Synthetic Approaches to Break the Chemical Shift Degeneracy of Glycans

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NMR spectroscopy is the leading technique for determining glycans' three-dimensional structure and dynamic in solution as well as a fundamental tool to study protein-glycan interactions. To overcome the severe chemical shift degeneracy of these compounds, synthetic probes carrying NMR-active nuclei (*e.g.*, ^{13}C or ^{19}F) or lanthanide tags have been proposed. These elegant strategies permitted to simplify the complex NMR analysis of unlabeled analogues, shining light on glycans' conformational aspects and interaction with proteins. Here, we highlight some key achievements in the synthesis of specifically labeled glycan probes and their contribution towards the fundamental understanding of glycans.

Introduction

Glycans, a.k.a. polysaccharides, are biopolymers ubiquitous in nature playing important roles as structural materials, for energy storage, and as mediators of many biological processes.[1] To gain a better insight into their function and establish structure-function correlations, it is important to understand glycans' three-dimensional structure. To date, the chemical complexity of polysaccharides hampered structural studies with standard characterization techniques. Unlike proteins and nucleic acids, glycans are non-linear polymers capable of adopting multiple conformational states separated by low energy gaps.[2,3] Low propensity to form single crystals with suitable dimensions hampers X-ray analysis, sensitivity to electron beam limits the use of electron microscopy (EM), and lack of chromophores prevents the use of circular dichroism (CD).[4,5] Single molecule imaging techniques have provided new tools to explore glycans' conformational space with sub-nanometer resolution, but to date they remained limited to the gas phase.[6,7]

Nuclear magnetic resonance (NMR) spectroscopy, supported by molecular dynamics (MD) simulations, is the leading technique for the structural characterization of glycans in solution phase.[8] Scalar *J*-couplings, NOEs, and residual dipolar couplings (RDCs) are NMR observables that are relatively simple to measure and useful for verifying theoretical models or derive experimental conformations.[9] Moreover, several NMR experiments are available to dissect glycans interactions with proteins.[10] Still, the severe chemical shift degeneracy of glycans (*i.e.*, signal overlapping) dramatically complicates the analysis.[11,12]

To facilitate the analysis of glycans by NMR spectroscopy, synthetic probes carrying NMR-active nuclei (*e.g.*, ^{13}C or ^{19}F) or lanthanide tags have been developed.[13,14,15] These strategies permitted to break the chemical shift degeneracy of glycans and revealed important aspects of glycan conformations and interactions. Furthermore, the insertion of NMR labels overcomes the poor sensitivity and resolution of standard NMR spectroscopy.[16] Still, the preparation of broad collections of specifically labeled glycans remains a synthetic challenge, often limiting these studies to small libraries of relatively simple glycans. Here, we discuss some synthetic developments to access specifically labeled glycan probes, highlighting their contribution in the structural and functional characterization of glycans.

^{13}C-Labeled Glycans

The large chemical shift dispersion of ^{13}C is a considerable advantage over ^{1}H for NMR studies. However, the low natural isotopic abundance of ^{13}C (1.1%) imposes the use of ^{13}C-enriched sample. ^{13}C-labeling has minimal impact on the chemical properties of the glycan[17] and it allows for a wide range of NMR experiments in combination with ^{1}H-NMR.[18] For these experiments, the problematic large ^{13}C-^{13}C couplings can be suppressed by virtual decoupling schemes.[19]

^{13}C-labeled glycan probes were prepared using chemical[20,17] and enzymatic[21] synthesis or metabolic[22] approaches. Uniformly labeled as well as site-specific labeled compounds have been reported, revealing important structural features of glycans and providing a detailed description of their interactions with proteins. A uniformly ^{13}C-labeled trimannoside Man_3 suggested the glycan binding epitope to the antiviral lectin cyanovirin-N.[23] The orientation of each hydroxyl group in the trisaccharide and the hydrogen-bonding pattern between the glycan and the protein in aqueous solution were identified.[24]

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^{13}C-labeled glycans offer an additional advantage in binding studies with heavily glycosylated proteins, avoiding interference with signals from the glycans attached to the protein. A α2,3- and α2,6- sialyl *N*-acetyllactosamine trisaccharides, ^{13}C-labeled at the Neu5Ac and Gal residues, identified the ligand for the spike glycoprotein in SARS-Cov-2 virus and permitted epitope mapping.[25]

