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MINI-REVIEW

Paramagnetic NMR probes for characterization of the dynamic conformations and interactions of oligosaccharides

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Abstract Paramagnetism-assisted nuclear magnetic resonance (NMR) techniques have recently been applied to a wide variety of biomolecular systems, using sophisticated immobilization methods to attach paramagnetic probes, such as spin labels and lanthanide-chelating groups, at specific sites of the target biomolecules. This is also true in the field of carbohydrate NMR spectroscopy. NMR analysis of oligosaccharides is often precluded by peak overlap resulting from the lack of variability of local chemical structures, by the insufficiency of conformational restraints from nuclear Overhauser effect (NOE) data due to low proton density, and moreover, by the inherently flexible nature of carbohydrate chains. Paramagnetic probes attached to the reducing ends of oligosaccharides cause paramagnetic relaxation enhancements (PREs) and/or pseudocontact shifts (PCSs) resolve the peak overlap problem. These spectral perturbations can be sources of long-range atomic distance information, which complements the local conformational information derived from *J* couplings and NOEs. Furthermore, paramagnetic NMR approaches, in conjunction with computational methods, have opened up possibilities for the description of dynamic conformational ensembles of oligosaccharides in solution. Several applications of paramagnetic NMR techniques are presented to demonstrate their utility for characterizing the conformational dynamics of oligosaccharides and for probing the carbohydrate-recognition modes of proteins. These techniques can be applied to the characterization of transient, non-stoichiometric interactions and will contribute to the visualization of dynamic biomolecular processes involving sugar chains.

Keywords Nuclear magnetic resonance spectroscopy · Paramagnetic effect · Conformational dynamics · Oligosaccharide · Lanthanide ion · Spin label

Introduction

Visualization of the dynamic behavior of biomolecules is currently an indispensable approach in every field of life science. This trend has been promoted by the development of biophysical methodologies, including various microscopic and spectroscopic techniques, along with computational simulation. It should be emphasized that progress resulting from the use of these methods has been boosted by the exploitation of chemical probes typified by fluorescent dyes and gold nanoparticles [1–4].

Nuclear magnetic resonance (NMR) spectroscopy is a potentially powerful technique for the atomic visualization of dynamic conformations and of interactions between biomolecules in solution. Needless to say, this technique provides glycochemists with valuable information regarding the covalent linkages and tertiary conformations of carbohydrate chains through chemical shifts, spin–spin couplings (*J* couplings), and nuclear Overhauser effects (NOEs) [5–8]. Protein structural biologists have reaped the maximum benefit of such NMR information. Through the development and improvement of hardware and software, along with sample preparation methodologies that include sophisticated stable isotope-

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labeling methods, they have successfully determined the atomic coordinates of proteins [9]. Carbohydrate NMR spectroscopy remains less mature in comparison with protein NMR studies [10]. In addition to the difficulties of sample preparation, carbohydrate NMR spectroscopists often suffer from the overlap of peaks due to the lack of variability of local chemical structures, as well as an insufficiency of conformational restraints provided by NOE data due to low proton density, compared with proteins. More essentially, the considerable number of degrees of freedom available to the internal motions of carbohydrate chains hampers traditional approaches for their conformational characterization [11].

Paramagnetism-assisted NMR approaches have recently been rekindled in the fields of NMR structural biology, including carbohydrate structural studies [12–15]. This revival has occurred in the context of the development of molecular biology-based sample preparation, in conjunction with the development of chemically designed paramagnetic probes as intelligence sources [16–23]. This article will outline how paramagnetic NMR techniques could be applied to the characterization of the conformational dynamics and interactions of sugar chains, so as to cope with the aforementioned difficulties in carbohydrate NMR spectroscopy.

