Dynamic aspects of antibody:oligosaccharide complexes characterized by molecular dynamics simulations and saturation transfer difference nuclear magnetic resonance

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Introduction

Cell surface carbohydrates can be considered as potent targets for recognizing pathogen infection or cancerous cells and are as such used as promising or already successful vaccine components against various pathologies (Jones 2005; Guo and Wang 2009; Hecht et al. 2009; Astronomo and Burton 2010). In the search for more potent vaccines, crystallographic investigations for finely describing carbohydrate protective epitopes were carried out (Cygler et al. 1991; Villeneuve et al. 2000; Vyas et al. 2002; Calaresu et al. 2003; Nguyen et al. 2003; Yuriev et al. 2005; Vulliez-Le Normand et al. 2008; Murase et al. 2009). Indeed, such a structural knowledge about carbohydrate:monoclonal antibody (mAb) interaction is thought to help in designing functional mimics of natural glycans for vaccination purpose. Alternatively, it could also help in improving the specificity of isolated anti-carbohydrate mAbs for diagnostic or therapeutic purposes (Chanteau et al. 2006; Pastan et al. 2007; Rachagani et al. 2009; Luallen et al. 2010), since this kind of mAbs is not necessarily easily obtained (Manimala et al. 2007).

The flexibility of carbohydrates when free is generally assumed (Demarco and Woods 2008; Landstrom and Widmalm 2010) and, consequently, it cannot be ruled out that parts of an oligosaccharide bound to a protein maintain some of their glycosidic linkages’ freedom (Canales et al. 2005; Clavel et al. 2007; Xu et al. 2009). Flexibility has been investigated on carbohydrate:Ab complexes on the Ab side (Krishnan et al. 2008) and, to our knowledge, only two studies using molecular dynamics (MD) simulations on such complexes have been published (Pathiaseril and Woods 2000; Kadirvelraj et al. 2006). The experimental validation of MD simulations of Ab:carbohydrate complexes may indeed be problematic (Woods and Tessier 2010), which probably does not encourage such approaches. In the meanwhile, saturation transfer difference (STD) nuclear magnetic resonance (NMR; MAYER M and MEYER B 1999, 2001) has been used...
successfully to study the protein recognition of carbohydrates (Angulo et al. 2006; Demarco and Woods 2008; Ernst and Magnani 2009), and more specifically the Ab recognition of carbohydrates in various situations (Johnson and Pinto 2008; Houlston et al. 2009; Enriquez-Navas et al. 2011). We thus reasoned that a quantitative analysis of experimental STD effects and their prediction using MD snapshots, thanks to the CORCEMA-ST approach (Jayalakshmi and Krishna 2002), could be efficient for structural description of oligosaccharide recognition by anticarbohydrate Abs. The aim of this paper is to demonstrate such feasibility and, therefore, to contribute to the confidence in the trajectories issued from MD simulations of protein:carbohydrate complexes.

