Gas-Phase Infrared Spectroscopy of Glycans and Glycoconjugates

Kim Greis[1,2†], Carla Kirschbaum[1,2†], Gert von Helden[2] and Kevin Pagel[1,2*]

[1]Institut für Chemie und Biochemie, Freie Universität Berlin, Arnimallee 22, 14195 Berlin, Germany

[2]Fritz-Haber-Institut der Max-Planck-Gesellschaft, Faradayweg 4-6, 14195 Berlin, Germany

[†]These authors contributed equally

Correspondence to: kevin.pagel@fu-berlin.de

Phone: +49-(0)30-838-72703

Email addresses of co-authors:

Kim Greis: greiskim@fhi-berlin.mpg.de

Carla Kirschbaum: ckirschbaum@fhi-berlin.mpg.de

Gert von Helden: helden@fhi-berlin.mpg.de

Abstract

Glycans are intrinsically complex biomolecules that pose particular analytical challenges. Standard workflows for glycan analysis are based on mass spectrometry (MS), often coupled with separation techniques such as liquid chromatography and ion mobility spectrometry. However, this approach does not yield direct structural information and cannot always distinguish between isomers. This gap might be filled in the future by gas-phase infrared (IR) spectroscopy, which has emerged as a promising structure-sensitive technique for glycan fingerprinting. This review highlights recent applications of gas-phase IR spectroscopy for the analysis of synthetic and biological glycans and how it can be integrated into MS-based workflows.

Introduction

The analysis of sugars is very challenging due to their tremendous structural complexity. They are assembled from monosaccharide building blocks, which exhibit multiple possible connection points. Therefore, monosaccharides are not assembled in a strictly linear fashion like amino acids in proteins but instead connected with a complex regio- and stereochemistry [1]. Moreover, several common monosaccharides such as glucose and galactose are isomeric and thus indistinguishable by mass spectrometry (MS). Knowledge about the structure of natural glycans including *N*-glycans, *O*-glycans, human milk oligosaccharides (HMOs) and glycosaminoglycans (GAGs) is of utmost importance as these glycans exhibit pivotal biological functions in humans [2]. However, no gold-standard method for their analysis has been established yet. MS is the most important technique for glycan analysis, often used in combination with additional stages of separation such as chromatography or ion mobility spectrometry (IMS) [1]. Unfortunately, MS alone cannot unambiguously assign glycan structures out of many possible isomers. Infrared (IR) spectroscopy on the other hand yields structural information and can be coupled to mass spectrometers as an additional dimension of information for structural assignment. Combined with computed spectra, 3D-structures of the probed glycans can be obtained. Here, we will discuss the recent developments in (cryogenic) gas-phase spectroscopy of glycans and its potential for glycomics.

Gas-Phase Infrared Spectroscopy of Ions

Gas-phase IR spectroscopy of ions relies on technology from the field of mass spectrometry, coupled with a suitable IR light source. In contrast to classical IR absorption spectroscopy, which is based on the detection of light attenuation, IR ion spectroscopy probes the impact of photon absorption on the ions, e.g. by monitoring fragmentation (Fig. 1). This approach is termed *action spectroscopy* and includes different experimental realizations [3]. Infrared multiple photon

dissociation (IRMPD) spectroscopy monitors light-induced fragmentation of molecular ions, which is only achieved after the sequential absorption of multiple photons. Multiple photon processes like IRMPD therefore require powerful IR light sources, most prominently free-electron lasers (FELs) or optical parametric oscillators (OPOs). Less powerful light sources are required for messenger tagging spectroscopy, in which a tag is weakly bound to the ion of interest at cryogenic temperatures and detached in a single photon absorption event. A third approach is cryogenic IR spectroscopy in helium nanodroplets (HeDrop spectroscopy). Here, ions are encapsulated in superfluid helium droplets, from which they are released in a multiple photon process. Contrary to IRMPD, the absorption of multiple photons does not lead to ion heating in helium droplets due to evaporative cooling, which significantly increases the spectral resolution.

