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Glycan structures and their interactions with proteins. A NMR view

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Carbohydrate molecules are essential actors in key biological events, being involved as recognition points for cell–cell and cell–matrix interactions related to health and disease. Despite outstanding advances in cryoEM, X-ray crystallography and NMR still remain the most employed techniques to unravel their conformational features and to describe the structural details of their interactions with biomolecular receptors. Given the intrinsic flexibility of saccharides, NMR methods are of paramount importance to deduce the extent of motion around their glycosidic linkages and to explore their receptor-bound conformations. We herein present our particular view on the latest advances in NMR methodologies that are permitting to magnify their applications for deducing glycan conformation and dynamics and understanding the recognition events in which there are involved.

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Introduction

Molecular recognition of glycans is a very complex process. The exquisite selectivity of their biological receptors (lectins, antibodies, enzymes) relies on solving the fragile balance between entropy (dynamics-rigidification, solvation-desolvation, hydrophobicity) and enthalpy elements (hydrogen bonds, CH-π and van der Waals, coulombic, water-receptor and ligand interactions) [1–4], also considering the role of features as presentation of epitopes and multivalency [5,6]. Understanding these features has many implications in chemical biology and for drug discovery [7].

There is a vast collection of NMR methodologies that can be employed, usually in combination with other NMR methods and/or additional techniques that allow investigating glycans' geometry and dynamics as well as their interactions (Table 1), from dissecting the solution conformation of the key reaction intermediate, the glycosyl oxocarbenium ion [8] to monitoring sugar recognition features with great detail [9–11]. Significant perspectives have been recently published [12,13], and therefore we herein focus on the ultimate developments (last two years) and why we guess they are affording new breakthroughs in glycosciences.

Technical advances in NMR are of paramount importance. The access to new magnets, at or beyond 1 GHz, will provide major enhancements in sensitivity and resolution [14]. Given the inherent low chemical shift dispersion of saccharide NMR spectra, this fact should afford a real advantage. New molecular biology strategies employing diverse prokaryotic and eukaryotic cell types permit accessing to key glycan receptors, including glycoproteins, also labelled with stable isotopes (^{2}H, ^{13}C, ^{15}N) to perform detailed NMR studies [15–17]. Fantastic developments are also taking place in synthesis, which now provide chemically complex pure glycans in sufficient amounts to provide an exceptional three-dimensional view of large biologically relevant glycan geometries and dynamics [18,19].

Study of complex saccharides: labelling strategies to overcome low sensitivity and resolution problems

The study of the conformation and dynamics of large and complex glycans is a challenging task. The ^{1}H NMR signals of the nuclei at the pyranose or furanose rings display a narrow range of variability and therefore, they show a large degree of overlapping, which challenges the non-ambiguous identification of the key NMR parameters with conformational and dynamic information. Although dispersion in two or three dimensions alleviates this problem, the inherent sensitivity issue of NMR precludes the use of heteronuclear methods, unless stable ^{13}C/^{15}N isotopes (or ^{19}F) are introduced in the glycan [20]. However, in the last few years, the use of paramagnetic NMR, taking advantage of the presence of paramagnetic lanthanide atoms, attached to the glycan through efficient lanthanide-binding-tags (LBTs), has revolutionized oligosaccharide conformational analysis

Table 1

NMR strategies applied to the study of the conformation and interactions of glycans