While uniformly ^{13}C-labeled glycans fueled the implementation of ^{13}C-NMR spectroscopy to study glycans, spectral overlap of the ^{13}C signals remains a challenge for homo-polysaccharides or highly repetitive glycans. In these cases, the preparation of site-specific labeled compounds is imperative to gain valuable information, but adds synthetic complexity. Site-specific ^{13}C-labeled glycans can be obtained *via* chemical synthesis[20,17] as well as chemo-enzymatic methods.[21] Automated glycan assembly (AGA)[26] is well-suited to generate collections of related glycans, incorporating ^{13}C-labeled residues in specific positions of the glycan chain (Figure 1A). A collection of linear β(1-6)-linked hexaglucosides, bearing one ^{13}C-labeled Glc unit in different positions of the chain, granted access to *J*-coupling values, supporting the helical model predicted by MD (Figure 1B).[14] This collection was further expanded to include oligomers with two ^{13}C-labeled Glc units, providing geometrical information on the relative orientation of the Glc residues along the glycan backbone, measured *via* ^{13}C-^{1}H residual dipolar couplings (RDCs) (Figure 1B).[27] A detailed NMR analysis using different aligning media demonstrated the high flexibility of these oligomers.

Site-specific labeled compounds were also exploited to identify the preferred protein binding epitope within a repeating oligomeric glycan (Figure 1C). A collection of ^{13}C-labeled poly-lactosamine hexasaccharides was prepared following a chemo-enzymatic approach. ^{13}C-labeled galactose units were introduced in particular positions of the tri-LacNAc compounds to distinguish the contribution of each LacNAc moiety during the binding with different galectins.[28]

Single-sites ^{13}C-labeling of pyranose rings of a collection of disaccharides gave experimental access to *J*-couplings across *O*-glycosidic linkages. These values were then quantitatively correlated to torsional angles using density functional theory (DFT) calculations. A statistical program (MA'AT) permitted to extract the conformation of the glycosidic linkages.[29,30] The results obtained for the isolated disaccharide were compared with the same disaccharide structure within an oligosaccharide to elucidate context effects.[31,32] An alternative approach to analyze glycosidic torsional angles was based on the replacement of ^{1}H on a uniformly ^{13}C-labeled residue with the ^{1}H-NMR-silent ^{2}H nuclei, giving access to inter-residue $^{3}J_{CH}$ and $^{3}J_{CC}$ coupling constants across the glycosidic linkage.[33] With these data, the glycosidic torsional angles ϕ and Ψ were determined, revealing the conformational preference of branched β(1,3)-glucan oligosaccharides.

These example stresses that the combination of ^{13}C labels with other NMR active nuclei could expand the scope of NMR analysis of glycans even further. Multiple labels were also useful for unambiguous resonance assignment, as demonstrated for a ^{15}N-,^{13}C-labeled α(2,8)-sialic acid tetrasaccharide.[34] The probe was obtained from a genetically engineered *E. coli* strain following isotopic enrichment of the capsular polysaccharide with ^{13}C and ^{15}N isotopes.[35] NMR analysis provided direct evidence of a left-handed helical conformation of the oligosaccharide in solution. A synthetic heparan sulfate octasaccharide uniformly labeled with ^{15}N- and ^{13}C reveled key interactions with hemagglutinin, providing valuable information for the structural characterization of the glycan-protein complex.[36]