The model chosen for this study is 

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\text{Shigella flexneri} \text{ 2a (SF2a), a Gram-negative enteroinvasive bacterium, known as the agent for endemic bacillary dysentery, or shigellosis (Kotloff et al. 1999; Kosek et al. 2010). The bacterial lipopolysaccharide is an important virulence factor, the polysaccharide moiety of which, the O-antigen (O-Ag), was identified as a major target of the host's protective immune response against reinfection (Levine et al. 2007; Kweon 2008). The SF2a O-Ag is built, to a large extent, by oligomerizing a pentasaccharide repeating unit (RU) AB(E)CD where A, B and C are } \alpha\text{-L-Rhap, } D \text{ is a } \beta\text{-D-GlcNAc and } E \text{ is an } \alpha\text{-D-Glc} \text{ (Figure 1; Simmons and Romanowska 1987). Non-stoichiometric O-acetylations of the basic RU were reported (Kubler-Kielb et al. 2007; Perepelov et al. 2009). In trying to identify fragments of SF2a O-Ag, which represent potential haptons of interest for vaccine development, the recognition of 20 synthetic O-Ag segments by 5 protective mAbs specific for this O-Ag was studied (Phalipon et al. 2006, 2009; Mulard and Phalipon 2008). In addition, the crystal complexes of mAb F22-4, a protective immunoglobulin G1 (IgG1) mAb, bound to [AB(E)CD]_2 and [AB(E)CD]_3 oligosaccharides (representing two and three RUs of the SF2a O-Ag, respectively) were obtained (Vulliez-Le Normand et al. 2008). Together with conformational studies on SF2a O-Ag, these crystal structures showed that mAb F22-4 is likely to recognize two O-Ag RUs of its cognate antigen in a conformation favored when free in solution (Theillet et al. 2011). In the following, we take advantage of the numerous structural and immunochromical data available for this model system, supported by additional physicochemical information provided herein, to probe the potency of a combination of STD-NMR and MD simulations, as a method to gain insights into the specificity and dynamics of interactions between a mAb and oligosaccharides related to its cognate antigen.

Results

Thermodynamic and kinetic data

In addition to IC_{50} data (Phalipon et al. 2006), the thermodynamic and kinetic constants of interactions between monoclonal IgG F22-4 and numerous synthetic SF2a O-Ag segments, whose synthesis were described previously (Costachel et al. 2000; Mulard et al. 2000; Belot et al. 2002, 2004; Segat-Dioury and Murlad 2002; Mulard and Guerreiro 2004; Table I) give essentially three important pieces of information. The first one is that the motif ECD is sufficient to reach sub-millimolar affinities. Second, when positioned at the non-reducing end side of ECD, thus generating the key SF2a branching pattern, residue B improves the binding free energy $\Delta G$ by about $-2$ kcal mol$^{-1}$, as observed from the comparison between ECD and B(E)CD or between ECDA' and B(E)CD. Finally, the measured rates with D_{0}AB(E)CD and AB(E)CD suggest that addition of the residue D_{0} at the non-reducing end of the biological RU increases the kinetic rates of dissociation $k_{off}$ by about a factor of 2.5. These two