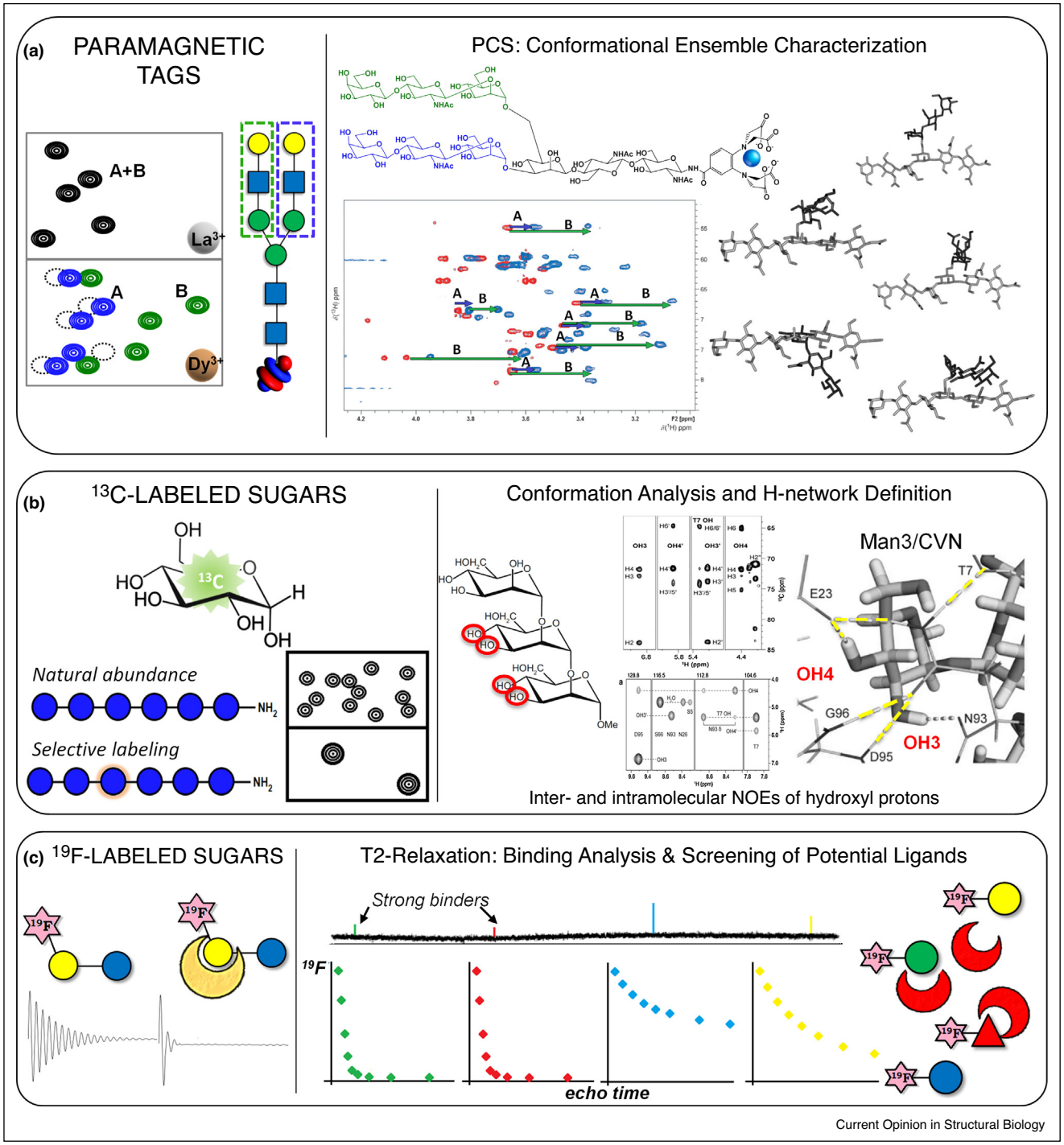
	NMR observables	NMR experiments	Specific applications
Conformational studies	Scalar couplings	1D-^{1}H	Ring conformations (puckering). Torsional angles. [Refs. 8,24••]
		1D-^{13}C	Ring conformations (puckering). Torsional angles. Glycosidic torsionals. ^{13}C-labelling required. [Ref. 9]
	Nuclear Overhauser and Rotating-frame Overhauser Effects	2D-NOESY/2D-ROESY	Ring conformations (puckering). Interglycosidic torsional angles. Intra-residue close contacts. [Refs. 26,28]
	Pseudocontact shifts (PCSs)	1D-^{1}H 2D-^{13}C-HSQC	Conformational space of complex structures deduced from anisotropic perturbations. [Refs. 21••,22•,23]
	Relaxation	^{13}C-{^{1}H}-CPMG	Structure dynamics and local molecular motions
Glycan-receptor interactions	Nuclear Overhauser and Rotating-frame Overhauser Effects	2D-Transferred-NOESY	Binding of small glycans to large receptors. Intermolecular ligand-receptor NOEs. [Refs. 11,42,58]
		2D-Transferred-ROESY	Chemical-exchange between free and bound ligands. [Ref. 64•]
		2D-^{13}C-HSQC-NOESY	Intermolecular glycan-receptor NOEs separated in the ^{13}C dimension to easily assign contacts through C-H preassigned pairs. [Ref. 30••]
