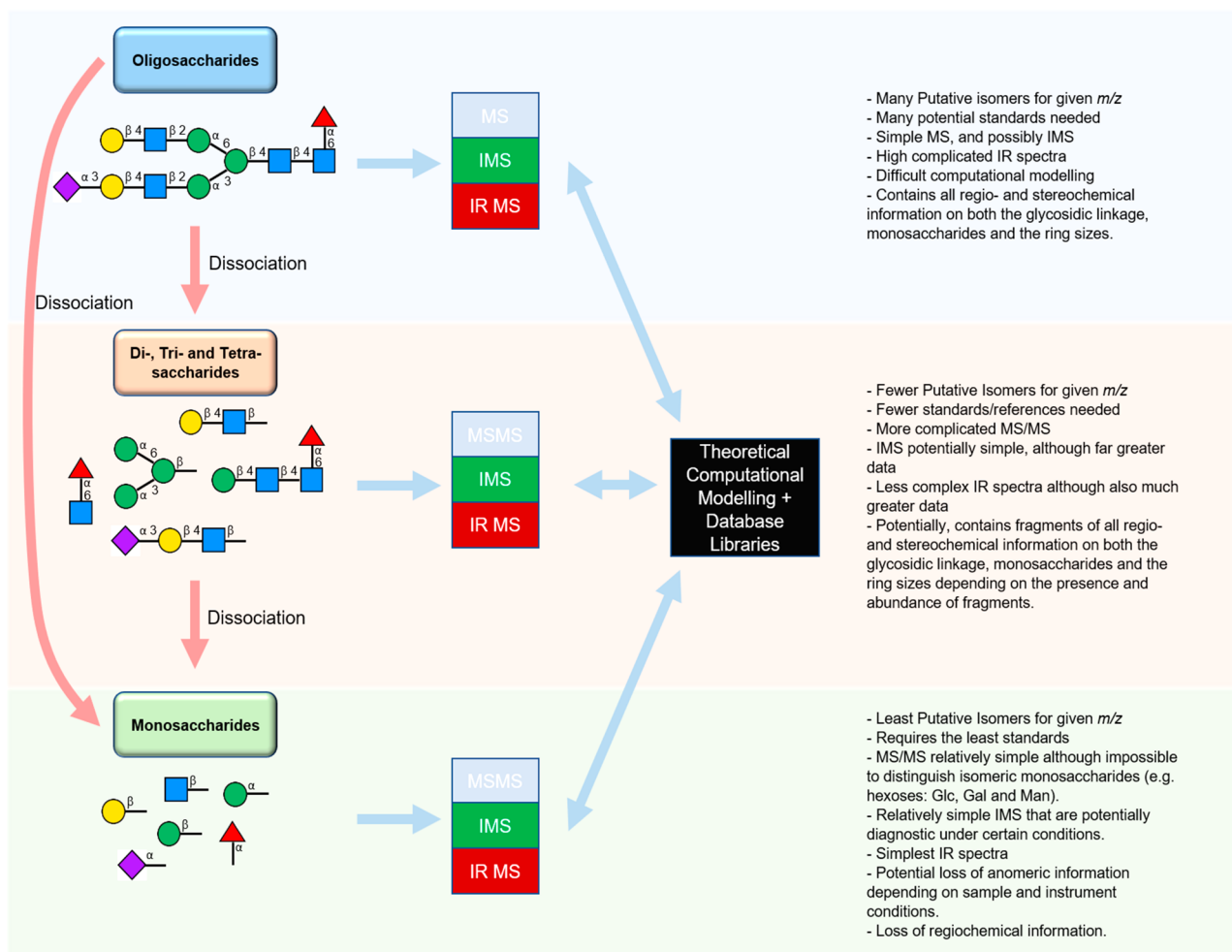
resonance assignments of unknown structures are carried out manually and for confirmation of oligo- and polysaccharides made by synthetic chemical or enzymatic approaches where a linker or spacer entity is often attached at the reducing end of the saccharide.