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\begin{align*}
\text{Table I. Thermodynamic and kinetic constants of interactions between mAb F22-4 and synthetic fragments of SF2a O-Ag} \\
\hline
\text{Ligand} & k_{on} \times 10^{-5} \text{ M}^{-1} \cdot \text{s}^{-1} & k_{off} \text{ (s}^{-1}) & K_{D} \text{ (mM)} & K_{D}^{*} \text{ (mM)} & N_{b} \text{ (binding sites)} & \Delta H^{\circ} \text{ (kcal} \cdot \text{mol}^{-1}) & \text{TAS}^{\circ} \text{ (kcal} \cdot \text{mol}^{-1}) \\
\hline
\text{ECD} & \text{n.m.} & \text{n.m.} & \text{n.m.} & 104.4 \pm 0.9 & 1.98 \pm 0.01 & \text{n.m.} & \text{n.m.} & \text{n.m.} \text{ n.m.} & \text{n.m.} & \text{n.m.} & 4.0 \\
\text{ECDA'} & 2.5^{\circ} & 20^{\circ} & \text{n.m.} & 87 \pm 1 & 1.97 \pm 0.01 & \text{n.m.} & \text{n.m.} & \text{n.m.} & \text{n.m.} & 5.6 \\
\text{ECDA'B} & 1.0^{\circ} & 25^{\circ} & \text{n.m.} & 232 \pm 2 & 1.96 \pm 0.01 & \text{n.m.} & \text{n.m.} & \text{n.m.} & \text{n.m.} & 6.0 \\
\text{B(ED)CD} & 20 \pm 1 & 7.5 \pm 0.6 & 3.8 \pm 0.5 & 3.48 \pm 0.04 & 1.98 \pm 0.01 & \text{n.m.} & \text{n.m.} & \text{n.m.} & \text{n.m.} & 5.0 \\
\text{B(E)CD} & 7.0 \pm 0.2 & 2.4 \pm 0.1 & 3.5 \pm 0.2 & 3.35 \pm 0.08 & 2.01 \pm 0.01 & \text{n.m.} & \text{n.m.} & \text{n.m.} & \text{n.m.} & 6.8 \\
\text{AB(ED)CD} & 6.3 \pm 0.3 & 5.8 \pm 0.2 & 9.3 \pm 0.7 & 16.8 \pm 0.2 & 1.9 \pm 0.1 & \text{n.m.} & \text{n.m.} & \text{n.m.} & \text{n.m.} & 6.1 \\
\text{D_{0}AB(ED)CD} & 3.2 \pm 0.4 & 15 \pm 1 & 43 \pm 4 & \text{n.m.} & \text{n.m.} & \text{n.m.} & \text{n.m.} & \text{n.m.} & \text{n.m.} & \text{n.m.} \\
\text{B(ED)AB(E)CD} & 0.36 \pm 0.01 & 0.053 \pm 0.005 & 0.175 \pm 0.005 & 0.44 \pm 0.09 & 2.07 \pm 0.07 & \text{n.m.} & \text{n.m.} & \text{n.m.} & \text{n.m.} & 11.8 \\
\text{[AB(ED)CD]_2} & 0.83 \pm 0.02 & 0.13 \pm 0.01 & 1.5 \pm 0.1 & 1.04 \pm 0.03 & 2.04 \pm 0.02 & \text{n.m.} & \text{n.m.} & \text{n.m.} & \text{n.m.} & 9.9 \pm 0.3 \\
\text{[AB(ED)CD]_3} & 1.7 \pm 0.1 & 0.11 \pm 0.01 & 0.65 \pm 0.1 & 1.16 \pm 0.03 & 1.95 \pm 0.09 & \text{n.m.} & \text{n.m.} & \text{n.m.} & \text{n.m.} & 9.7 \pm 0.6 \\
\text{D_{0}AB(ED)AB(E)CD} & 0.59 \pm 0.3 & 0.29 \pm 0.02 & 5.0 \pm 0.5 & 4.8 \pm 0.2 & 1.99 \pm 0.02 & \text{n.m.} & \text{n.m.} & \text{n.m.} & \text{n.m.} & 10.6 \\
\hline
\end{align*}
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\text{n.m.: not measured.}
\text{\textsuperscript{a}Measured by stopped-flow fluorimetry (except for ECDA' and ECDA'B').}
\text{\textsuperscript{b}Measured by ITC.}
\text{\textsuperscript{c}Too fast for stopped-flow measurements.}
\text{\textsuperscript{d}Best fit of computed STD intensities to NMR experiments using } K_{D} \text{ measured by ITC.}
\text{\textsuperscript{e}Synthesis to be published elsewhere.}

\text{Fig. 1. The basic chemical structure of the RU of SF2a O-Ag (Simmons and Romanowska 1987).}
last results cannot be explained by analyzing the crystal structure of the F22-4:[AB(E)CD]2 complex (Vulliez-Le Normand et al. 2008), where residue B does not contact F22-4 and where residue D0 is not present in the ligand (Supplementary data, Figure S1 and Table S1).

In contrast, the crystal structure allows to understand why introduction of the B'(E')C' segment at the reducing end of a given ligand improves binding. Indeed, upon B(E)CDA' elongation with B'(E')C', residue E' establishes hydrogen bonds with the Ab (Supplementary data, Figure S1 and Table S1), which provoke a decrease in the $k_{\text{off}}$ rate of more than an order of magnitude.