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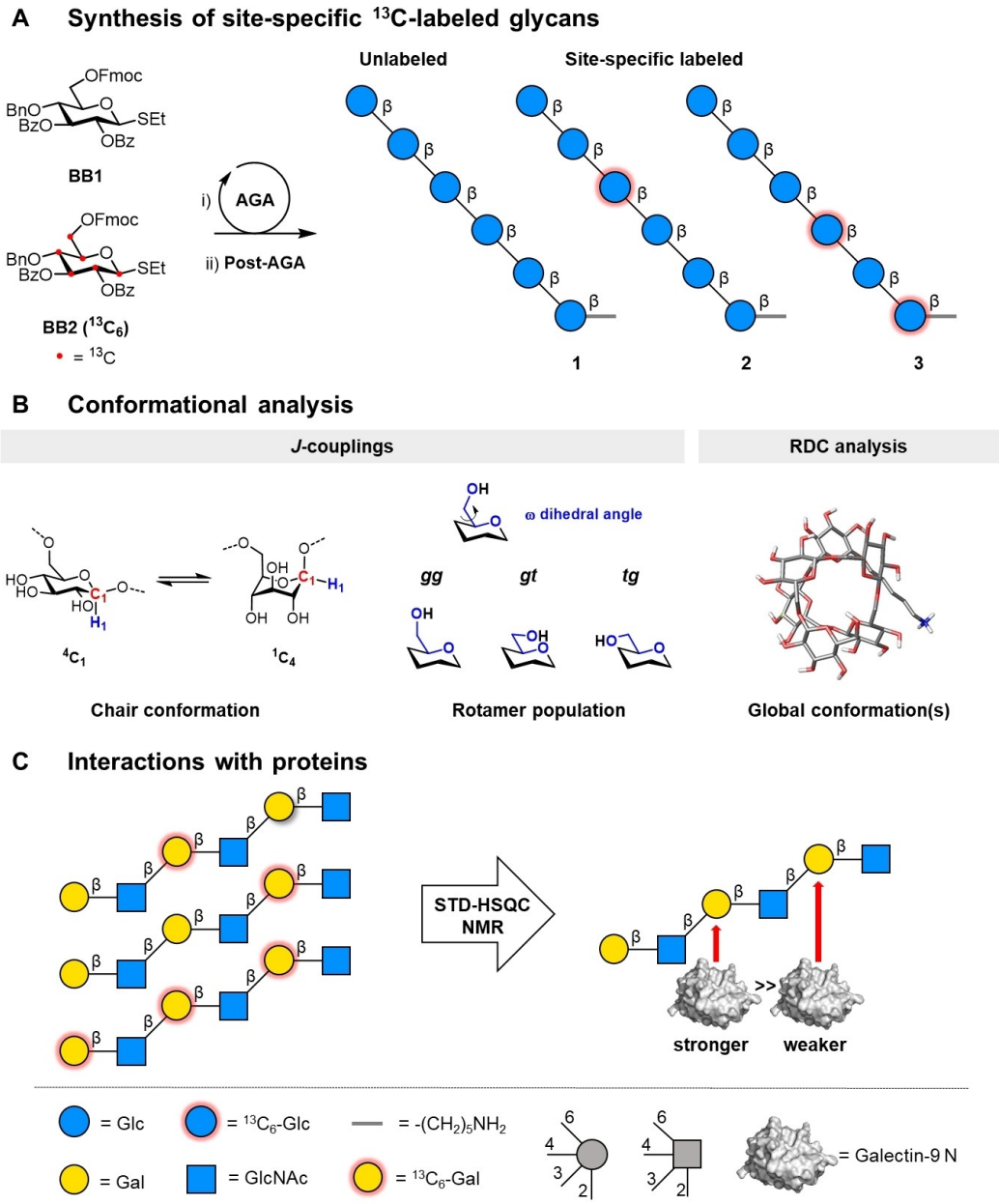

Figure 1. A .The introduction of ^{13}C-labeled residues in specific positions of the oligosaccharide breaks the chemical shift degeneracy of the unlabeled analogue. Site-specific ^{13}C-labeled oligosaccharides can be prepared by AGA using protected monosaccharide building blocks (**BB1** and **BB2**). B. ^{13}C-labeling facilitates glycan conformational studies providing access to local as well as global conformational information (i.e., J- coupling constants and RDCs). C. Site-specific ^{13}C-labeled tri-Lac-NAc hexasaccharides permitted to identify the preferred binding epitopes for a panel of human galectins by STD-HSQC NMR (the 3-D model of galectin-9 N is represented, PDB 2d6 m). The monosaccharides are represented following the symbol nomenclature for glycans (SNFG). For additional details we refer to Refs. [14,27,28].

^{19}F-Labeled Glycans

An attractive chemical modification of carbohydrates is the introduction of ^{19}F. The absence of background signals in biological systems makes ^{19}F-labeled glycans ideal probes for detecting interactions with biomacromolecules. Short acquisition times, high sensitivity and large chemical shift range are additional advantages offered by the ^{19}F nucleus. Hence, (deoxy)fluorination of carbohydrates is an effective approach to study protein-carbohydrate interactions.[37,38,39] ^{19}F chemical shift perturbation and line broadening effects in ligand-observed NMR can be exploited to detect binding. Simple spectra analysis offers the possibility of studying events in real-time, with potential applications for in vivo studies,[40] or to monitor enzymatic reactions.[41] Synthesis of site-specific 19F-labeling can also be combined with ^{13}C-labeling to provide additional geometrical information for conformational analysis.[27] As drawback, the synthesis of ^{19}F-labeled glycans can be challenging, often demanding different protocols depending on the position(s) to be labeled on the glycan residue.[42,43] The

enzymatic incorporation of ^{19}F-labeled monosaccharides in glycoconjugates is an interesting avenue to access complex glycan probes,[44] but ^{19}F-monomers are not always accepted by enzymes.[41]