		CNH-NOESY	Close contacts between ^{13}C nuclei from the ligand and ^{15}N nuclei from the protein. Requires ^{13}C-labelling and ^{15}N-labelling, respectively. [Ref. 29]
	Chemical shifts	2D-^{13}C-HSQC	Epitope mapping from the glycan point of view. Requires ^{13}C-labelling. Binding constants and binding dynamics (titration). [Ref. 29]
			Epitope mapping from the receptor point of view (hot-spot labeling). Used for complex/large systems. [Refs. 16,17]
		2D-^{15}N-HSQC	Epitope mapping from the receptor point of view. Requires ^{15}N-labelling. Binding constants and binding dynamics (titration). [Ref. 48]
	Pseudocontact shifts (PCSs)	2D-^{13}C-HSQC	Detailed analysis of the ligand epitope mapping in complex glycans. [Ref. 21••]
	Paramagnetic Relaxation Enhancement (PRE)	2D-^{15}N-HSQC	Epitope mapping from the receptor point of view. [Ref. 35]
	Diffusion	2D-DOSY	Changes in relative molecular sizes by complex formation. [Ref. 35]
	Relaxation	^{19}F-{^{1}H}-CPMG	Identification of binders. Screening of ^{19}F-labelled compound libraries. [Refs. 38–42]
		STD	Identification of binders. Epitope mapping from the glycan point of view. [Refs. 65,71•]
	Other	2D-STD-TOCSY	Epitope mapping from the glycan point of view, applied to complex glycans displaying acute signal crowding. [Ref. 37]