**Interactions as observed by NMR**

While the nuclear overhauser effects (nOes) of the free oligosaccharides were null to slightly positive at 600 MHz, negative transferred nOes (trnOes; Clore and Gronenborn 1982) are observed with pentasaccharides AB(E)CD, B(E)CDA' and ECDAB' in solution with mAb F22-4 (Supplementary data, Figure S2). The proximity of the H-6 of rhamnose C to H-3 of glucose E is detected when AB(E)CD and B(E)CDA' interact with F22-4—not observable for ECDAB' due to overlays of the resonances—whereas no proximity between H-6 of rhamnose C and H-1 of glucose E can be noticed. This is consistent with the Ab recognition of these two pentasaccharides in a conformation similar to the one adopted by the non-reducing end unit of [AB(E)CD]2 in the crystal complex.

Although some relatively low $k_{\text{off}}$ rates and the high molecular weight of IgG F22-4 induce significant spin diffusion, the kinetic characteristics of the studied interactions allowed to discriminate contacting and non-contacting ligand protons by STD-NMR measurements for the interactions between F22-4 and SF2a O-Ag fragments AB(E)CD, B(E)CDA', ECDAB', ECD'A' and D0AB(E)CD, respectively (Figure 2, Supplementary data, Table S2). Alkyl proton resonances of residues E, C and D are indeed clearly more perturbed than those of residues A, B, A', B' and D0, so that differences of STD intensities between protons give average absolute deviations of ~0.2. They equal exactly to 0.19, 0.17, 0.22, 0.14 and 0.22 for interactions implying AB(E)CD, B(E)CDA', ECDAB', ECD'A' and D0AB(E)CD, respectively. It means that residues E, C and D experience closer and more continuous contacts to mAb F22-4 during the interaction of every evoked oligosaccharide. This is again consistent with an interaction in the part of the Ab binding site recognizing the non-reducing unit of [AB(E)CD]2 in the crystal complex, where residues E, C and D are the most buried in the mAb binding site, establishing the important interactions with the Ab.

**MD simulations**

Taking into account the high intrinsic flexibility of oligosaccharides, one can suspect that the ligand maintains some flexibility within the complexes. Therefore, we performed MD simulations in explicit solvent of the interactions between mAb F22-4 and AB(E)CD, B(E)CDA', ECDAB', ECD'A' and D0AB(E)CD, respectively, using the AMBER suite of programs and its affiliated force fields PARM99 and GLYCAM04 (Case et al. 2005; Kirschner et al. 2008). Using as starting structures truncated versions of the crystal complex F22-4:[AB(E)CD]2, with the oligosaccharides in the part of the Ab recognizing the non-reducing unit of [AB(E)CD]2, we observed in every MD trajectory that the ECD motif is constantly tightly bound to the mAb via persistent hydrogen bonds and remains in the same conformation as in the co-crystal, whereas the residues A, B, A', B' and D0 show a higher degree of flexibility (Figures 3 and 4). These residues do not seem to establish strong interactions with the Ab during the MD simulations.

Besides, these last residues display similar linkage flexibilities in the MD trajectories of the free oligosaccharides and of the complex (Figure 4). Moreover, MD simulations of these SF2a oligosaccharides free in solution reveal that the E-C linkage conformation in the complex has a higher relative conformational energy in “linear” ECD, ECD'A' and ECDAB' than in branched AB(E)CD, B(E)CDA' and D0AB(E)CD ($\Delta$ $\approx$ 1 kcal. mol$^{-1}$). This linkage is also more flexible in linear oligosaccharides and thus induces higher conformational entropic losses upon interaction with F22-4, which is likely to reach 1 kcal. mol$^{-1}$ per linkage (Megavini et al. 2005). The conformational behaviors of the branched oligosaccharides are similar to the one of the SF2a O-Ag, as reported previously (Theillet et al. 2011).