Paramagnetic effects as sources of long-distance information

Magnetic dipole–dipole interactions between nuclei and an unpaired electron induce a variety of paramagnetic effects that modulate NMR signals according to their geometric relationships [24, 25]. For example, an effect known as paramagnetic relaxation enhancement (PRE) can dramatically increase NMR relaxation rates, resulting in line-broadening of signals from the nuclei spatially proximal to the paramagnetic center (Fig. 1) [26]. Pseudocontact shift (PCS), observed as a modification of chemical shift values, is caused by paramagnetic metal ions with anisotropic magnetic susceptibility (Fig. 1) [27, 28]. Table 1 lists the lanthanide probes often used during paramagnetic NMR analyses of biomacromolecules [14]. For example, Gd^{3+} is used as a strong PRE sauce but not for PCS observations because of its isotropic magnetic susceptibility. By using highly paramagnetic lanthanides such as Dy^{3+}, PCS can be measured for nuclei even around 40 Å from the ion. However, they often provide unfavorable relaxation enhancement for the spatially proximal nuclei, resulting difficulty of observing their NMR signals. Little paramagnetic ions provide less PCS and PRE and therefore can offer proximal distance information. The intensity of these perturbations shows an r^{-3}- (PCS) and r^{-6}-dependence (PRE) upon the distance (r) between individual nuclei and the unpaired electron. The unpaired electron, which has a large magnetic moment, can affect the NMR signals of nuclei surrounding it in a wide area through through-space dipole interactions [29]. Hence, when characterizing biomolecular conformations and interactions, PRE and PCS measurements provide long-distance information that is independent of NOE- and J coupling-derived information, which, in contrast, offer information regarding local conformations.

In addition to these benefits, the anisotropy of the magnetic susceptibility associated with paramagnetic ions tightly attached to the biomolecule causes its weak molecular alignment with the high magnetic field. This enables observation of internuclear residual dipolar couplings (RDCs), which depend on the orientation of individual vectors connecting two nuclear spins. Thus, RDC can be a source of information for characterizing the conformation and internal motion of biomolecules [30, 31].

$$R_2^{\mathrm{PRE}} = \frac{1}{15}\left(\frac{\mu_0}{4\pi}\right)\frac{\gamma_I^2 g^2 \mu_B^2 S(S+1)}{r^6}\left\{4\tau_c + \frac{3\tau_c}{1+(\omega_I\tau_c)^2}\right\} \quad (1)$$

$$\Delta\delta^{\mathrm{PCS}} = \frac{1}{12\pi r^3}\left[\Delta\chi_{\mathrm{ax}}(3\cos^2\theta - 1) + \frac{3}{2}\Delta\chi_{\mathrm{rh}}\sin^2\theta\cos 2\phi\right] \quad (2)$$

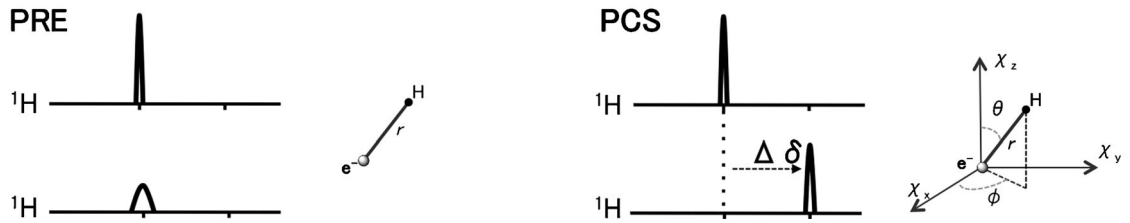

Fig. 1 NMR spectral perturbations caused by paramagnetic effects. The R_2 enhancement through dipole–dipole interactions is shown in Eq. (1), where μ_0 is the permeability of vacuum, γ_I is the gyromagnetic ratio of the nucleus, $\omega_I/2\pi$ is the Larmor frequency of the nucleus, g is the electronic g-factor, μ_B is the Bohr magneton, S is the spin, r is the distance between the paramagnetic center and the nucleus, and τ_c is the correlation time. The correlation time is defined as $1/\tau_c = 1/\tau_r + 1/\tau_e$, where τ_r and τ_e are the rotational correlation times of the molecule and the effective electron relaxation time, respectively. In Eq. (2) for PCS, $\Delta\chi_{ax}$ and $\Delta\chi_{rh}$ are the axial and rhombic components, respectively, of the anisotropic magnetic susceptibility ($\Delta\chi$) tensor. The polar coordinates of the nucleus, r, θ, and φ, are defined with respect to the paramagnetic center and the principal axis of the $\Delta\chi$ tensor. Adapted from Zhang *et al*. 2013 [12] with permission from The Chemical Society of Japan

