How molecular modelling can better broaden the understanding of glycosylations

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Abstract:

Glycosylations are among the most ubiquitous post-translational modifications (PTMs) in proteins, effects of their perturbations are seen in various diseases such as cancers, diabetes, arthritis to name a few. Yet they remain one of the most enigmatic aspects of protein structure and function. On the other hand, molecular modelling techniques have been rapidly bridging this knowledge gap since the last decade. In this review, we discuss how these techniques have proven to be indispensable for better understanding of the role of glycosylations in glycoprotein structure and function.

Introduction

Glycosylations are one of the most ubiquitous post-translational modifications, evident from the fact that nearly 10% of all structures in PDB are found to be glycosylated.While they play vital roles in diverse physiological processes and pathologies, they remain one of the poorly understood biomacromolecules. We discuss in following minireview how glycans and glycoconjugates influence such processes, and how molecular modelling techniques can better contribute to current understanding of glycosylations and to better understand the role of glycans.

Types of glycoconjugates

Glycans can be broadly classified into both N- and O- type glycans. N-glycans typically originate from Asparagine residues (Fig. 1). The core structure of N-glycans consist of a pair of N-acetylglucosamine (GlcNAc) residues, connected with a bisecting pair of mannose (Man) residues. The GlcNAc residues are linked by β1-4 linkage, while mannose residues are connected to the terminal GlcNAc through α1-3 and α1-6 linkages. The bisecting mannose residues may often have a third/fourth antenna (Fig. 1), or it may also have an additional 'stalk' of GlcNAc residue.

The antenna may be of complex-type, where each antenna have a chain of GlcNAc and galactose (Gal) residues in varying number of repeats, or it may be of oligomannose-type, where the antennae have only mannose-residues arising from them. The third type is hybrid, where antennae consist of a mix of complex as well as oligomannose-types. All these varieties are related to different pathologies and the understanding of their structural roles is crucial to decipher their roles.

O-glycans arise from serine/threonine residues. The core structure of O-glycan consists of a N-acetylgalactosamine (GalNAc) residue, which has bisecting Gal and GlcNAc branches (Fig. 1). However, the core GalNAc structure is also present without further branches in several proteins. The bisecting branches can in turn have various combinations of subsequent saccharide residues arising from them.

Last, Glycosaminoglyacns (GAGs) are another category of saccharide-based PTMs, where saccharide residues arising from N- as well as O-glycosylation positions have sulphate residues covalently attached to them. GAGs are classified into keratan sulphate, dermatan sulphate, chondroitin sulphate and heparan sulphate (Fig. 1). These are composed of alternating repeats of GlcNAc or GalNAc residues with Glucoronic acid (GlcA), Iduronic acid (IdoA) or Gal residues (in keratan sulphate). The sulphate groups are covalently attached to GlcNAc/ GalNAc residues at 6' carbon position, or to 4' carbon in case of GalNAc in dermatan sulphate. Additionally, they are also covalently attached to IdoA at 2' carbon position (in dermatan sulphate and heparin), as well as to GlcA at 6' carbon position [1].

In addition to GAGs, Hyaluronan is another class of polysaccharide that consists of repeats of GlcNAc and GlcA linked via β1-4 and β1-3 linkages respectively (Fig. 1). While these are not covalently attached to any protein, they are present in extracellular matrices, and fulfil various types of structural roles. ~~Similarly, cellulose is the structural polysaccharide present in plants. They are polymeric forms of glucose, where glucose molecules are attached via β1-4 linkages.~~