**STD predictions based on rigid and dynamic structural models**

Using the CORCEMA-ST approach (Jayalakshmi and Krishna 2002) on artificially truncated forms of the crystal complex corresponding to the trnOe results, average differences of 0.11, 0.08, 0.16 and 0.14 were obtained between predicted and experimental STD-NMR normalized intensities for AB(E)CD, B(E)CDA', ECDAB' and ECD'A', respectively (Figure 2, Supplementary data, Table S2). The STD intensities of residues A' and B' are systematically slightly underestimated by the prediction, as well as those of residues E and C but to a lesser extent. The best score obtained for B(E)CDA' is partly due to the weakest dissociation constant $k_{\text{off}}$ provoking large spin diffusion and low disparity among the different protons STD intensities.

STD intensities calculated by averaging results from MD snapshots were in better agreement with NMR measurements than those based on the fixed coordinates taken from the crystal structure. Mean differences between MD predicted STD intensities and experimental data equal to 0.09, 0.08, 0.07 and 0.09 for interactions implying AB(E)CD, B(E)CDA', ECDAB' and ECD'A', respectively (Figure 2, Supplementary data, Table S2). Since the glycosidic linkages D-A' and A'-B' explore a large conformational space across the MD trajectories, residues A' and B' come close to some saturated Ab methyl groups and thus experience more saturation in these modeled dynamic complexes than anticipated from their fixed position in the crystal complex. Compared with the prediction from the crystal structure, a sparsely enhanced saturation of residues E and C, which are almost fixed during the MD trajectories, is also observed, which is probably due to the mobility of the Ab amino acids side chains.
Kinetic constants for interaction between ECDA′ or ECDA′B and mAb F22-4 were obtained from best fit of the STD prediction to the NMR experimental results, using the $K_D$ measured by ITC. Indeed, they were not measurable by stopped-flow fluorimetry for any of the ECD, ECDA′ and ECDA′B′ ligands, indicating that the dissociation constant $k_{off}$ is greater than 15 s$^{-1}$. Interestingly, it would thus appear that addition of residue B induces a decrease in the dissociation constant $k_{off}$ and an increase of the association constant $k_{on}$.

We also performed MD calculations with D0AB(E)CD docked in the part of the F22-4 binding site contacting the non-reducing unit of decasaccharide [AB(E)CD]$_2$ (Figure 3C). The mean difference between the MD predicted and experimental STD intensities of 0.09 (Supplementary data, Figure S5 and Table S2) strongly suggests that the mAb F22-4 binds this hexasaccharide mainly in this part of the mAb binding site, which implies that F22-4 is likely to recognize internal epitopes of the SF2a O-Ag.

### Discussion

**STD prediction from MD simulations**

To our knowledge, successful averaged prediction of STD intensities from snapshots of MD simulations has not yet been reported, although studies combining these two
techniques were published. Qualitative interpretation of MD trajectories can indeed provide support for the interpretation of STD-NMR results (Haselhorst et al. 2007; Cabeca et al. 2009) or averaged structures can be used for the CORCEMA-ST calculations (Yuan et al. 2008) when ligand motion amplitudes are not too large. In the case of SF2a O-Ag oligosaccharide fragments interacting with mAb F22-4, the significant flexibility observed for some oligosaccharide residues prompted us to calculate and then average the STD intensities of multiple snapshots of MD trajectories. The consistency of STD intensities obtained from MD trajectories in comparison to the NMR experimental results appears convincing to us, especially in the case of ECDaB’ and ECDa’B’. This may be due to the large conformational space explored by the glycosidic linkages D-A’ and A’-B’ and to the various densities of Ab saturated methyl groups in the area where the corresponding residues are located.