Table 1 Magnetic properties of major metal ions used in paramagnetism-assisted NMR techniques

Paramagnetic				Diamagnetic
Relaxation reagent	Shift reagent (Little paramagnetic)	Shift reagent (Moderately paramagnetic)	Shift reagent (Highly paramagnetic)	
Gd^{3+}, Mn^{2+}	Eu^{3+}, Ce^{3+}, Sm^{3+}	Er^{3+}, Yb^{3+}	Tb^{3+}, Dy^{3+}, Tm^{3+}	La^{3+}, Lu^{3+}, Ca^{2+}

Paramagnetic probes of biomolecular NMR

Appropriate paramagnetic probes, *i.e.*, paramagnetic metal ions or spin labels, are required to observe paramagnetic effects. Paramagnetic NMR spectroscopy has traditionally been developed for the characterization of metalloproteins because they possess endogenous paramagnetic probes and have intrinsic metal ion-binding sites that can be used to incorporate paramagnetic ions [32, 33]. Many lectins harbor metal ions such as Ca^{2+} at their carbohydrate recognition sites [34] and, therefore, would be well-targeted by this approach [35–38]. Metal coordination by Lewis X was probed using paramagnetic effects induced by Mn^{2+} or Co^{2+} ions that were substituted for the physiologically coordinated Ca^{2+} ion [39].

NMR shift reagents containing lanthanide ions or paramagnetic relaxation reagents such as Gd^{3+} have been employed as exogenous paramagnetic probes to resolve peak overlap problems and identify peaks originating from the probe-accessible sites [40]. Thus, modulation of the NMR spectrum upon addition of these reagents into a solution containing a target molecule facilitates spectral analysis. Moreover, paramagnetism-induced spectral changes can offer useful information about molecular conformation, given that the probe preferentially binds a specific site on the molecule [41].

Sophisticated paramagnetic approaches based on a more general method for the site-specific attachment of paramagnetic probes onto target recombinant proteins have recently been developed. These probes are typically attached through disulfide formation at a mutationally introduced cysteine residue [42]. A variety of paramagnetic tags containing an ion-binding derivative, such as a metal-binding peptide [18, 21, 23] or a synthetic metal chelator, have been created to immobilize the paramagnetic center on diamagnetic proteins [16, 17, 20]. Stable organic radicals, such as the nitroxide radical, are also widely utilized for the site-directed spin labeling of proteins [22].

Paramagnetic probes can be site-specifically incorporated into the target protein by modification of their specific ligands, thereby providing information concerning microenvironments surrounding the bound ligand [43, 44]. A pioneering study by McConnell and coworkers achieved the detailed characterization of an antigen-binding site of a monoclonal antibody