In contrast to other isotopic labels, the introduction of ^{19}F can have significant effects on the physicochemical properties of the glycan.[45,46] The replacement of a hydroxyl group of the ring by a fluorine atom generates a C–F group, smaller in size compared to the C-OH group. Being the C–F bond highly polarized, the electron density of the whole structure is altered,[47,48] with repercussions on glycan conformation and interactions.[39] In addition, the extent and the site of fluorination can affect the glycan lipophilicity[49,50] and *in vivo* stability.[51,52,53] Thus, a careful placement of the ^{19}F atom in the glycan is key to avoid interference with the parameter under investigation (*e.g.*, conformation or binding), while preserving excellent sensitivity and spectral resolution.[46]

Recently, a collection of ^{19}F-labeled Lewis type 2 glycan antigens was prepared by AGA and screened against mammalian and bacterial lectins, as well as enzymes, in a simple ^{19}F-NMR assay.[38,46] Chemical shift perturbations or changes in peak intensity upon addition of the protein allowed to qualitatively assess the strength of the interaction (Figure 2A). The antigens were designed with the ^{19}F reporter in the lactose inner core subunit, distal from the binding site, to minimize the effects of the fluorine atom during the binding event. Control experiments demonstrated that the ^{19}F reporter did not affect the binding (Figure 2B) and could be placed far from the glycan binding site, while preserving excellent sensitivity (Figure 2C).

Deoxyfluorination implies a drastically weaker H-bond donation ability, compared to the hydroxyl group. Thus, the OH/F exchange could identify the contribution of individual hydroxyl groups engaged in hydrogen bonds with a protein.[54,55,56] This strategy was used to pinpoint the hydroxyl groups of Glc, Man, and Gal involved in lectin binding and highlighted those that can be chemically modified without affecting binding.[57] The simultaneous screening of the ^{19}F-monosaccharide library in the presence of a lectin was performed using a ^{19}F-NMR T_2 filtering-based assay. The drastic reduction of the ^{19}F peak intensity in the presence of the protein indicated the bound compounds (Figure 3).