Fig. 4. Conformational energy maps of glycosidic linkages calculated from MD simulations of (a) D0AB(E)CD and (b) ECDaB’ in complex with mAb F22-4 and (c) D0AB(E)C and (d) ECDaB’ free in solution. Dihedral angles are defined as $\Phi = \text{O5-C1-O1-C}_x$ and $\Psi = \text{C1-O1-C}_x-\text{C}_{x-1}$. Contours are separated by 0.75 kcal. mol$^{-1}$. The represented conformational behaviors are representative of those observed with other branched and linear oligosaccharides.
Further improvements of MD predictions would require a better definition of the saturated protein protons and therefore knowledge of their chemical shifts. Besides, we cannot exclude the possibility that other unknown minor binding sites exist. Nevertheless, the level of agreement between calculated and experimental data is a substantial support for the MD results. This validation of MD simulations should be fruitful in numerous studies about protein recognition of flexible ligands. It also reminds that carbohydrate flexibility in a protein binding site may have to be taken into account in docking (Kerzmann et al. 2008; Agostino et al. 2009, 2010) or when refining ligand bound conformation (Jayalakshmi et al. 2004; Jayalakshmi and Krishna 2005).

**Rationalization of interaction constants and structural information**

To characterize ligand chemical groups taking an important part in the Ab:carbohydrate recognition, affinity measurements of different fragments of the native antigens, chemically modified or not, are often used together with structural information (Villeneuve et al. 2000; Vyas et al. 2002; Oberli et al. 2010). We present here improved structural details with the dynamic aspects of the studied complexes, by use of a strategy that does not require any chemically modified ligand.

In every simulation performed for the different complexes between SF2a O-Ag fragments and IgG F22-4, MD trajectories show the trisaccharide ECD interacting specifically and strongly with the mAb, notably through numerous hydrogen bonds also present in the F22-4-[AB(E)CD]$_2$ crystal structure. On the contrary, the A, B, A', and B' residues display similar conformational features in the complex as in the free oligosaccharides (Figure 4) and do not establish strong and persistent interactions with mAb F22-4. Hence, we assume that B(E)CD and ECD experience similar interactions with F22-4, although the corresponding binding free energies differ by 2 kcal · mol$^{-1}$.

This may be explained by partial pre-organization of the E-C linkage in B(E)CD: the recognized conformation is adopted by B(E)CD at lower conformational energy cost and with lower entropic losses than by ECD. This is consistent with a higher $k_{\text{off}}$ for B(E)CD than for ECD even if B(E)CD is a bigger molecule. Moreover, we argue that the presence of residue B constrains residue E during the interaction with F22-4, which is consistent with a slightly higher flexibility of the glycosidic linkage E-C in the MD trajectory of F22-4: ECD and which would explain the higher $k_{\text{off}}$ of ECD.

The same argument could be used with ECDA' and B(E)CD'A', by just adding the contribution of residue A'. Indeed, by comparing B(E)CD and B(E)CD'A', we can see that residue A' slows down the association rate by increasing the size of the molecule, but also slows down the dissociation rate by establishing many transient interactions with the mAb as observed in the MD trajectories. The comparison between B(E)CD and AB(E)CD leads approximately to the same conclusions, with A establishing few transient interactions with the mAb. Thus, it is hypothesized that an Ab with mutated amino acids designed to improve the binding to A, B, A' or B' residues could also bind SF2a O-Ag better. As an example, such property might be of interest when considering the search for potential diagnostic tools.

The interpretation of results concerning the interaction of D$_0$AB(E)CD and the mAb F22-4 is less straightforward: according to the MD simulations, the presence of residue D$_0$ cancels some interactions between residue A and the Ab, but it is probably not sufficient to explain the difference of $k_{\text{off}}$ between AB(E)CD and D$_0$AB(E)CD. However, the good consistency between experimental STD intensities and those predicted by the MD simulations gives confidence in the docking of D$_0$AB(E)CD in the part of the mAb F22-4 binding site recognizing the non-reducing RU of [AB(E)CD]$_2$ in the corresponding crystal complex. In this situation, D$_0$AB(E)CD mimics an internal RU bound to mAb F22-4, in agreement with the available antigenicity data and in support to previous hypothesis (Vulliez-Le Normand et al. 2008). MAb F22-4 is thus likely to recognize both non-reducing end and internal epitopes of the SF2a O-Ag, with a preference for the most exposed terminus of the O-Ag. This is in contrast to the binding mode of mAb SYA/J6, which recognizes intrachain epitopes located on the S. flexneri Y O-Ag (Vyas et al. 2002).