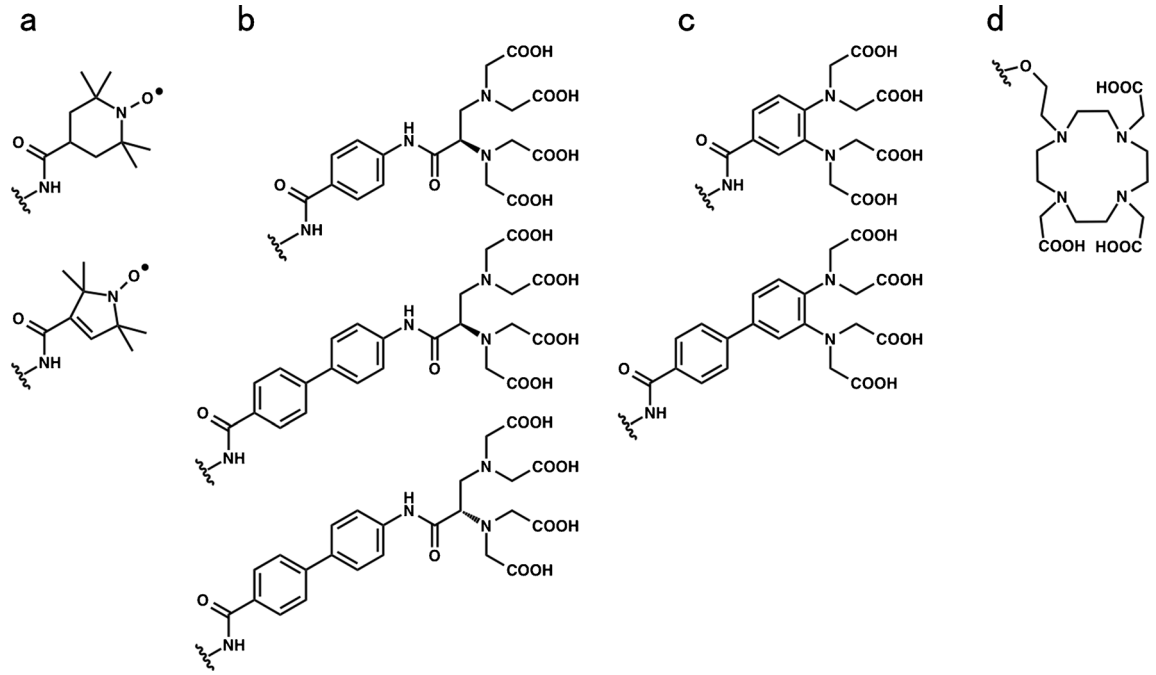

Fig. 2 Synthetic tags for sugar chains subjected to paramagnetism-assisted NMR analysis. **a** Nitroxide radicals for spin labeling [48, 49, 55, 78], **b** and **c** lanthanide-chelating units based on ethylenediamine [57–59, 61] and phenylenediaminetetraacetic acid [50, 53, 54, 56, 60], respectively, and **d** a DOTA (1,4,7,10- tetrakis (carboxymethyl)-1,4,7,10-tetraaza-cyclododecane) derivative [79]

directed against a spin-labeled dinitrophenyl hapten [45–47]. Thus, paramagnetic NMR methods are also useful for probing biomolcular interactions. Prestegard and co-workers employed spin-labeled ligands, for example an *N*-acetyllactosamine attached with a TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl) moiety (Fig. 2) at its reducing terminus, for mapping the carbohydrate recognition domain of galectin 3 (Gal-3 CRD) and the substrate-binding site of α-2,6-sialyltransferase (ST6Gal-I) [48, 49]. A geometric model of the disaccharide accommodated in the active site was constructed according to a complementary approach that included PRE, saturation transfer difference, and transferred NOE observations between the substrates and the protein. Lanthanide-tagged lactose has recently been used as a sensitive tool to monitor carbohydrate-lectin interactions via PCS detection [50].

Reciprocally, carbohydrate-binding proteins could be paramagnetically labeled in order to investigate the conformations of bound ligands. For sugar-lectin docking simulations, the Gal-3 CRD was fused to a lanthanide-binding peptide to provide PCSs and RDCs as conformational constraints for the bound lactose, complementing a single intermolecular NOE observed for the complex [51].

Conformational analyses of sugar chains based on paramagnetic effects

Paramagnetic tagging of oligosaccharides has recently been proposed for their conformational analysis. Immobilization of the paramagnetic center is crucial for accurate quantitation of observed paramagnetic effects in this approach, because motional freedom of the tag moiety causes ambiguities during data interpretation [52]. Hence, a selective amination reaction at the reducing terminus and subsequent formation of a rigid amide linkage is often employed for paramagnetic tagging of oligosaccharides. This method is applicable not only to totally