(Figure 1, panel a). The paramagnetic metal causes pseudo-contact shifts (PCS) in the NMR resonance signals of its adjacent nuclei. PCS contain conformational information, since they depend on the inverse of the third power of the metal-nucleus distance, permitting the estimation of the corresponding distances. On this basis, the conformational ensemble of bi-antennary [21••] and tetra-antennary complex-type [22•] and high mannose *N*-glycans have been deduced [23], expanding the knowledge on the conformational properties of these molecules.

Alternatively, automated glycan assembly (AGA) synthesis has permitted to study various well-defined hexasaccharides that display selectively ^{13}C-labelled glucose moieties at chosen positions (Figure 1, panel b) [24••]. This elegant scheme also breaks the chemical shift degeneracy, facilitating the specific NMR investigation of each glycosidic linkage and residue, including the individual $^{1}J_{CH}$ values that encode information on the relative population of axial/equatorial geometry of the anomeric proton. The authors experimentally determined $^{1}J_{CH}$ values, which are linked to the axial/equatorial orientation of the corresponding proton, and thus assessed the presence of minute amounts of the $^{1}C_{4}$ (D) chair conformations in the internal Glc residues of the Glcβ(1-6)-linked hexasaccharides.

It has been assumed that oligosaccharides do not display well-defined conformational patterns. However, Schubert *et al.* have demonstrated that all the Le^{X}-type branched saccharides show a well-defined three-dimensional structure, which is related to the presence of a C–H···O 'non-conventional' hydrogen bond that is in turn linked to the stabilizing stacking of a Fuc moiety versus a non-vicinal

Figure 1



Sugar labelling as NMR strategy for analyzing glycan conformation and their interaction with proteins. **(a)** The use of paramagnetic tags breaks the NMR signal degeneracy of sugars of the *N*-glycan branches and the PCS analysis, carrying distance restraints, provides information about glycan conformation. **(b)** The introduction of ^{13}C-labelled sugars reduces signal overlapping and gives access to key protein-carbohydrate intermolecular NOEs through isotope-edited experiments. **(c)** ^{19}F tags combined with T2 relaxation experiments as high throughput ligand screening method. Ligand binding increases ^{19}F relaxation rates and this effect can be exploited to differentiate binders and non-binders.

pyranose [25•]. As a required feature to provide this hydrogen bond, these two moieties should be attached to a third common one through α(1-3) and β(1-4) or α(1-4) and β(1-3) glycosidic linkages. The common pyranose is either Glc/GlcNAc, while the residue involved in the interaction with the Fuc unit may be Gal, GalNAc, Glc or GlcNAc. The presentation of the corresponding epitopes is clearly related to the achieved 3D-shape [26].

An intelligent use of ^{13}C-labelled sugars has been employed to address the influence of aromatic stacking on glycoside reactivity. Thus, the solvolysis and glycosylation of specifically designed ^{13}C-glycosyl donors and acceptors bearing aromatic platforms was evaluated and proved the ability of aromatic moieties to stabilize the intermediate glycosyl oxocarbenium ion [27]. Regarding molecular recognition, the presence of stable isotope labelling allow using ^{15}N and/or ^{13}C filtered NOESY experiments, competent experiments to study sugar-protein interactions, as mastered in the analysis of the recognition of heparan sulfate by a heparin binding protein. The conformation of the bound heparan sulfate and several intermolecular distance restraints were determined and used in the MD simulation of the complex that finally revealed the key electrostatic and hydrophobic protein-carbohydrate contacts [28]. Thus, the interaction between a ^{13}C-labelled Manα(1-2)Manα(1-2)ManαOMe trisaccharide (Man_3) and the virucidal lectin cyanovirin-N (CV-N) P51G (^{13}C/^{15}N double labelled), has been explored by Nestor *et al.* [29]. Although the system is rather challenging, since the interaction takes place in the slow-exchange regime in the chemical shift timescale, the authors wisely exploited a combination of ^{1}H-^{13}C and ^{15}N-^{13}C heteronuclear correlation experiments to determine carbohydrate-protein intermolecular NOE-derived distances and thus, to decipher the key lectin-Man_3 contacts.

Additionally, the same group was able to detect the NMR signals of the OH protons of the ^{13}C-labelled Man_3 sample at room temperature [30••], allowing the access to key recognition features of the molecule, impossible to characterize with unlabelled ligands. Thus, a comprehensive and unprecedented analysis of the OH directionality as well as the intra- and intermolecular hydrogen bond network present in a sugar-lectin complex in solution was provided taking advantage of intra- and intermolecular NOEs. Alternatively, an elegant NMR method to deduce the existence of inter-residual hydrogen bonds in oligosaccharides has recently been devised [31], which, in turn, has underscored the contribution of this feature to the glycan 3D conformational shape. As a technical advance, a simple and robust NMR strategy for observing the saccharide hydroxyl groups in a supercooled aqueous solution has been conceived [32].