Altogether, we explored the ability of free oligosaccharides to mimic the original antigen and the intermolecular interactions occurring during the binding. This complementary information from biophysical methods, NMR and modeling show its potency in deciphering the molecular events responsible for improved or hampered mimicry and increased or decreased affinity. For instance, ECDA'B' appears to be a good mimic of SF2A O-Ag when binding to F22-4, but a poor mimic when free in solution, and the opposite is true for D$_0$AB(E)CD. This kind of information is of interest in the course of vaccine design, since one should probably select carbohydrate mimics presenting a conformational behavior similar to that of original antigen. This would allow to educate the humoral immune system in recognizing conformations adopted most of the time by this natural antigen.

**Conclusion**

In summary, using the well-studied mAb F22-4:SF2a O-Ag system, we have demonstrated that the combination of STD-NMR and MD simulations could provide important knowledge about the recognition abilities of a protective mAb toward a set of synthetic fragments of its cognate antigen. Moreover, this study brought into light information on the dynamics of the corresponding Ab:carbohydrate complexes that is not available from the X-ray structure, nor from NMR analysis only. The information derived from this study was in full agreement with previously available structural and antigenicity data. However, although predictions of immunogenicity would be desirable, such predictions are difficult based on Ab:carbohydrate interactions alone, especially if the study is limited to a single mAb. Indeed, investigations on the binding mode of protective anti-carbohydrate mAbs give information about protective epitopes, i.e. oligosaccharides that are recognized by the effectors of the immune system in a certain immunization context. This observed likelihood does not necessarily mean that these oligosaccharides would elicit a protective response in another context, e.g. when used as part of a glycoconjugate immunogen.
Last, we argue that this combined approach of MD simulations and STD-NMR analysis would be powerful to study carbohydrate-specific Abs more precisely, if one aims at improving Ab specificity in order to get diagnostic and/or therapeutic tools. Alternatively, the approach disclosed herein may find wide applications in the search for highly specific drugs targeting the carbohydrate recognition domain of selected receptors (Ernst and Magnani 2009). Along these lines, one could think for example about pattern recognition receptors of the innate immunity system for which crystal structures were obtained (Sorme et al. 2005; Attrill et al. 1999, 2001) of the pentasaccharide in the presence of mAb F22-4 were recorded after 16,384 scans as reported previously (Theillet et al. 2009). A 10 ms T1g filter was inserted before acquisition in order to remove F22-4 signals. No water suppression was added because of the low concentration of H₂O and for obtaining a maximum signal to noise ratio. Selective on-resonance irradiations of Ab resonances were performed at 0.8 ppm and off-resonance at 30 ppm, using series of 20 Gaussian shaped pulses of 270° (50 ms, 1 ms delay between pulses, excitation width approximately 2γB1/2π of 50 Hz) for a total saturation time of 1 s, so that the direct irradiation of oligosaccharides was negligible in the absence of any Ab.

Subtraction of saturated spectra from reference spectra was obtained by phase cycling (Mayer M and Meyer B 2001). To quantify the STD effect, the intensities of the observed signals were measured with respect to the corresponding signals in the reference spectra of the pentasaccharides free in solution, using the VNMR software. Mixing times varying from 100 to 800 ms and water presaturation were used for trNOe experiments.

**MD of free oligosaccharides**

We performed the MD simulations of the free oligosaccharides following the protocol described in Theillet et al. (2011). The dihedral angles Φ and Ψ are defined following the IUPAC convention, i.e. O5-C1-O1-Cx and C1-O1-Cx-Cx-1, respectively.