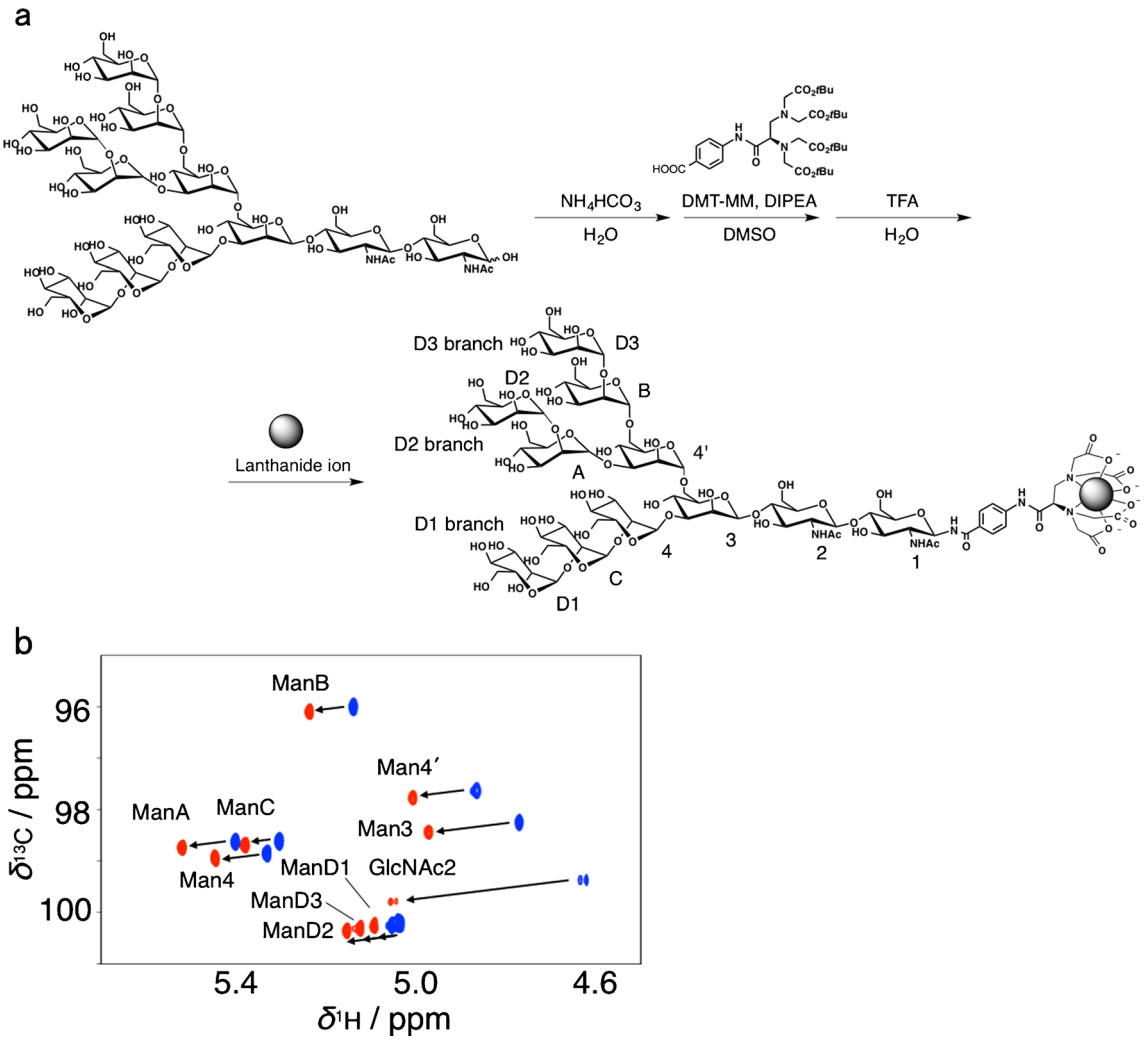

Fig. 3 PCS observation of high-mannose-type oligosaccharide M9. **a** Introduction of the paramagnetic lanthanide-chelating tag. **b** Anomeric region of the ^{1}H–^{13}C HSQC spectra of the tagged sugar complexed with Tm^{3+} (*red*) and La^{3+} (*blue*). Chemical shift differences induced by PCS are indicated by *arrows*. Adapted from Yamaguchi *et al*. 2014 [61] with permission from John Wiley and Sons

synthetic oligosaccharides [53, 54], but also to oligosaccharides obtained from natural glycoproteins and glycolipids [55, 56].