The LBT-based paramagnetic approach has also been used for monitoring glycan-lectin recognition, even in multi-antennary glycans [21••,22•]. Since the PCS are different for the 'same' sugar residue at the different arms, it is possible to discriminate each residue at every branch using simple 1H-13C HSQC NMR spectra and examine the interacting glycan epitope through regular line width analysis or through standard STD experiments. This methodology has been successfully applied to study the interaction of complex-type glycans with model lectins and a variant of influenza hemagglutinin [21••], expanding the limits of application of NMR to this relevant biomedical problem [33]. An alternative strategy, using either a paramagnetic ion or a spin-label now attached to the receptor permits measuring additional NMR data. The analysis of the paramagnetic relaxation enhancements (PRE), which show a $1/r^6$ distance dependence, can be achieved by attachment of the TEMPO radical, and allow studying the conformational features of glycans and their interactions. Using this approach, Moure *et al.* have mastered the interaction between the Robo1 human protein and heparan sulfate [34]. The PRE provoked by TEMPO, which could be easily measured from the reduction in intensity of the protein cross-peaks in typical ^{1}H-^{15}N HSQC spectra or the ligand in 1D experiments, combined with MD simulations, allowed describing the dynamics and binding features of the process. Alternatively, by employing diffusion ordered spectroscopy experiments (DOSY) the authors selected the signals of the bound ligand to deduce, by means of the obtained PRE constraints, the location and orientation of the tied sugar. The attachment of the TEMPO radical to the sugar may also open new avenues to monitor interactions, with possible added values if the sugar is labeled [35].

Fluorinated sugars are also largely employed in chemistry and biology [36]. Obviously, the spectral dispersion of the ^{19}F-NMR signals facilitates the NMR study of fluorine-containing saccharide molecules and their interactions (Figure 1) [37]. Moreover, the relaxation properties of ^{19}F, with a high chemical shift anisotropy, make fluorinated sugars ideal for detecting weak interactions. The changes in linewidth of the ligand ^{19}F NMR signals of monofluoroacetamide and difluoroacetamide GlcNAc-containing oligosaccharides upon WGA binding have been used to efficiently monitor the lectin-chitooligosaccharide interactions [38]. Alternatively, T_2-filtered ^{19}F NMR relaxation experiments, which take advantage of the increased relaxation rate $R_{2,obs}$ of protein binders, have allowed screening a library of 2-deoxy-2-trifluoroacetamido-α-mannoside analogues to Langerin and deducing secondary binding pockets at the lectin surface, in the proximity to the canonical calcium binding site [39]. The same experiment has been used to monitor the binding of a library of monofluorinated sugars by DC-SIGN [40] and LecA [41], related C-type lectins. Specifically, the hydroxyl groups of the natural sugars were replaced one by one with fluorine, which permitted establishing the fundamental

protein-carbohydrate polar interactions. Strikingly, unexpected binding of DC-SIGN to specific fluorinated Gal moieties was observed, facilitating the derivation of a second binding pose of the histo blood human B-type antigen to this lectin related to the immune system [42].

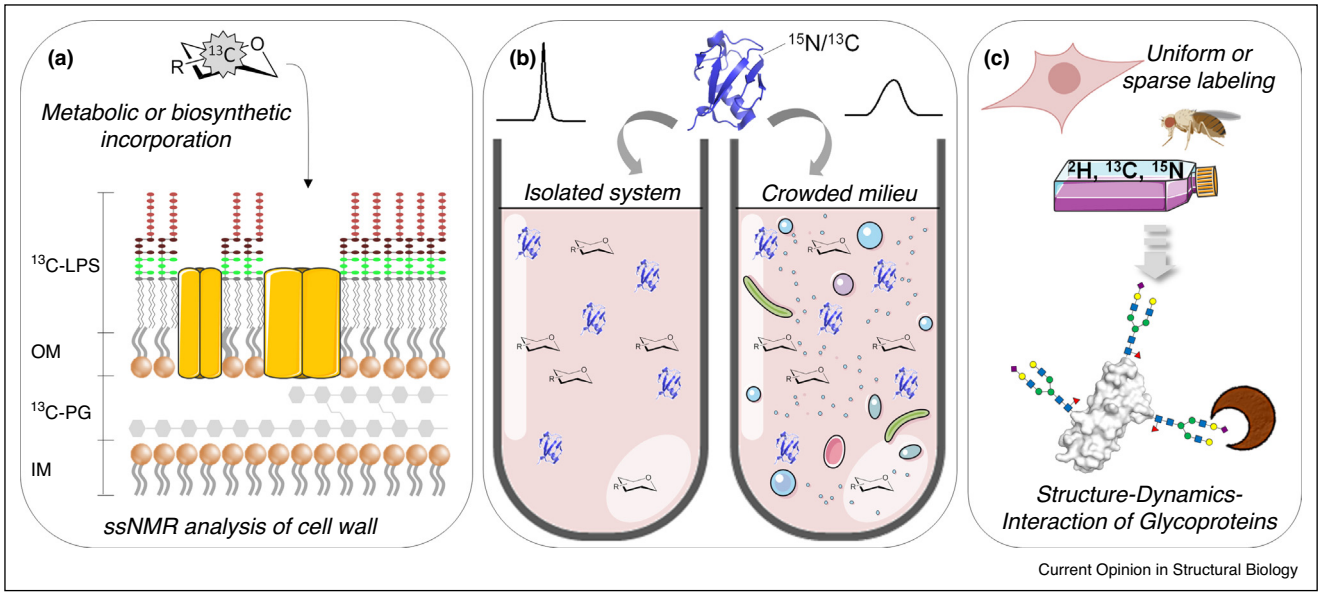
Addressing sugar functions in the cell microenvironment