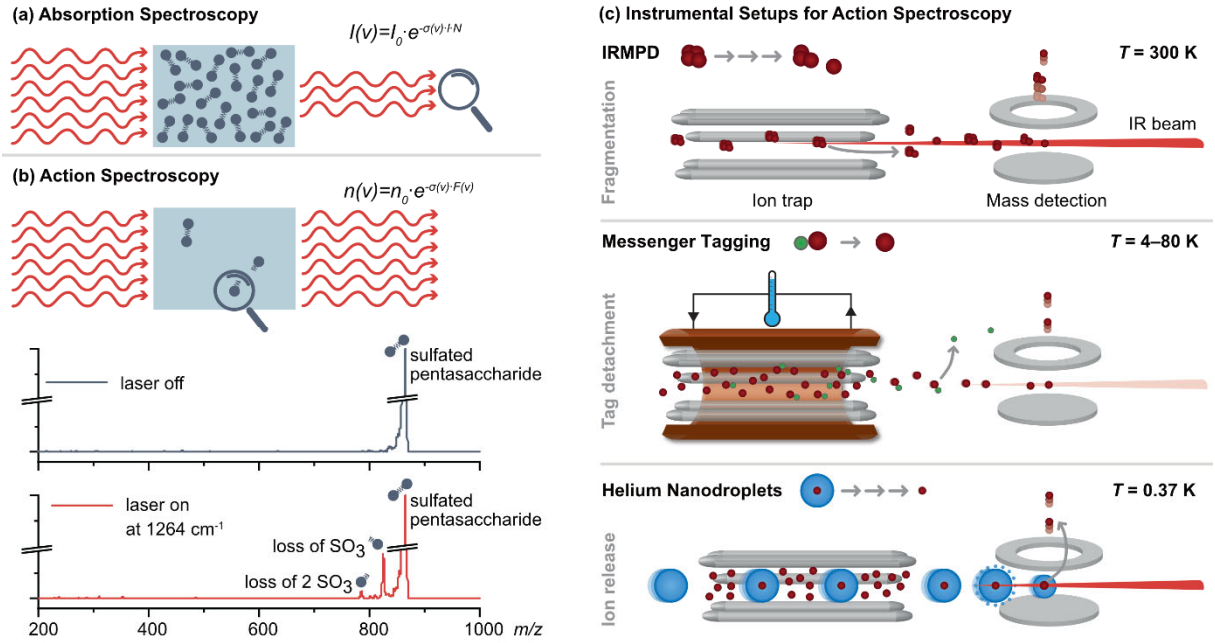

Figure 1. Infrared spectroscopy of ions in the gas phase. **(a)** Absorption spectroscopy measures light attenuation behind a sample of high molecular density. **(b)** Action spectroscopy monitors light-induced changes occurring on the molecular level, such as fragmentation. **(c)** Action spectroscopy has been implemented in different experimental setups including infrared multiple photon dissociation, messenger tagging, and helium nanodroplet spectroscopy. All approaches rely on ion traps, tunable light sources and a mass detection scheme. Reprinted from ref. [1]. Creative Commons Attribution 4.0 International License.

Applications for Model Glycans and Synthesis

In the past few years, it has become apparent that gas-phase IR spectroscopy, particularly cryogenic IR spectroscopy, can distinguish isomeric model glycans exhibiting only subtle structural differences. Pioneering studies on neutral monosaccharides in argon jets by gas-phase IR spectroscopy showed promising results to determine sugar structures [4,5]. However, studies on

larger carbohydrates with IRMPD spectroscopy showed that the IR signatures of disaccharides are already significantly congested, yielding limited diagnostic information [6-8]. The advent of cryogenic IR spectroscopy for glycan analysis, on the other hand, showed the enormous potential of this technique and its variations. A first ground-breaking study on model glycans showed that isomeric protonated and sodiated mono-, tri- and tetrasaccharides, differing in composition, connectivity and configuration, can be unambiguously distinguished based on their unique HeDrop-IR fingerprints in the 1000–2000 cm^{-1} region [8]. Likewise, it was confirmed that isomeric sodiated disaccharides and pentasaccharides exhibit distinct IR patterns in the 3000–4000 cm^{-1} region using messenger spectroscopy (H_2 and N_2 tags) [9]. IRMPD investigations on lithiated [10] and sodiated acetylated monosaccharides as well as hexose phosphates showed the capability of the technique to study smaller glycans, but also highlighted the spectral congestion in deprotonated disaccharides [11]. A workflow was developed in which liquid chromatography (LC)-MS and IRMPD can be directly coupled to identify two regioisomeric GlcNAc disaccharides from one solution [12]. IRMPD was used to show that isomeric protonated and sodiated glycan epitopes containing α2,3- and α2,6 linked sialic acids can be unambiguously distinguished based on their IR signatures in the 3000–4000 cm^{-1} region [13].