The rigidity of the ion-coordinating units and the choice of ion species are also important factors for appropriate PCS measurements with quantitative data interpretation. Several paramagnetic tags capable of chelating a metal ion have been developed based on phenylenediamine- or ethylenediaminetetraacetic acid (EDTA) and used to perform PCS measurements of oligosaccharides (Fig. 2) [50, 53, 57–59]. Lanthanide ions, such as Tm^{3+} and Dy^{3+}, introduced onto the reducing termini of target oligosaccharides using these reagents serve as useful paramagnetic probes for determining the tertiary conformations of oligosaccharides.

PCSs have been observed for *N*,*N*′-diacetylchitobiose covalently attached to an EDTA-based lanthanide-chelating tag at its reducing end [57, 58]. Using two-dimensional ^{1}H-^{13}C heteronuclear single quantum coherence (HSQC) spectra, PCS values were estimated as the differences in ^{1}H and ^{13}C chemical shifts between the carbohydrate complexes with a diamagnetic reference La^{3+} ion and those with a paramagnetic ion, *e.g.*, Tm^{3+}. The experimentally obtained PCS values exhibited excellent agreement with those estimated by back-calculation based on the reported 3D conformation of *N*,*N*′-diacetylchitobiose. This demonstrates that paramagnetism-assisted NMR could be applicable to the determination of the 3D conformations of oligosaccharides in solution, if the molecule could be sufficiently rigid. Furthermore, the introduction of lanthanide tags causes distinct PCSs even for identical non-reducing terminal groups in multiantenary oligosaccharides, resolving their severely overlapping peaks and facilitating spectral analysis [60, 61].

Exploration of the conformational dynamics of oligosaccharides

Unlike *N*,*N*′-diacetylchitobiose, most oligosaccharides are inherently flexible. To explore the conformational spaces of such flexible biomolecules, computational approaches such as molecular dynamics (MD) simulations are potentially very powerful. Note that simulation results depend heavily on calculation protocols, including initial structures and computational times. NMR spectroscopy can be employed for experimental evaluation of the results of calculation.

Erdélyi *et al*. successfully characterized the conformational space of lactose using an approach based on cross validation of selection of the conformations by inspecting Monte Carlo conformational search against their experimental PCS and RDC data [59]. This highlighted the utility of paramagnetic NMR approaches to give improved insight into the dynamic behavior of this disaccharide in solution.

MD-derived conformational ensembles occupied by a series of sialyl oligosaccharide moieties of gangliosides, including the GM3 trisaccharide, the GM2 tetrasaccharide, and the GM1 pentasaccharide, were also evaluated using the lanthanide tagging approach [53, 54, 56]. The PCS-validated conformational ensemble models of the GM1 and GM2 oligosaccharides share striking similarities, indicating that the outermost galactose residue has no significant impact on the conformation of the remaining parts of these glycans. By contrast, the GM3 trisaccharide exhibited a distinct conformational ensemble for the sialyl linkage due to the restriction of conformational freedom by the *N*-acetylgalactosamine branch.

PCS-based conformational analysis has also been applied to complex-type and high-mannose-type oligosaccharides