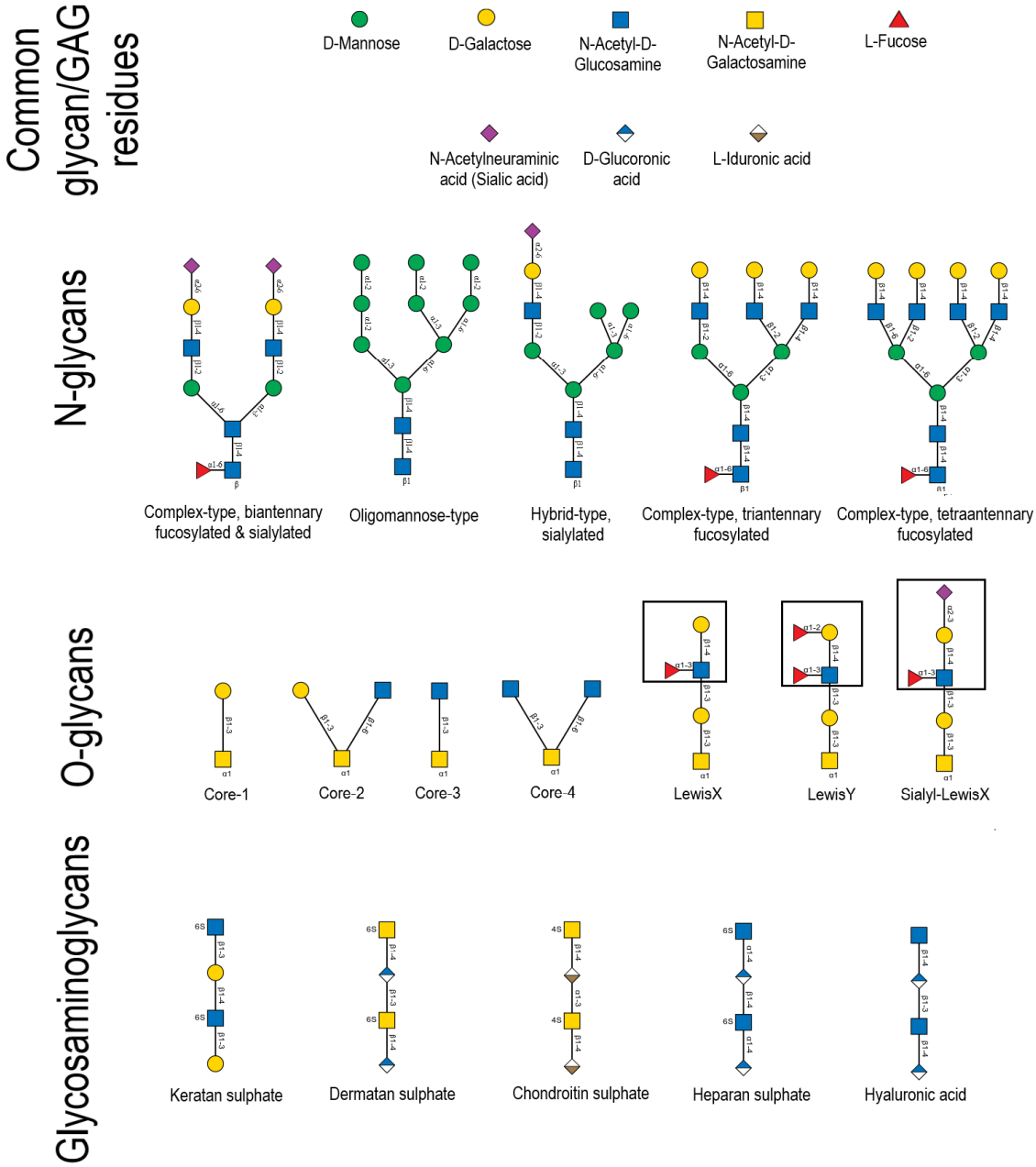

Figure 1. Types of glycosylations commonly observed in various human proteins. Residues of glycans and glycosaminoglycans are depicted according to the SNFG scheme [2], the visualization was constructed using the Glycoglyph program [3].

How do glycoconjugates influence physiological processes and pathologies?

Glycoconjugates participate in a wide variety of physiological and pathological processes. We discuss some of the prominent cases here. A large body of experimental studies show the importance of glycosylations in the context of cancers. Their role is evident from the fact that aberrant glycosylations have been used as biomarkers for the detection of cancers [4], and are also proposed to be the new set of targets for anti-cancer therapies [5]. One of the recurring theme of aberration is increase in branching, sialylation and core-fucosylation of N-glycans [6] and increase in O-

glycosylations such as Tn, sialylated Tn and T-antigens [6]. They are observed to have a role in a variety of cancers, such as Neuroblastoma, liver, colon, breast, lung cancers to name a few [7–10].