Cryogenic IR-IR double resonance spectroscopy was employed to explore the conformational heterogeneity of protonated glucosamine [14] and sodiated glucose [15] anomers in the 3000–4000 cm^{-1} region using He- and D_2 tags respectively. Structures for lossless ion manipulations (SLIM) ion mobility (IM) MS were combined with N_2-messenger spectroscopy to separate and identify sodium adducts of disaccharide anomers [16]. The structures of fragment ions from deprotonated disaccharides were studied using IRMPD spectroscopy in the 1000–2000 cm^{-1} region, which yielded important information about the fragmentation mechanism [17,18]. The same technique was applied in combination with *ab initio* molecular dynamics to investigate conformational changes of lithiated [19] and protonated [20] monosaccharides. Further, N_2-messenger spectroscopy was employed to investigate the fragmentation mechanism of sodiated β-cyclodextrin. The IR spectra indicate that the mechanism is consistent for the observed fragments [21].

IRMPD spectroscopy was used to record the unique fingerprints of glycosidic ring size isomers, based on which protonated, lithium- and ammonium-adducted pyranosyl and furanosyl glycans can be unambiguously differentiated in the 3000–4000 cm^{-1} region (Fig. 2A) [22-24]. Previously, the approach has been successfully employed to distinguish isobaric 6-sulfated and 6-phosphated glucosamines [25].

The power of IR spectroscopy for glycan sequencing has been demonstrated in recent studies, which showed that glycosidic *C*-fragments from α- and β-linked glycans yield a diagnostic IR signature in the 3000–4000 cm^{-1} region using IRMPD [26] and messenger spectroscopy (Fig. 2B) [27]. Here, the configuration at the anomeric carbon is retained after fragmentation. For *B*-fragments, on the other hand, the stereochemistry at the anomeric carbon is not retained. However, a first study using IRMPD spectroscopy on unprotected glycans indicates that distinct IR patterns are obtained depending on whether the *B*-fragments originate from the cleavage of an α- or β-glycosidic bond [28]. For *B*-fragments of protected galactosides investigated by HeDrop spectroscopy such an effect was not observed [29].

In addition to the co-existence of isomers, the sequencing of glycans using tandem MS techniques is further complicated by a phenomenon called fucose migration [30,31]. Here, the fucose residue in protonated glycans can migrate to other positions within the molecule, which may lead to erroneous structure assignments. Studies on fucosylated glycans using HeDrop spectroscopy suggest that fucose migration is a general phenomenon in MS and not only limited to tandem MS. The rearrangement of deprotonated isomeric blood group epitopes carrying fucose residues at different sites into a common structure was confirmed by the close similarity of their IR spectra [32]. In a subsequent study, it has been found that migration also occurs in ammonium-adducted glycans. This finding indicates that a mobile proton rather than a direct protonation is required for the rearrangement [33].

In another application, IR spectroscopy of glycans has been employed to study the elusive intermediate of glycosynthesis, the glycosyl cation. In chemical glycosylation, this cationic intermediate is formed by activation of glycosyl donors, carrying a leaving group at the anomeric center (C1). Subsequent attack of a nucleophile, such as a further monosaccharide building block, can occur from both sides of the glycosyl cation, yielding an anomeric mixture of products. Direct characterization of the intermediate has been challenging due to its short-lived nature and required stabilization in super acids [34]. Using both IRMPD and HeDrop spectroscopy in the 1000–2000 cm^{-1} region in combination with density functional theory (DFT) computations, it was possible to study the structure of bare glycosyl cations. The experimental data suggests that acetyl protecting groups at the C2 position (neighboring group participation) [35,36] and at the C3- or C4-positions (remote participation) [37-39] can stabilize the positive charge at the anomeric carbon by formation of a covalent bond with the carbonyl oxygen. The studies confirmed that the structure of the intermediate correlates strongly with the stereoconfiguration of the products in glycosynthesis. Furthermore, HeDrop spectroscopy has been employed to determine the structure of the Ferrier cation, the intermediate of the Ferrier rearrangement reaction, which is important

for the synthesis of unsaturated glycosides. In this reaction, the C3-acetyl protecting group of an 1,2-unsaturated glycoside is cleaved, leading to the positively charged intermediate. Upon attack of a nucleophile at the C1-position a 2,3-unsatured glycoside is formed. The data confirmed that the Ferrier cation is stabilized by neighboring group participation of an acetyl group at the C4 position (Fig. 2C) [40].