Probably, one of the frontiers of the application of NMR methods for understanding glycan recognition is the development of robust in-cell protocols. Different approaches are being developed toward monitoring these processes using analogous conditions to those *in vivo* (Figure 2). For instance, the combination of solid-state NMR (ssNMR) methods with Dynamic Nuclear Polarization (DNP) has been employed to elucidate the LecA lectin residues involved in galactose recognition without isotope labeling production [43•]. The methodology used a ligand attached to a paramagnetic tag that under DNP conditions allowed scanning the protein-sugar interface and located the binding site. The PG and lipopolysaccharide (LPS) components of the Gram-negative bacterial cell wall have been also analyzed by solution [44,45] and ssNMR methods [46•]. Significant findings related with the transport and recognition of LPS have been achieved. Moreover, the recognition of the histo-blood group oligosaccharides by virus-like particles has been examined through standard STD-NMR experiments, illuminating their specific recognition features by key proteins on the capsid [47].

Trying to mimic the cell environment, the interaction between lactose and human galectin-3 (hGal3) has been studied in a crowded *milieu* [48], containing fairly large concentrations of the albumins (BSA-I, and HSA-I), as well as synthetic Ficoll and PEG 3350 polymers. Viscosity-driven effects were observed in the ^{1}H-^{15}N HSQC spectrum of hGal3 upon addition of the polymers, while broadening of the galectin signals was observed in the presence of HSA and BSA, which were explained by the existence of quinary interactions generated by the formation of large complexes between hGal3 and some glycoproteins that accompanied the albumin preparations.

Other milestone of the application of NMR in glycosciences is the study of intact glycoproteins [49–51]. Their intrinsic heterogeneity, which may be linked to its function makes the NMR analysis rather challenging. A paradigmatic example is the derivation of the *N*-glycan structures of antigenic variants of the major capsid protein from chlorovirus PBCV-1, a major task achieved by the combination of NMR/MS, as key step to deduce the corresponding virus-encoded glycosyl transferases [52]. Nevertheless, in the last few years, NMR methods have been applied to get information on the structure and dynamics of the glycans within glycoproteins [53], as exemplified in the study of the glycosylated IgE

Figure 2



Major approaches employed for the study of glycan structures and their interactions in mimicking cell environments by NMR. **(a)** The metabolic or biosynthetic incorporation of ^{13}C labelling combined with ssNMR methods allows the direct observation of intact PG and LPS in membrane-like environments (OM, outer-membrane; IM, inner-membrane). **(b)** The study of protein-carbohydrate interactions in crowding media highlighted protein quinary interactions with the cellular *milieu*. **(c)** Recombinant overexpression of isotope labelled glycoproteins allows analyzing the glycan effects on the structure, dynamics or molecular recognition of the entire glycoconjugate.