Glycosylations also regulate cellular homeostasis and participate in developmental processes. Glycosylations in proteins such as ~~stromal interaction molecule~~, Cyclin-M, Carboxypeptidases and Torsin-A [11–13] are found to be important in the context of maintenance of different homeostasis in cells. Similarly, various glycosylations participate in crucial processes that are part of the development of organisms. These involve recognition of sperm by the egg and vice-versa [14,15], differentiation of intestinal villi [16], myogenesis to name a few [17].

Importance of glycosylations in mounting of immune response is one of the more well-studied functions of the glycosylations. This includes innate, adaptive as well as humoral immune systems. Many of the pattern recognition molecules, one of the primary components of the innate immune system are glycosylated. Some of these glycosylations are demonstrated to be important for recognition of pathogens [18]. In addition, they are also shown to modulate protein-protein interactions that activate complement systems [19]. Similarly, numerous cell-surface receptors of white-blood cells that are components of adaptive immune systems, such as Toll-like receptors [20] and chemokine receptors [21], have glycosylations which influence their functioning by modulating signalling cascades and protein-protein interactions. Glycosylations of immunoglobulins (Ig) are among the more critical components of its functioning [22]. In IgG, they play a critical role in stabilization of the protein fold. Furthermore, glycosylations also facilitate interactions with protein partners such as Fc-γ receptors [22,23]. This property is one of the reasons IgG is a popular system for development of therapeutic monoclonal antibodies [22].

In addition to modulating homeostasis and immune response, glycosylations, particularly glycosaminoglycans (GAGs) maintain the normal organisation of tissues. These chains are attached to a family of proteins called small leucine-rich proteoglycans [24]. The negative charge of GAGs significantly contribute to the structural integrity by enabling strong interactions with structural proteins such as collagen, but also confer load-bearing capability to connective tissues by attracting a large number of water molecules [25], creating differences in turgor pressure.

Both bacterial and viral pathogens use their glycosylations to evade detection and gain entry into the host. In bacteria, certain glycosyltransferase enzymes act as toxins by glycosylating important host proteins and obstructing their activity [26]. On the other hand, viral glycosylations act as a shield that contributes to evasion from host immune response, such as in influenza viruses [27], HIV [28] and the more recent SARS-CoV2 [29]. In addition to this, they are also demonstrated to stabilize the viral capsid proteins, and facilitate protein-protein interactions crucial for gaining entry into the host [27].

Why is structural characterization of glycoconjugates difficult?

Numerous experimental methods are used for analysis of composition and structures of glycosylations. In this section, we discuss some of these methods, their advantages and limitations.

Mass spectrometry is among the most commonly used methods to determine glycosylation composition. In this method, glycoprotein samples undergo N-glycosidase/β-alkaline reduction treatments, after which they are purified typically using gas chromatography. A mass/charge spectrum is generated, where each peak corresponds to a different glycan. The glycan composition is determined from charge-mass ratio [30]. Another major advantage of this method is that the intensity of the peak allows one to determine the abundance of each type of glycan from the sample.

NMR is another method used to decode glycosylation composition, structure and dynamics. Treatment of glycoprotein samples under paramagnetic field, then perturbation of this magnetic field results in nuclear overhauser effect (NOE). The chemical shift generated from NOE enables derivation of distance restraints, which can be used to construct the model of the sample. While this technique has been very successful in decoding the structures of proteins, there continue to be many

challenges while using it for studying glycosylations. One of the most common problems is the fact that there is a considerable overlap of chemical shifts due to the presence of repeating chemical units, particularly for glycoconjugates with large number of repeating units like GAGs [31]. This is accompanied with degeneracy of chemical shift, due to the similar nature of numerous saccharide residues, resulting in large overlaps in the 2D spectra and ambiguity in the assignment of atoms. Furthermore, due to the highly dynamic nature of oligosaccharides, reliable assignment of residues becomes difficult [31]. But despite these challenges, more recent advancements in NMR methodologies, such as improvements in magnets and use of lanthanide tags for glycan residues have alleviated many of the above challenges [32].