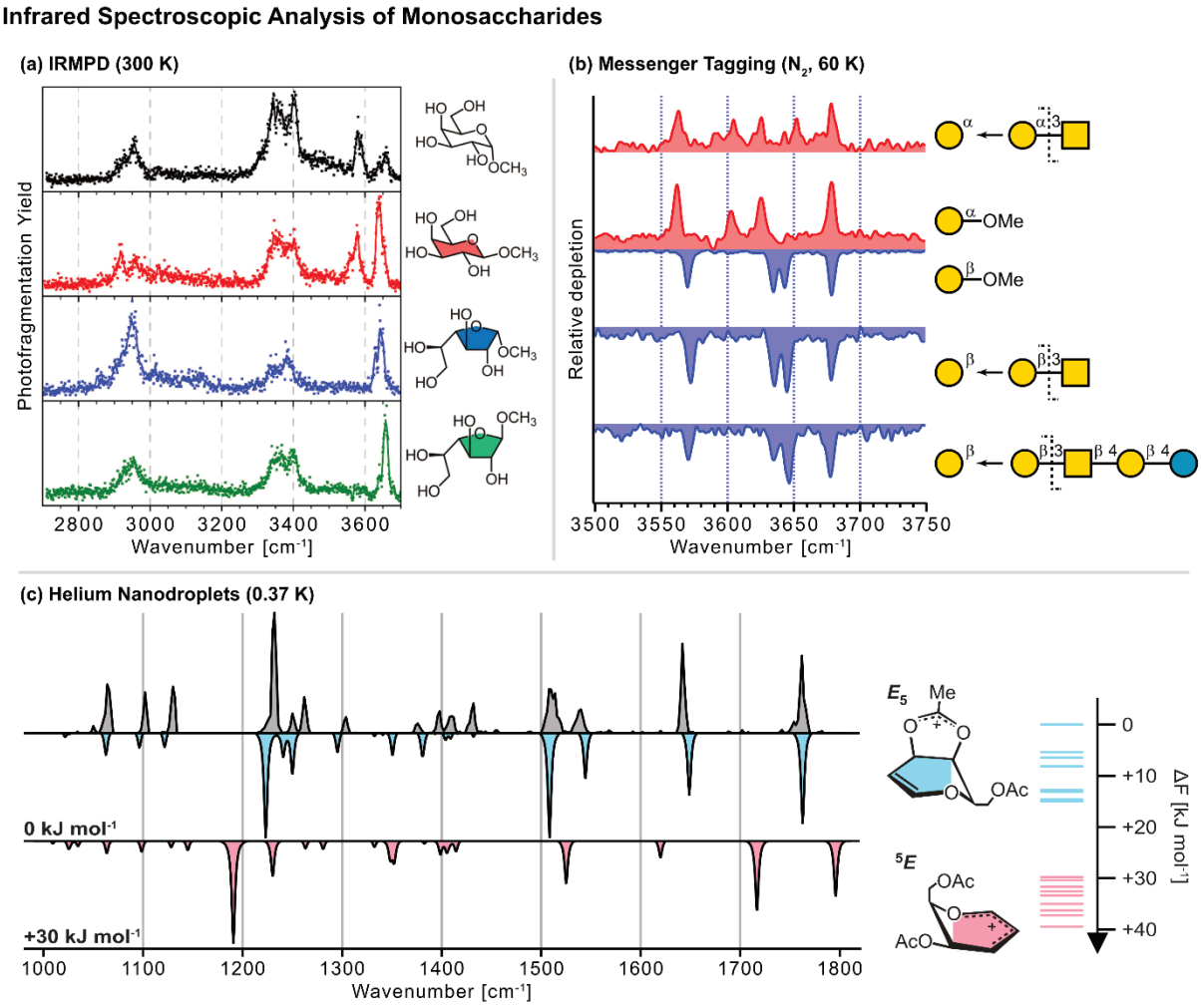

Figure 2. (a) Ring-size isomers and anomers of galactose can be differentiated by IRMPD spectroscopy in the 3000–4000 cm^{-1} region (adapted with permission from ref. [24]. Copyright **2021** American Chemical Society). **(b)** Cryogenic vibrational spectra (N_2-messenger spectroscopy) of anomeric C_1 fragments of galactose-containing glycans are distinguishable and contain striking similarities to methylated anomers (adapted with permission from ref. [27]. Copyright **2020** American Chemical Society). **(C)** (left) Experimental cryogenic infrared spectra (HeDrop spectroscopy) of Ferrier galactosyl cations (gray trace) adopt dioxolenium-type structures (blue) rather than oxocarbenium-type structures (red). (middle) Low-energy structures and puckers. (right) Free energies of reoptimized low-energy structures at 90 K (adapted with permission from ref. [40] Copyright **2020** American Chemical Society). Infrared spectroscopy in helium nanodroplets unarguably leads to the narrowest absorption bands but is experimentally challenging to implement. IRMPD spectroscopy, on the other hand, is experimentally less challenging but the broadness of absorption bands can impede answering certain analytical questions.

Biological Glycans

In addition to synthetic model glycans, glycans from biological sources can be analyzed with gas-phase IR spectroscopy. Similarly to other glycan analysis techniques, however, the analytical potential can vary drastically between individual glycan classes. N_2-messenger spectroscopy was used in the 3000–4000 cm^{-1} region to identify isomeric HMOs ranging from tri- to hexasaccharides as sodium adducts. The distinguishable IR patterns lead to an unambiguous identification that is difficult to achieve by drift-tube IM-MS alone [41]. Later, the findings were extended to glycan mixtures [42] and fragment-based approaches [43] by coupling the methodology with SLIM-IM-MS. Here, the glycans can be separated on-line before recording the IR spectrum. In a follow-up paper the authors built a database containing experimental IR fingerprints of five HMOs to identify isomeric glycans in mixtures [44]. The necessity of this endeavor is underlined by the inability of computational chemistry to predict IR spectra of biological glycans well enough for unambiguous structural assignments [1].