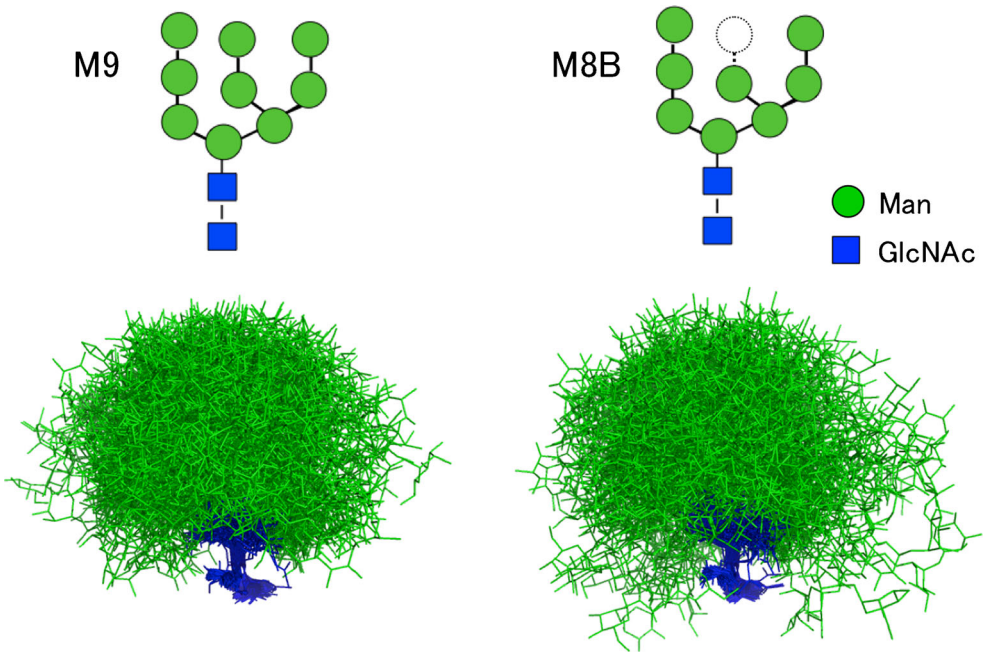

Fig. 4 Superimpositions of 240 conformers derived from NMR-validated replica exchange MD simulations of the high-mannose-type M9 (*left*) and M8B (*right*) oligosaccharides. Adapted from Yamaguchi *et al*. 2014 [61] with permission from John Wiley and Sons

[60, 61]. A triantennary oligosaccharide containing nine mannose residues, M9, as well as its derivative M8B was overexpressed in genetically engineered yeast cells as fully ^{13}C-labeled forms [62, 63] and attached to an EDTA-based lanthanide-chelating tag (Fig. 3a). The experimental PCS values obtained for these oligosaccharides (Fig. 3b) were poorly simulated by conventional MD simulation techniques, even though the total calculation time periods were expanded to 3 microseconds. By contrast, simulation results obtained employing a generalized-ensemble algorithm finally satisfied the experimental data. The NMR-validated conformational ensembles of M9 and M8B indicated that the removal of the non-reducing terminal mannose residue from the D2 branch results in a significant expansion of the conformational space, with an increased population of the foldback conformations, in which the D2 and D3 termini gain access to the reducing-terminal residue (Fig. 4). These results are qualitatively consistent with previous reports based on chemical shift, *J* coupling, NOE, and PRE data [55, 62, 64–70] and, furthermore, give quantitative insights into conformational fluctuations of this biologically important class of oligosaccharides [71, 72].

Perspectives

Paramagnetism-assisted NMR spectroscopy provides long-range atomic distance information that can be used to characterize the overall conformations of oligosaccharides and their dynamics in solution, complementing local conformation information derived from *J* couplings and NOEs. We have already mentioned that paramagnetically labeled ligands can probe the carbohydrate binding-sites of proteins, offering conformational information about the bound ligands. Finally, we should stress that paramagnetism-assisted NMR technique can be useful for characterizing non-stoichiometric interactions as well as transient interactions. Iwahara *et al.* successfully characterized transient encounter complexes formed between DNA and a transcription factor by inspecting PRE data [73]. This line of approach could be applicable for probing dynamic interaction processes involving sugar chains. In this sense, membrane systems will be fascinating targets for applying paramagnetic NMR approaches. The PRE-based techniques have been utilized to determine the conformations of ganglioside GM1 embedded in phospholipid bicelles [74] and for characterizing the interaction of amyloid β with GM1 clusters [75]. Paramagnetic probes are currently developed for magnetic resonance analysis of biomolecules in cellular environments [76, 77]. Design and creation of novel paramagnetic probes will open up new avenues for in-cell and on-cell NMR approaches, as well as magnetic resonance imaging toward the visualization of dynamic biomolecular processes of glycobiological interest.

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Conflict of interest The authors declare that they have no conflicts of interests.

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