high-affinity receptor (FcεRIα) expressed in human HEK 293 cells. Chemical shifts and differences in signal line-broadening between folded and unfolded states were used as reporters of glycan solvent accessibility that allowed building a 3D model of the glycoprotein. The study was developed even further and included the direct detection of molecular recognition processes between the *N*-glycans of the intact glycoprotein and hGal3 [54••] Obviously, from the technical perspective, the methodology uses uniformly isotope labelled samples generated in insect or mammalian cells [55]. Interestingly, a new methodology based on the comparison of NMR data with MD predicted observables has been recently proposed to characterize sparsely labelled large glycoproteins [56]. A label-free approach has been proposed [57•], and applied to deduce the glycan composition analysis of therapeutic monoclonal antibodies, which requires however protein denaturation [15].

Standard ligand-based or protein-based NMR methods continue being essential to decipher glycan molecular recognition features [58–62] and established protocols are being further developed [63•]. A comprehensive study combining these NMR protocols (STD, HSQC, EXSY) with ITC and modelling has been used to explore the recognition of the histo-blood group antigens by hGal3 [64•] The authors deduced the key role of the conformational entropy of the ligands in the interaction process. Fittingly, the Fuc residue in the tetrasaccharide antigens does not contact with the lectin. However, the tetrasaccharides display much higher affinity than the parent trisaccharides. The role of the Fuc is to rigidify the glycosidic linkages, thus providing the proper presentation of the sugar epitope without a major entropy penalty, which is much larger for the constituent LacNAc, H-type II or galili fragments.

As additional examples, the interactions of different oligosaccharides, glycopeptides and glycomimetics, versus C-type lectins have been described [65]. In this context, selective glycomimetics versus different C-type lectins are being developed using a NMR fragment-based approach [66], which demonstrated the presence of allosterism in langerin and DC-SIGN [67]. A combined X-Ray/NMR approach has demonstrated that fluorination of biphenyl mannosides allows the establishment of perfect π–π stacking interactions with the tyrosine gate of FimH, a relevant biomedical target [68]. As additional examples, the binding sites for the alginate trisaccharide on the surface of β-lactoglobulin have been identified using a chemical shift perturbation analysis [69], highlighting the possible existence of a protein dimer-monomer equilibrium dependent on pH.

Other recent studies related to this issue have been focused on antigen/antibody interactions. In this context, NMR may be used as a tool to elucidate the structural details that play a key role in molecular recognition events in solution involving glycans [70]. As paramount example, in the quest of saccharide-based vaccines versus tropical diseases, a detailed multidisciplinary study using synthetic, NMR, and immunological methods has demonstrated that the key epitope of the Sp1 oligosaccharides displays a helix structure when interacting with the corresponding monoclonal antibodies. A minimum repeat of 9 residues is required to achieve a significant immune response [71•]. Glycans are not only relevant to humoral immunity by modulating antigen/antibody recognition but could also be important for antibody/receptor interactions [72], which are both glycosylated. Recent studies have reported NMR approaches for addressing the glycosylation profiles in these systems and their potential relevance in molecular recognition [51,54••].

Conclusions

Structural biology is undergoing the cryo-EM revolution. Fantastic advances are expected in the molecular recognition field, including unraveling details on essential glycan's interactions in biology. However, given the genuine mobility of the glycosidic linkages of glycans, NMR will continue occupying a predominant role for accessing structural and dynamic details of glycans in their free and bound states. The combination of magnets beyond the GHz with the explosion of robust methodologies in chemical biology allowing stable isotope labelling, complex N-glycan synthesis and the access to glycoproteins, including therapeutic glycosylated antibodies, together with the democratization of novel technologies in NMR will produce a burst of NMR applications in glycosciences, going into the cell. The future is already here, and the continuous development of new methodologies in solid state, DNP and in-cell NMR ensures addressing the precise glycan roles in nature.

Conflict of interest statement

Nothing declared.

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