N-glycans are commonly enzymatically cleaved from *N*-glycopeptides/-proteins prior to MS analysis. Using the same methodology as for the HMOs described above, it was found that the IR signatures obtained from protonated and sodiated complex *N*-glycans from enzymatic cleavage are identical to those of reference standards. [45]. This study indicates that a library of spectra from fragment ions can be used to assign structural features in more complex glycans that are not implemented in the library yet. The approach was extended to protonated fucosylated complex *N*-glycans. The integration of SLIM-IM-MS messenger spectroscopy in analytical workflows was proven to be feasible, as a diagnostic spectrum can be recorded within 55 s [46]. Recently, the methodology was employed to identify positional isomers of complex fucosylated *N*-glycans cleaved from monoclonal antibodies. The sodiated isomers, in which the galactose residue is either located on the α1,3 or on the α1,6 arm, can be separated by SLIM-IM-MS and their IR signatures subsequently probed and compared to synthesized standards, leading to exact identification of the regioisomers (Fig. 3A) [47].

A particularly challenging class of biological glycans are glycosaminoglycans (GAGs). GAGs are highly abundant linear oligosaccharides that consist of a repeating disaccharide backbone featuring a distinct hexuronic acid stereochemistry and a highly diverse sulfation pattern. In comparison to other glycans, GAGs are difficult to analyze using MS based techniques, as fragmentation usually leads to release of SO_3 and with that a loss of the most crucial sulfation information [1]. In early studies, monosaccharides relevant for GAGs were analyzed by IRMPD. The IR spectra of rubidiated iduronic and glucuronic acid (IdoA and GlcA) were recorded in the 3000–4000 cm^{-1}

region and contain significant differences [48]. In some cases, the use of anharmonic computations is required to model experimental IR spectra, as shown for glucosamine 6-sulfate in the 1000–2000 cm^{-1} region [49]. IRMPD spectra of deprotonated IdoA, GlcA and a hyaluronic acid tetrasaccharide (HA-dp4) were measured in the 3000–4000 cm^{-1} region. The setup allowed recording IR spectra of fragments of HA-dp4, confirming the presence of GlcA in HA-dp4 [50]. In a subsequent study, the positional isomers glucosamine 6-sulfate and glucosamine 3-sulfate were differentiated as protonated and deprotonated species. Here, IR spectra of four deprotonated isomeric heparan sulfate (HS) and chondroitin sulfate (CS) disaccharides that differ significantly were also recorded [51]. Moreover, deprotonated GalNAc4S and GalNAc6S isomers were differentiated based on their IR patterns in the 3000–4000 cm^{-1} region. With Y- and B-fragment ion IR spectra of CS disaccharides and dermatan sulfate tetrasacharides, assignments on the position of the sulfate can be made [52]. H_2-messenger spectroscopy was used to identify a set of isomeric HS and CS disaccharides as sodium adducts[53].