On the other hand, X-ray crystallography is the most widely used method for structural characterization of glycosylations. Out of 9,522 protein structures in PDB on which glycosylations have been detected, a staggering 89.4% (8,508 structures) of these were solved using X-ray crystallography, as of October 2021 (Fig. 2) [33]. However, a closer examination of the data reveals that this has not been without challenges and problems. It may be noted that a vast majority of detected N-glycosylations (68.5%) in various glycoproteins have either two or three detected residues (Fig. 2) [33]. This suggests that most of the glycosylations documented in PDB are incomplete. One of the primary reasons for this is because of the presence of multiple reactive groups in glycosylations, they tend to get digested during the protein purification steps, and the information pertaining to glycosylations is lost. Even if the glycosylation composition is conserved during the purification steps, the highly flexible nature of saccharide residues poses significant obstacles to their crystallization and extraction of electron densities. For the saccharides whose electron densities are resolved, there are major concerns about their reliability. As many as 30% of glycoprotein structures in PDB had errors associated with the carbohydrate electron densities [34], and large number of errors were associated with poor fit of pyranose residue assignments [35]. However, there have been numerous efforts to alleviate these problems. Numerous tools have been developed to search for such inconsistencies, assign scores related to reliability of carbohydrate residues in PDB and in some instances, correct the errors associated with residue assignment. Tools such as PDB-CARE, PDB-REDO, Carbohydrate Structure Suite and Rosetta [36], [37–39] have all contributed towards more reliable structural annotation of saccharide residues. Recent PDB updates have also attempted to address these issues with the introduction of validation of carbohydrate raw data, fitting atoms with electron densities and analyzing for anomalies in properties such as bond lengths, angles and dihedral angles [40].