Devoid of any information to complement structural determination of an oligo- or polysaccharide, NMR identification amounts to a humongous task since there are well over a 100 different monosaccharides described to date.<sup>117</sup> However, if any information on sugar components by other chemical analyses or bioinformatics—including their absolute configuration, anomeric configuration, and linkage position as well as substituents—can be supplied as input to the NMR-based structural elucidation, the task becomes manageable as the number of permutations are significantly reduced. Thus, further developing and streamlining this approach<sup>118</sup> is judged to be highly beneficial to the structural glycochemistry and glycobiology research fields.

## COMPUTATIONAL ASPECTS: INFORMATICS, DATABASES, AND MODELING

As carbohydrate analytical approaches (as well as sample preparation) improve their throughput and become hybridized with complementary instrumentation, the sheer amount of data will rapidly increase. For example, a single tandem mass spectrum of a glycan precursor ion will produce multiple different fragments; within a given MS scan, multiple glycan precursors could be selected for dissociation; and if the sample has undergone HPLC separation, there will be thousands of MS(/MS) spectra. This complexity could increase by more than a magnitude when each MS measurement is hyphenated to IMS or gas-phase IR spectroscopy for the precursors/product ions. As a result, manual interpretation for these larger datasets becomes very challenging. Therefore, suitable informatics and databases are (and will continue to be) crucial for determining the most probable structure of a glycan analyte. A number of diverse informatics tools have been developed that facilitate the interpretation of (U)/HPLC, MS, MS/MS,  $\text{MS}^n$ , and NMR data from a range of different carbohydrate classes. Some of these that specifically focus on structure identification, are listed in Table S1 in the [Supporting Information](#). The LC tools generally assign GU values to separate labeled glycans and compare them to database values.<sup>24,119</sup> Some of these are also integrated with expected GU shifts upon actions of specific exoglycosidases, providing additional indirect structural information.<sup>120</sup> Given that this is a very new field, there are currently no gold-standards yet for glycan bioinformatics, and the field is still continually evolving.<sup>121</sup> Many of these tools (both LC and MS) specialize for *N*-linked glycan or glycopeptide analysis. Some of these bioinformatic tools merely match experimental data points to standard datasets whose structures are known.<sup>24,60,119,120,122–124</sup> Alternatively, knowledge of *N*-glycan biosynthetic pathways also has allowed for theoretical *N*-glycan/glycopeptide structure libraries to be built, whose theoretical MS and MS/MS spectra can be used to help identify experimental MS and MS/MS data.<sup>125</sup> The presence of glycans/glycoconjugates is often facilitated by looking for monosaccharide oxonium ions,<sup>126</sup> diagnostic *m/z* shifts corresponding to neutral losses of glycans<sup>127</sup> and/or identifying the presence of conserved core structures. MS/MS *N*-glycan/glycopeptide approaches can build up a topological tree from *m/z* shifts from this core structure constricted by biosynthetic routes.<sup>127,128</sup> However, these approaches are disadvantageous when attempting to discern the structures of unknown glycans. Other algorithms are more complex and seek to *de novo* characterize glycan structures such as GlycoDeNovo, Glyco-Peakfinder, and Branch-and-bound.<sup>59,129,130</sup> These tools are typically restrictive to a certain class of glycans, facilitating analysis time and reducing the number of candidate structures.<sup>127</sup> Many tools, however, merely provide information on the glycan composition and provide no topological information.<sup>131–135</sup>

There have been multiple databases for a range of native and modified carbohydrates, including *N*-/*O*-linked glycans,<sup>24,136–139</sup> bacterial exopolysaccharides (EPS),<sup>140</sup> and GAGs,<sup>141,142</sup> containing a range of information from the taxonomy/tissue in which the glycan was found and the glycan's retention properties under a specified set of standard conditions and instrumentation and MS(/MS) spectra (also under specified conditions and instrumentation), to name a