HeDrop spectroscopy was used in the 1000–2000 cm^{-1} region to study protonated and sodiated fondaparinux, a highly sulfated pentasaccharide that is clinically used as heparin substitute. The spectra contain highly resolved and narrow absorption bands and comparison to IRMPD spectra indicates that HeDrop spectroscopy yields more diagnostic data with a broad applicability for database assignments. Based on their IR patterns, deprotonated sulfated and non-sulfated HA-dp4 can clearly be distinguished [54]. The HeDrop IR signatures of four diastereomeric heparan sulfate tetrasaccharides X-GlcNAc6S-X-GlcNAc6S-L (with X = IdoA or GlcA in all four possible combinations, and L = aminopentyl linker) in the negative ion mode show that even changes in a single stereocenter in comparably large glycans are clearly distinguishable (Fig. 3B). Interestingly, the linear combinations of IR spectra of the heterogenous and the homogenous diastereomers each lead to similar patterns [55]. Further studies are needed to evaluate if such correlations also occur for other structural motifs in GAGs, as this approach would enable increment based structural assignments. In a further study, deprotonated CS disaccharides with all possible sulfation motifs were analyzed. Higher charge states lead to more defined IR signatures, whereas the signatures of singly sulfated CS are more crowded. Hence, the energetically accessible structures for the multiply charged species are reduced due to charge repulsion, which in turn limits the degree of folding [56].