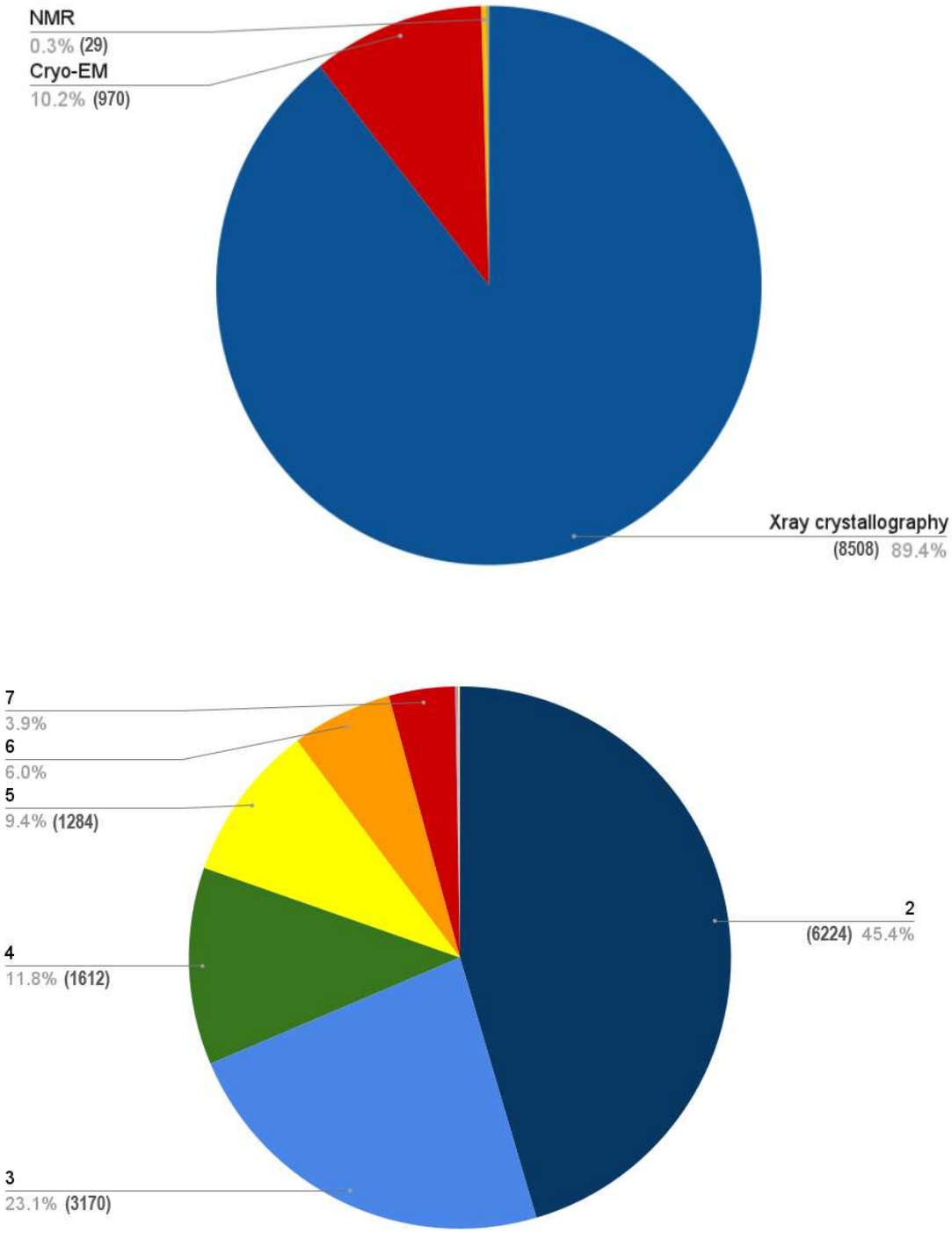

Figure 2. Top: Charts showing share of glycoproteins in the Protein Data Bank [33] solved by different experimental methods. **Bottom:** Chart showing the share of chain length from all the detected oligosaccharides in the Protein Data Bank [33]. Values represented as percentage, with absolute values shown in brackets. Data represented as of October 2021.

How molecular modelling can better contribute towards understanding of glycosylations?

In light of the above-discussed limitations of experimental techniques, we argue here that molecular

modelling is a valuable way to bridge the knowledge gap on glycan structural biology. The first studies on glycans using molecular dynamics were performed in 1987 on oligomannose-type glycans [41]. Subsequently, similar studies were conducted on complex-type biantennary glycans, where different modes of glycan conformation were characterized [42]. These studies coincided with early developmental efforts on carbohydrate force fields [43,44]. Since then, numerous studies have added interesting insights on glycans, including their additional conformational states [45,46] as well as impact of changes in glycosylation composition [47,48]. At the same time, numerous modelling studies on glycoprotein systems highlighted their importance on protein functioning. One of the most well-known examples is the IgG Fc domain. Successive simulation studies, coupled with solving of Fc domain structures demonstrated ability of IgG glycans to modulate Fc domain interactions with Fcγ-Receptor [49] as well as the importance of saccharide residues in the stability of Fc domain [50]. Particularly, the latter set of studies demonstrated how changing composition of glycosylations can have a drastic impact on Fc domain dynamics, by means of disruption of protein-carbohydrate interactions.

Apart from the immunoglobulins, glycoproteins of retroviruses have also witnessed extensive studies using molecular simulations in the recent past. One of the earliest studies demonstrated how oligosaccharides on the hemagglutinin of influenza virus are crucial for establishing virus-host interactions [51]. In addition to this, they also conferred species-dependent specificity by means of species-specific interaction networks via hydrogen bonds and electrostatic contacts [52]. Apart from modulating protein-protein interactions and conferring specificity, influenza glycosylations were also found to contribute to immune evasion by obstructing binding of drug molecules with surface proteins like Neuraminidase [53]. Similar roles were also observed for HIV glycoproteins. Apart from stabilization of the protein fold, which was observed for the V3 region of gp120 glycoprotein of HIV-1 [54], glycans of gp120 were also observed to galvanise virus-host interactions [55].

In addition to Influenza virus and HIV, recent emergence of SARS-CoV2 led to numerous modelling studies that led to unravelling of important insights into its entry in a short period of time. Insights obtained from these studies have been instrumental in development of vaccines against SARS-CoV2. The group of Robert Woods generated one of the first glycosylated models of spike glycoprotein in the initial stages of the pandemic, along with simulations that showed the role of glycan shielding in the immune evasion capability of the virus [56]. This was supported by studies comparing the glycan shields of SARS-CoV1 and SARS-CoV2 that showed denser shielding by the glycans [57]. However, among the most important, breakthrough study on the spike glycoprotein was performed by the groups of Rommie Amaro and Elisa Fadda, which demonstrated how glycans on residues Asn165 and Asn234, apart from providing the shielding effect, also facilitate interactions between receptor binding domain (RBD) of the spike with human angiotensin converting enzyme-2 (ACE2) receptor [29]. Moreover, in this study, the importance of the dynamics of numerous glycans may be shown on the overall structure of the spike protein and shielded the accessibility to the protein parts as seen in Figure 3 [29]. A similar work showed that the glycan on Asn343 modulates conformational behaviour of RBD by acting as a 'gate' [58]. These modelling studies have made significant contributions to our understanding of the SARS-CoV2, which may have been much more difficult to accomplish using experimental methods.