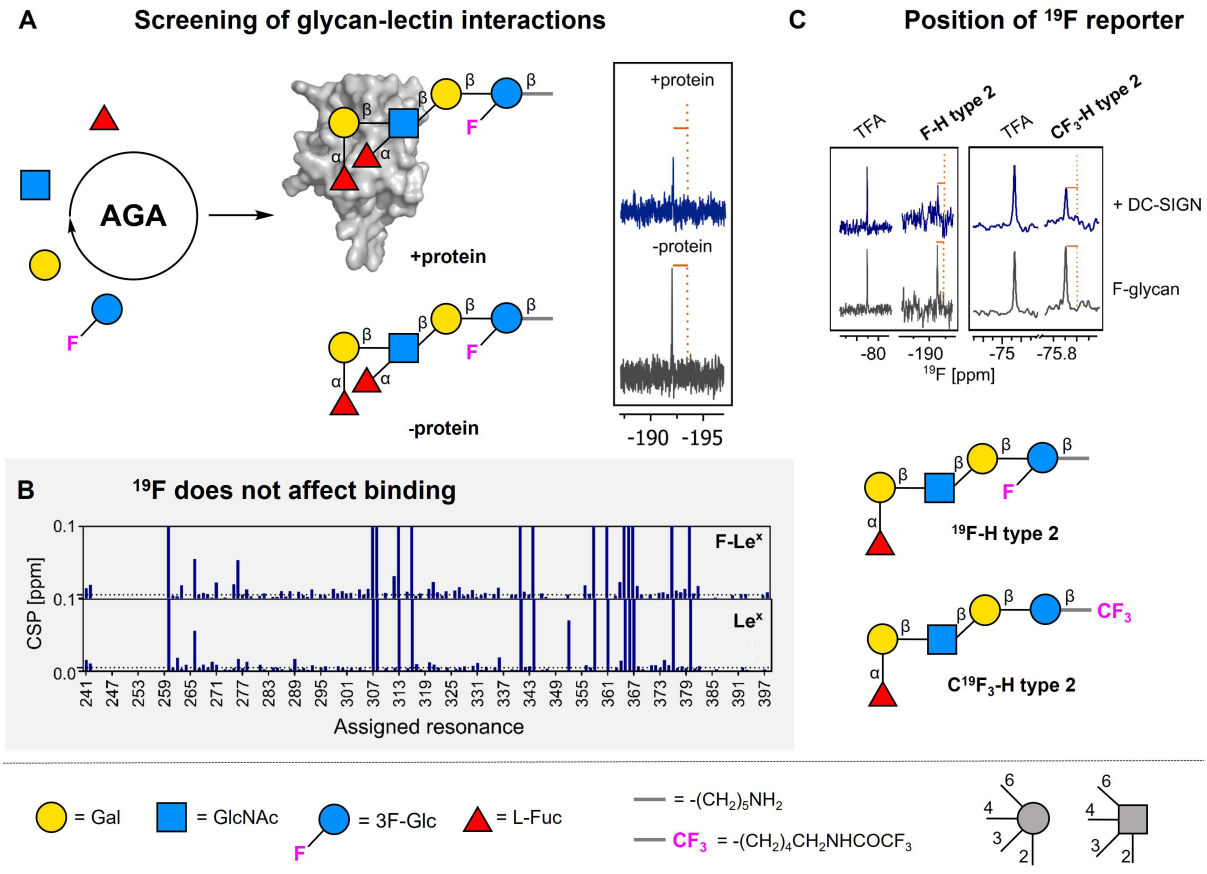

Figure 2. A. A collection of ^{19}F-labeled Lewis type 2 antigen analogues was prepared by AGA. The F-glycans were screened against proteins, including mammalian and bacterial lectins, as well as enzymes. B. Chemical shift perturbation plot showing that **^{19}F-Le^x** perturbed resonances similarly to the non-fluorinated **Le^x**. C. Carr-Purcell-Meiboom-Gill (CPMG) NMR spectra of **^{19}F-H type 2** and **C^{19}F-H type 2** alone (grey) and in presence of the mammalian lectin DC-SIGN (blue). DC-SIGN binds to both ^{19}F-glycans as shown by a decrease in peak intensity in presence of protein (orange lines). The monosaccharides are represented following the symbol nomenclature for glycans (SNFG). Figure adapted from Ref. [46].

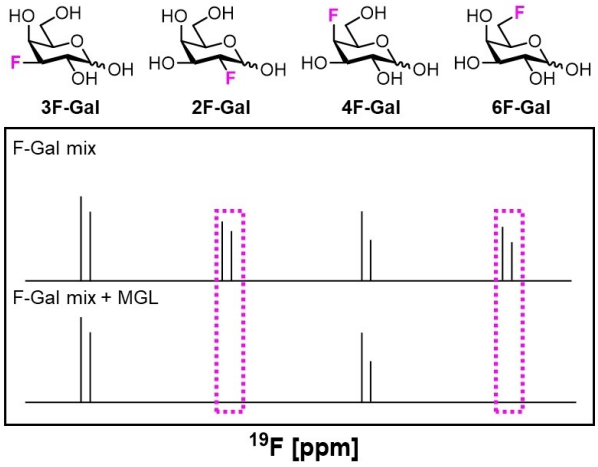

Figure 3. Systematic OH/F substitution of Gal monosaccharide generates a library of eight monofluorinated molecules to be screened by ^{19}F-NMR (top). Cartoon representation of ^{19}F-NMR spectra of the F-galactose mixture in absence (upper panel) and in presence (lower panel) of the human Macrophage Galactose-type Lectin (MGL) (bottom). For additional details we refer to Ref. [57].