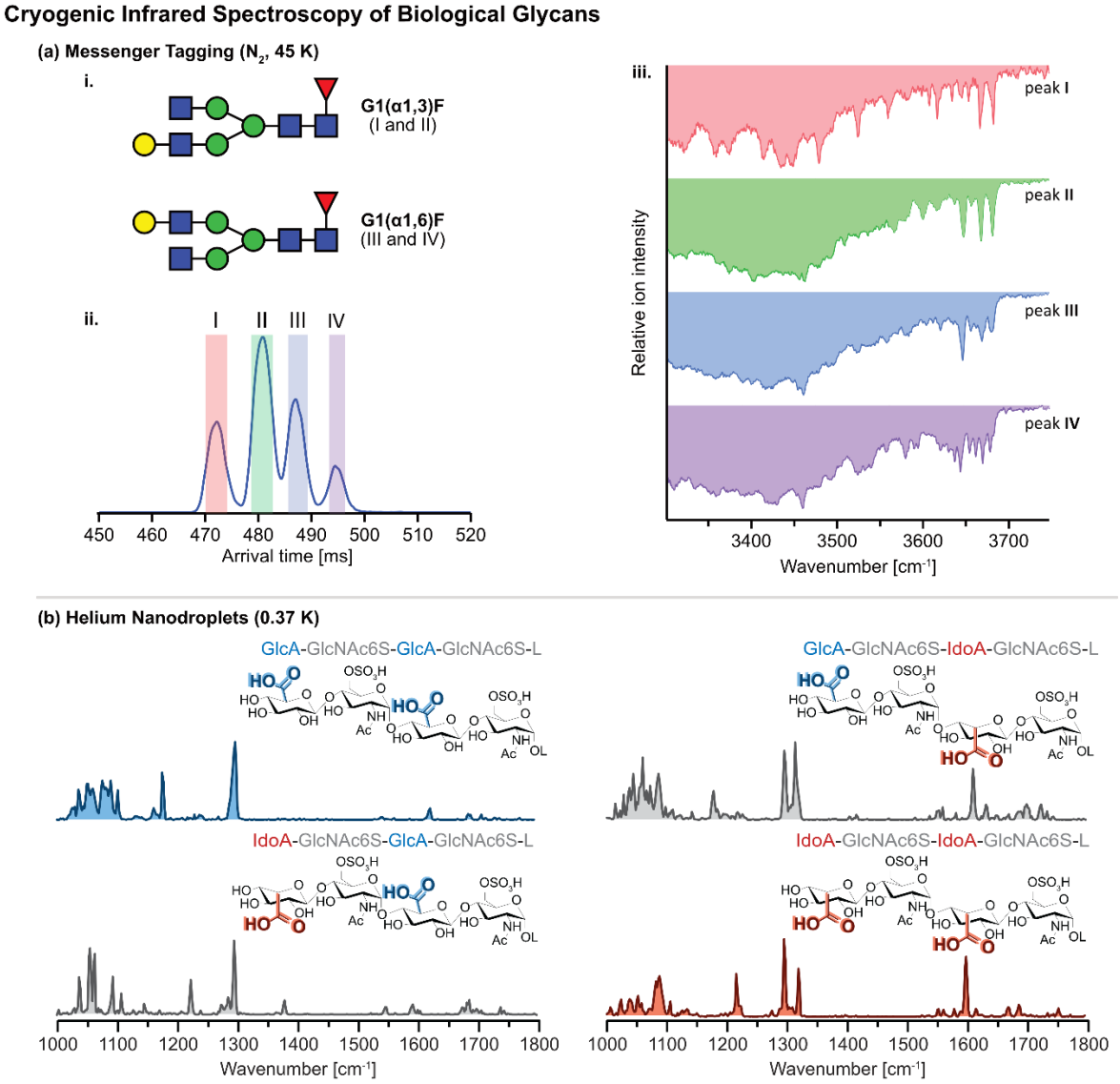

Figure 3. (a) (i) Structure of the two positional isomers of the complex *N*-glycans **G1F**. (ii) Arrival time distribution of **G1F** after 60 m SLIM-IM-MS separation, representing a anomeric mixture of the G1(α1,3)F (I and II) and G1(α1,6)F (III and IV) positional isomers. (iii) Cryogenic IR spectra (N_2-messenger spectroscopy) of each peak observed in the arrival time distribution of **G1F** (reprinted from ref. [47]. Attribution-Non Commercial 3.0 Unported Licence). **(b)** Cryogenic IR spectra (HeDrop spectroscopy) of heparan sulfate tetrasaccharides, investigated as doubly deprotonated anions (adapted with permission from ref. [55]. Copyright **2020** American Chemical Society).

Glycoconjugates

One of the remaining challenges in the field of glycomics is the analysis of glycoconjugates, which are composed of a glycan and a non-glycan part, the aglycone. Such promiscuous molecules pose fundamental analytical challenges due to the diverging properties of the polar glycan and nonpolar aglycone and the overall increased size and complexity. While released *N*-glycans have been investigated by gas-phase IR spectroscopy, the complexity and congestion of IR spectra increases tremendously for intact glycoproteins and -peptides, rendering the overall information gain very

sparse [57]. For smaller glycolipids, however, the cryogenic IR spectroscopic approach lately yielded promising results allowing for clear differentiation of anomers, regio- and stereoisomers in mono- and trisaccharide glycosphingolipids [58]. The isomers are also distinguishable by nuclear magnetic resonance (NMR) spectroscopy, the gold-standard for structural analysis. However, IR spectroscopy requires 2–3 orders of magnitude less sample, which renders the analysis of biological mixtures possible (Fig. 4) [58]. One drawback of IR spectroscopy is the need for reference spectra of glycolipid standards for deconvolution of isomeric mixtures, which are not commercially available for glycans larger than one monosaccharide unit. Therefore, we expect fragmentation methods prior to IR spectroscopy to become one of the main future developments. Future applications of IR spectroscopy will thus largely depend on the availability of spectral libraries.