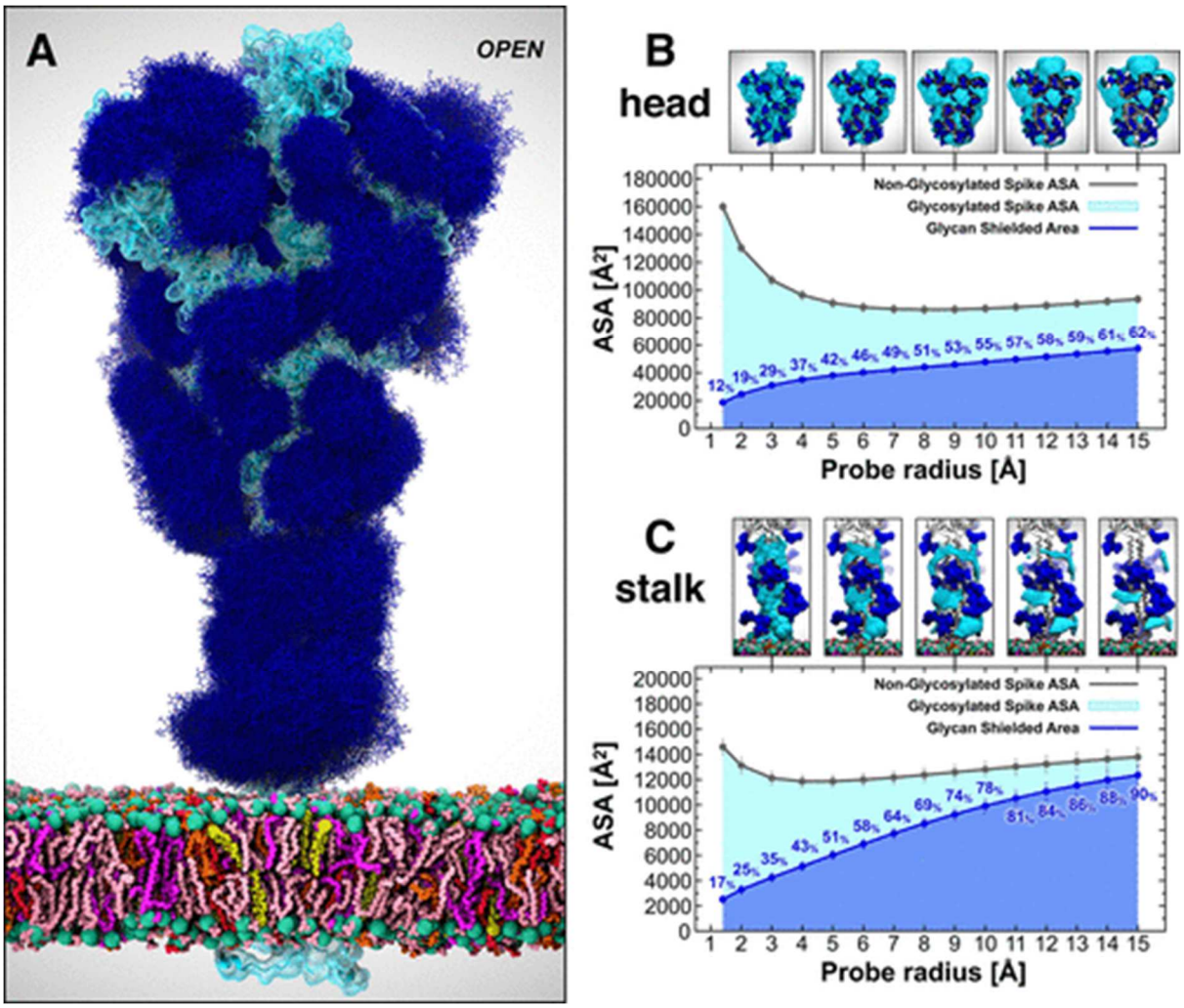

Figure 3: Glycan shield of the SARS-CoV-2 S protein. (**A**) Molecular representation of the Open system. Glycans at several frames (namely, 300 frames, one every 30 ns from one replica) are represented with blue lines, whereas the protein is shown with cartoons and highlighted with a cyan transparent surface. Color code used for lipid tails (licorice representation): POPC (pink), POPE (purple), POPI (orange), POPS (red), cholesterol (yellow). P atoms of the lipid heads are shown with green spheres. Cholesterol's O3 atoms are shown with yellow spheres. (**B, C**) Accessible surface area of the head (**B**) and stalk (**C**) and the area shielded by glycans at multiple probe radii from 1.4 (water molecule) to 15 Å (antibody-sized molecule). The values have been calculated and averaged across all replicas of Open and are reported with standard deviation. The area shielded by the glycans is presented in blue (rounded % values are reported), whereas the gray line represents the accessible area of the protein in the absence of glycans. Highlighted in cyan is the area that remains accessible in the presence of glycans, which is also graphically depicted on the structure in the panels located above the plots (reproduction authorized by ACS from ref [29])

While extensive modelling studies have been performed on immunoglobulins and retroviral glycoproteins, similar studies are conducted on other systems as well. Numerous focussed efforts have been made on transmembrane receptors, such as N-methyl aspartate receptors (NMDA receptors). Modelling studies on NMDA receptors have shown that glycosylations on the GluN1 domain stabilize clamshell conformation, which is its physiologically active state [59]. A similar role of glycans was also observed in the epidermal growth factor receptor [60]. In insulin receptor, modelling studies have shown that the removal of sialic acids from its glycans results in significant perturbations in insulin

receptor dynamics and interactions, ultimately resulting in insulin resistance [61].

Conclusions, Outstanding knowledge gaps and general future directions

In the previous decade, there has been tremendous progress in our understanding of glycoconjugate structure, dynamics and its impact on glycoprotein functioning. Molecular modelling and simulation techniques have significantly contributed towards this progress. But despite all the progress, several knowledge gaps persist, which are discussed here.