**Figure 7.** Assessment of current state-of-art of fragment-based approaches, employing the three gas-phase techniques—mass spectrometry (MS), ion mobility spectrometry (IMS), and gas-phase IR spectroscopy—for various sizes of saccharides after gas-phase dissociation of an oligosaccharide precursor. Oligosaccharide precursors possess the full complement of structural information; however, mass spectrometry cannot discern their connectivity, regiochemistry, or stereochemistry (although potentially Hex/HexNAc compositions) and IR spectra are very complex. IMS is less complex and measures collisional cross sections that can be compared to standards. Di- to tetrasaccharide fragments can potentially retain all structural information in fragments which can be built back up. Tandem mass spectrometry still does not typically yield stereochemical information, although regiochemistry and connectivity can be discerned. IR spectra of fragments are still very complicated, but simpler than of the precursor, and could yield all structural information. IMS spectra may be simple and could be diagnostic compared to a reference library. In combination, exhaustive amounts of data are present which might allow full assignment with high confidence. Finally, monosaccharide fragments can be obtained by extensive fragmentation. Employing just tandem mass spectrometry results in loss of all information associated with connectivity and provides only composition information. IMS allows for the potential differentiation of monomers, even if isobaric, and also has the potential to determine the stereochemistry of the glycosidic bond because of memory effects. IR spectra are less complex than for oligomers and can also be diagnostic for stereochemistry of the glycosidic bond. These experimental approaches can be facilitated by combination with theoretical computation modeling that could generate theoretical IMS CCSs, tandem mass spectra, and IR spectra that could evaluate experimental data. Additionally, databases of libraries of spectra could facilitate structural identification. These libraries would need to be exhaustive if they are going to possess information and spectra for all isomeric precursors/oligomer fragment, whereas they become more concise the smaller the fragment size being analyzed.

few. These databases of known compounds continue to be expanded and show iterated improvements as they become defunct and subsequent databases grow from them. A series of carbohydrate-linked databases and the information they provide are listed in Table S2 in the [Supporting Information](#). The ability to compare known database standards for retention times (and corresponding  $m/z$ ) has been a real cornerstone of (U)/HPLC *N*-glycomics in being able to identify rapidly the presence or absence of certain glycoforms including those from clinical samples.<sup>24,119,141</sup> Comparison of libraries of (LC-)MS/MS spectra for known compounds has been touted as a means of identifying glycans as well.<sup>137,141,143,144</sup> To the best of our knowledge, only a single database (GlycoMob) containing IMS

CCSs has been produced, primarily focusing on precursor ions.<sup>67</sup> However, it could be hugely beneficial to record CCSs for both the precursor and diagnostic product ions, especially since it would require fewer “standards” for matching product ion data, and they have been shown to be capable of retaining stereochemical information.<sup>53,95</sup> Currently there are no databases for gas-phase IR spectra, where again both precursor and product ion data could be hugely beneficial. This though is primarily due to the technique being relatively new and highly specialized and requiring custom non-commercial instrumentation.

It is impracticable to have vast information-dense libraries of “all possible” carbohydrates (including any derivatization) with

a full complement of analytical information, and consequently it will remain a struggle to identify unknown sequences (in fact unknowns may be misidentified). Therefore, these databases work well for previously identified carbohydrate structures of systems where the biological pathways are well understood, characterized, and highly conserved. It is hoped that framework initiatives such as the *minimum information required for a glycomics experiment* (MIRAGE) that put in place guidelines for reporting results and methodologies for various qualitative and quantitative glycomics experiments will lead to situations where newly published glycan structures can be readily curated into databases including the analytical methods and sample preparation.<sup>121,145,146</sup>

Finally, computation modeling is potentially hugely impactful in numerous ways for glycan. For example, being able to predict *in silico* the precise MS/MS fragmentation pathways would greatly facilitate structure assignment as opposed to current approaches that often just generate mass lists from all possible fragments. Similarly, being able to predict the conformational families an oligosaccharide analyte forms is useful for generating theoretical CCSs or theoretical IR spectra. These can be compared to experimental data to improve confidence in assignments and ideally eventually would allow assignments of unusual sugars that have no experimental standards. As *in silico* CCS calculations and IR spectra also improve, these possibilities become more viable. Computational modeling tools were very recently reviewed (during the submission of this manuscript).<sup>147,148</sup>