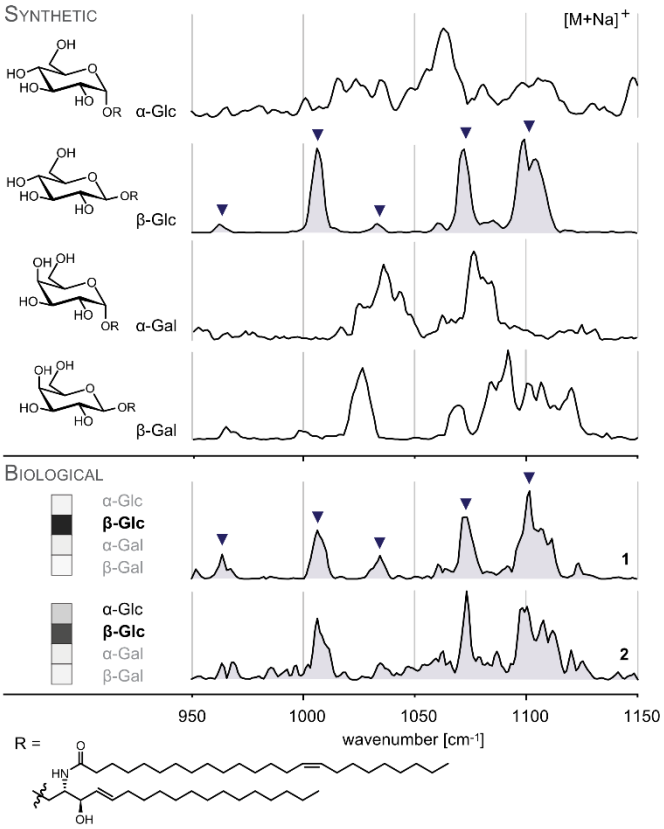

Figure 4. Spectral deconvolution of biological glycosphingolipid mixtures. β-Glucosylceramide is assigned as the main component with the help of reference spectra. The biological extract 2 from alpha-glucosidase deficient mice contains an elevated amount of α-glucosylceramide due to the missing catabolizing enzyme (reprinted from ref. [58]. Creative Commons Attribution 4.0 International License).

Summary and Outlook

The complexity and technical requirements for glycan analysis depend on the respective glycan class. Some *N*-glycans for example are relatively straightforward to characterize using commercialized LC-MS-based workflows, whereas positional isomers can only be reliably differentiated using gas-phase spectroscopy techniques. Sufficient characterization of other classes

such as mucins and GAGs using established techniques is even harder. The sequencing of a small heparan sulfate oligosaccharide is already a formidable task that requires a lot of time and effort. Especially in these more challenging areas of glycobiology, gas-phase spectroscopy may help to resolve a couple of long-standing problems and thus contribute to the implementation of more reliable glycan sequencing workflows by providing an additional level of confidence. An increasing body of literature demonstrates that cryogenic ion spectroscopy can identify subtle structural details in both synthetic and biological glycans. By combination with computational modelling, direct information on the structure and conformation can be obtained; however, a *de novo* assignment based on theory is difficult, if not impossible at the present stage. Therefore, a library-based approach is currently required for the assignment of larger structures. Online coupling of IR spectroscopy with LC-MS workflows has been hampered by the acquisition time of IR spectra usually exceeding 10 min, which is too slow for a direct hyphenation to LC [12]. However, tremendous technical improvements are currently being achieved, as demonstrated recently by an instrument that allows recording IR spectra of multiple species simultaneously over a range of 450 cm^{-1} in as little as 15 s [59]. Such setups, in which spectra can be measured within a few seconds using a commercial light source are expected to render IR spectroscopy more broadly available and compatible with chromatography-based high-throughput analytical workflows in the future.

Annotated References

(••) [58] This study shows that isomeric glycolipids can be unambiguously identified using HeDrop IR spectroscopy and that complex isomeric mixtures of biological origin can be deconvoluted.

(••) [37] Both papers demonstrate for the first time that remote participation exists in glycosyl cations and that the structure of the intermediate correlates directly with the stereoconfiguration of the products in glycosynthesis.

(••) [39] See annotation to Ref. [37].

(••) [27] The paper shows that the anomeric memory of C-fragments is a general phenomenon that is independent of the fragment or the precursor ion size or branching.

(••) [59] An instrumental setup is presented that allows for probing multiple species at the same time (multiplexing) within a few seconds, which is needed for incorporating cryogenic infrared spectroscopy in analytical workflows.

(•) [24] This study shows that IRMPD spectroscopy can be used to differentiate between anomeric galactohexoses and -furanoses. The findings are important for sequencing bacterial glycans.

(·) [47] Here, a combination of SLIM-IM-MS and cryogenic IR spectroscopy is used to differentiate between two positional isomers of a nonasaccharide in a biological mixture of complex *N*-glycans.

(·) [55] In this paper, the authors differentiate between four diastereomeric GAG tetrasaccharides by probing their IR signatures in helium droplets. A strong relationship between the structures and the spectra was found.

(·) [18] The authors investigate the structure of fragment ions of a disaccharide using IRMPD spectroscopy. The findings help to elucidate the fragmentation mechanism and explain the abundance of certain fragment ions in tandem mass spectra.

(·) [20] This study deals with conformational changes of protonated monosaccharides and yield first evidence for the formation of an unprotected oxocarbenium ion in the gas phase.

Declaration of Interest

The authors declare no conflict of interest.

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Keywords

Mass Spectrometry, Infrared Spectroscopy, Glycomics, Biomolecules, Glycans

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