While most of molecular modelling studies on glycans and glycoproteins have been performed on two types of systems, namely immunoglobulins [49,50] and retroviruses [29,51,54], there is a lot of potential for similar studies in other systems of biomedical and economic interest. One example is the glycoproteins involved in various cancers, given the fact that numerous glycosylations are associated with cancers, and they represent a lucrative target for the design of novel anti-cancer drugs. In addition to cancers, there is also a lot of potential for such studies in the context of other non-communicable diseases as well, such as cardiovascular diseases, autoimmune diseases to name a few [62]. There has also been significant progress in our understanding of plant and microbial glycans [63]. More modelling studies on such systems will further add interesting insights that will help furthering our understanding of glycosylations.

It also needs to be noted that such advancements must be accompanied with similar progress in development of methodologies related to molecular modelling as well. While there has been considerable development on modelling of glycosylations on proteins [64,65] and on various carbohydrate force fields [66], one of the areas of potential concern is the need for further refinements to improve accuracy of force fields, considering that recent studies that compared carbohydrate force fields have highlighted this [67,68]. These studies demonstrated that despite strong sampling of carbohydrates, significant divergences exist with respect to carbohydrate dynamics sampled with different force fields. Divergences were particularly observed with respect to binding free-energies and free-energies associated with dihedral angles [67,68].

There is also an evident need for similar development of coarse-grained force fields. While there has been considerable recent progress with this respect [69–72], there is a need for further development of parameters on molecules such as GAGs. Progress in this direction will be particularly advantageous, since coarse-graining enables robust conformational sampling of large, complex protein systems at a fraction of computational resources consumed by all-atomic simulations. Apart from the force fields themselves, there is also a need for development of techniques for analysis of saccharide dynamics from simulations. While some initial efforts have been made in this direction [2,73–75], there is a need for analysis methodologies that enables study of global conformational dynamics of glycans.

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