## ■ OUTLOOK AND CONCLUDING REMARKS

Unequivocal characterization of all stereochemical information within a given carbohydrate remains an extraordinary challenge for analytical chemists. HPLC or UHPLC strategies to reduce the vast complexity of biological samples prior to more detailed structural interrogation will no doubt still be hugely important especially as techniques head toward automation. It is apparent from research over the past decade that much of the advancement in gaining structural information has revolved around MS analysis and the production of gas-phase ions, as a wide variety of oligosaccharides (and glycan conjugates) can be ionized and manipulated sensitively. MS/MS alone provides incomplete structural information (compositional and sometimes regiochemical), as it is usually “blind” to stereoisomerism. More elaborate MS/MS, MS<sup>n</sup> and energy-resolved MS/MS approaches that sometimes employ different fragmentation techniques, derivatization or varying adducts have been shown to be capable of distinguishing a variety of isomeric standards. However, these techniques still need to explicitly demonstrate that they can be extended to the detailed structural characterization of more complex precursors. Incorporating IMS gives another dimension of separation to MS analysis and allows assignment sensitive to the three-dimensional structure. Akin to currently exploited chromatographic strategies, identification of glycan precursors from databases of known CCSs, limits its application to unknown glycans. Fortunately, progress in chemical and enzymatic synthesis is beginning to make it possible to prepare large collections of well-defined glycan standards.<sup>149</sup> Some classes of glycans can be synthesized in an automated fashion,<sup>150</sup> and chemoenzymatic methods are emerging that can readily provide glycans with highly complex architectures.<sup>151</sup> Studying the CCS of fragments and comparing these smaller species to previously acquired standards may circumvent this limitation,

especially since it has been shown that fragments retain some “memory” of the precursor ion structure. Moreover, derived tandem mass spectra from multiple types of dissociation strategies are rich in information providing compositionally specific fingerprints. Current resolution of commercial IMS instrumentation still limits this strategy as it is challenging to get baseline separation of all isomeric mono-/disaccharides etc. However, the challenge of improving IMS resolving power without much loss in sensitivity is a very active area of investigation.

To overcome some of the limitations of MS and IM-MS, the addition of gas-phase IR action spectroscopy has been shown to provide fine structural information, since both precursor and product ions can be studied in most instrument set-ups. Data so far suggest that Vibrational spectra are highly sensitive to structural and conformational variations. Combined with theoretically generated IR spectra (or CCS for IMS measurements) of computationally optimized three-dimensional structures, it may be possible in the future to increase confidence in structural assignments, but considerable development is required since calculations are not straightforward. Cold-ion IR spectroscopy is raising the resolution of these measured vibrational spectra, increasing the likelihood of observing unique identifiable species. This is still a budding area, and it will be very interesting to see how it develops. Currently, its main restrictions are the requirement of complex custom-built instrumentation and FEL facilities for certain experiments. Also, the time required to acquire gas-phase vibrational spectra is not currently suitable for high-throughput analyses.

Combinations of these complementary MS-hyphenated techniques in the gas-phase will likely provide the greatest amount of information and increase confidence in glycan structure assignments (Figure 7). Advanced, informatics tools that can integrate these diverse and information-rich datasets to pull out and annotate significant features and predict probable structural assignments will be hugely important.<sup>152</sup>

Future developments call for an improvement of the accuracy of NMR chemical shift predictions, where this in part can be made by calculation of the potential of mean force (PMF) surface of oligosaccharides by molecular dynamics (MD) simulations,<sup>153</sup> using a high-quality force field for carbohydrates, from which a set of conformations are selected and for which their NMR chemical shifts are calculated by quantum chemical methods and weighed according to the corresponding Boltzmann population distribution. In this way, data may be obtained where synthesis or isolation of oligosaccharides is either not possible, or too difficult to carry out. A combined workflow that includes information from techniques such as MS, IR or any other technique that complements the NMR-based structural elucidation, which ultimately may need to be employed if no other technique suffices, as well as bioinformatics integration, will need to be developed and implemented in a web-based application and made available to the glyco community to drive significant further advances in this area.

## ■ ASSOCIATED CONTENT

### 📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.9b06406.

Two tables listing software and databases that facilitate glycan analysis (PDF)

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### Notes

The authors declare no competing financial interest.

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