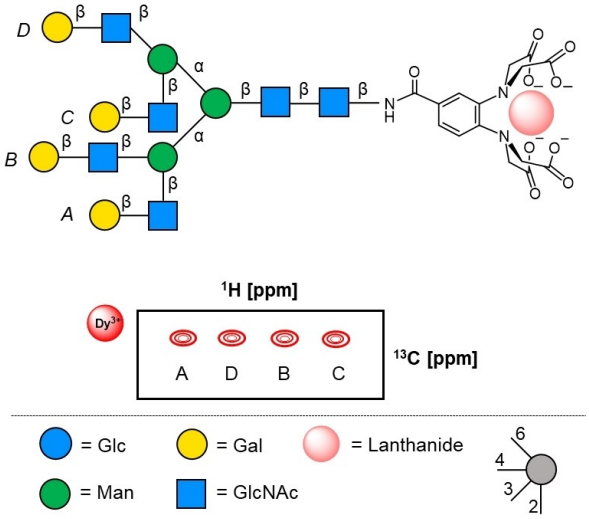

Figure 4. Tetra-antennary lanthanide-tagged N-glycan and cartoon representation of a ^{1}H-^{13}C HSQC NMR spectra of the tetra-antennary N-glycan conjugate loaded with dysprosium (paramagnetic) showing distinct NMR signals for each Gal of the N-glycan. The monosaccharides are represented following the symbol nomenclature for glycans (SNFG). For additional details we refer to Ref. [15].

Glycans with Lanthanide Tags

An alternative strategy to isotopic labeling is the use of lanthanide tags. The glycan is functionalized with a tag capable of complexing a paramagnetic ion, which induces significant chemical shift changes in the glycan due to dipolar interactions with the metal unpaired electrons (*i.e.* pseudo-contact shifts, PCS).[58] This strategy increases chemical-shift dispersion facilitating chemical-shift assignment.[59] The PCSs depend on the distance and the angle between the nuclei and the metal; thus, they contain conformational information over a range of 30 Å.[60] Rigid tethers between the glycan and the lanthanide tag, often based on aromatic moieties, are preferred as they minimize paramagnetic averaging effects.[61,62] The tethers are generally installed at the reducing end of the glycan in a late stage of the synthesis, followed by complexation with the lanthanide ion (*e.g.*, La^{3+}, Dy^{3+}, Tm^{3+}, Tb^{3+}). PCS-derived chemical shift changes permitted the discrimination of the individual branches in complex bi-antennary,[60,63] tetra-antennary (Figure 4),[15] and high-mannose-type *N*-glycans.[64] The involvement of each individual branch of the tetra-antennary *N*-glycan in the recognition by two *N*-acetyl-lactosamine-binding lectins could be described with unprecedented resolution, confirming major interactions between the Gal and GlcNAc residues at the A, C, and D arms with the *Datura stramonium* seed lectin.[15]

Conclusions and Outlook

NMR spectroscopy is the leading method for determining the three-dimensional structure and dynamic of carbohydrates in solution phase and a fundamental tool to study protein-glycan interactions.[11,12,65] However, as molecular mass and complexity increase, NMR spectra become progressively more difficult to interpret and unambiguously assign due to spectral crowding. We demonstrated that labeled glycans or lanthanide tags are valid approaches for addressing these problems and simplify the analysis.[60,63]

To date, the complexity of glycan synthesis has often limited these NMR studies to small collections of short and relatively simple glycans. Recent advances in synthetic methodologies as well as the advent of automated techniques granted unprecedented access to oligo- and polysaccharides. We imagined that these technologies will fuel the production of broad libraries of complex labeled glycans, permitting to monitor a specific site of a glycan within the bigger structure. So far, much effort was directed to the study of mammalian glycans. In the future, we expect that this approach could be extended to explore complex bacterial polysaccharides or glycosaminoglycans.[36]

Among all NMR-active nuclei, the ^{19}F nucleus stands out due to its unique properties.[37] Broad collections of ^{19}F-glycans with different chemical shifts could be designed for rapid real-time detection of binding to a protein. The installation of ^{19}F reporters in polysaccharides extracted from natural sources could also be exploited as an alternative strategy to labeling with UV-Vis active chromophores. Furthermore, the absence of ^{19}F in biological samples could open the way to *in cell* NMR studies, often hampered by high background signals.

With increasing molecular complexity, the implementation of multiple labels could facilitate the NMR analysis even further. These analyses should be carried out with proper control studies to confirm that the modification, albeit small, does not influence the results. Molecular dynamics simulation could provide some guidelines for such studies.[66] On the other hand,

force fields for MD are in a constant refinement process to overcome the pitfalls (*e.g.* chair conformational equilibria) and experimental validation is a fundamental tool to improve the accuracy of their prediction.[4,66]

Lastly, labeled glycans could be useful probes for solid state NMR, revealing important aspects of glycan interactions[67] and aggregation.[68]

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Conflict of Interest

The authors declare no conflict of interest.

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