**MD simulations of Fab F22-4 with SF2a O-Ag segments**

The decasaccharide present in the X-ray structure of the Fab/[(AB(E)CD)₅] complex (PDB ID: 3BZ4, resolution 1.80 Å; Vulliez-Le Normand et al. 2008) was processed using the Conformational Analysis Tools (CAT; Frank 2005) software in order to assign atom and residue names that match the GLYCAM04 building blocks as well as information on Cys S-S bonds and carbohydrate branching. With this information available, further processing of the pdb file was performed using LEAP following the standard protocols (Case et al. 2005). All His residues were assumed to be neutral and were protonated at the ε position. Hydrogen atoms were added to the protein using LEAP. Counter ions were added to counterbalance the overall charge of the system. For periodic boundary condition simulations, the system was solvated in a box of TIP3P water with approximate dimensions of 90 Å × 70 Å × 90 Å. Initial MD simulations were performed using the full Fab and a truncated form for comparison. Since no significant differences could be detected with the truncated form.
(consisting of the variable regions of the light and heavy chains), this latter form was used for the simulation of the various complexes in order to save CPU time.

The MD simulations were performed with the all-atom AMBER force field (Case et al. 2005) using the PARM99 parameters for proteins augmented with GLYCAM04 parameters for oligosaccharides (Kirschner et al. 2008). The MD simulations were carried out by using the SANDER module and were performed using periodic boundary conditions and the particle-mesh Ewald (PME) approach to account for long-range electrostatic effects. A 10 Å cutoff was used for calculating non-bonded interactions. One to four electrostatics and non-bonded interactions were scaled by the default values of 1/1.2 and 1/2.0, respectively. Potentially unfavorable contacts in the initial structure were removed by 2500 cycles of energy minimization; 1000 cycles of steepest descent were followed by 1500 steps of conjugate gradient. Equilibration of the system was carried out as follows: after energy minimization the temperature of the system was raised from 0 to 300 K over 20 ps by coupling the system to a heat bath with a time constant of 1 ps using a weak-coupling algorithm (ntt = 1) while keeping the volume constant. The pressure was adjusted by isotropic position scaling to one bar (pressure relaxation time = 2 ps) and the water density to 1.01 g cm$^{-3}$ during the next 80 ps of the equilibration period. Position restraints were applied during the initial equilibration period to all protein backbone atoms using a force constant of 5 kcal mol$^{-1}$.

Production dynamics of 5 ns were performed at 300 K using a 2 fs time-step, with the SHAKE algorithm applied to all hydrogen-containing bonds (Ryckaert et al. 1977). For the PME simulation, the AMBER default options were used. During the MD simulation, snapshots of the coordinates were taken every ps. The resulting 5000 snapshots were analyzed with the CAT program (Frank 2005).

Calculations of STD intensities
We performed calculations of STD intensities on truncated structures from crystal complex and on snapshots of MD simulations of different complexes collected every 100 ps. In this last case, each snapshot has been processed by CORCEMA-ST and the resulting predictions were then averaged. Only the Ab residues within a 8 Å radius of the oligosaccharides were considered for the CORCEMA-ST treatment. Concentrations, field strength, delays, kinetic and thermodynamic constants were set to experimental conditions or results from biophysical studies. Correlation times of 60 and 1.5 ns were used for IgG Ab (bound state of the ligand) and oligosaccharides (free state of the ligand), respectively. Only methyl groups of leucine, isoleucine and valine were considered to be fully saturated.

Supplementary data
Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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Conflict of interest
None declared.

Abbreviations
Ab, antibody; CAT, Conformational Analysis Tool; mAb, monoclonal antibody; MD, molecular dynamics; Ig, immunoglobulin; NMR, nuclear magnetic resonance; nOe, nuclear overhauser effect; O-Ag, O-antigen; PME, particle-mesh Ewald; RU, repeating unit; SF2a, Shigella flexneri 2a; STD, saturation transfer difference; trnOe, transferred nOe.

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