# Studying glycobiology at the single-molecule level

*Abirami Lakshminarayanan, Mylène Richard and Benjamin G. Davis* *

Abstract | Attempts to elucidate the roles of carbohydrate-associated structures in biology have led to the distinct field of glycobiology research. The focus of this field has been in understanding the evolution, biosynthesis and interactions of glycans, both individually and as components of larger biomolecules. However, as most approaches for studying glycans (including mass spectrometry and various binding assays) use ensemble measurements, they lack the precision required to uncover the discrete roles of glycoconjugates, which are often heterogeneous, in biomolecular processes. Single-molecule techniques can examine individual events within challenging mixtures, and they are beginning to be applied to glycobiology. For example, single-molecule force spectroscopy (SMFS) by atomic force microscopy (AFM) has enabled the molecular interactions of sugars to be studied, single-molecule fluorescence microscopy and spectroscopy have led to insight into the role of sugars in biological processes and nanopores have revealed interactions between polysaccharides and their transporters. Thus, single-molecule technology is becoming a valuable tool in glycoscience.

Glycobiology explores a variety of biomolecules, including conjugates containing carbohydrate moieties and those that are discrete monosaccharides, oligosaccharides and polysaccharides. The roles of such sugars and glycoconjugates (that is, carbohydrates that are covalently linked to another chemical moiety) in cell biology are varied. For example, these moieties are involved in recognition[1], structure, modulation and signalling mechanisms[2,3] (FIG. 1). These functions highlight glycosylation as one of the most important and diverse post-translational modifications at the heart of fine-tuning biological mechanisms. Indeed, there has been widespread interest among researchers in understanding and modulating glycobiology. However, there has been a lack of progress in the field due not only to its intrinsic complexity and diversity but also to, as we discuss here, a lack of requisite tools and techniques to study glycoconjugates[2,4,5].

The structural heterogeneity of glycoconjugates, which arises from branching, interlinking and different anomeric stereochemistries, has made it difficult to delineate individual structures and their roles in cellular pathways[5,6]. The study of glycobiology would therefore benefit from approaches that allow the elucidation of precise structures within dynamic mixtures and the study of single events (that are mediated by broad populations of biomolecules) within diverse mixed interactions[7–9]. Single-molecule methods can, in principle, address these goals by providing discrete analysis of molecules, and the events in which they are involved, in the context of the biological spectrum; this motivates the use of such methods in the study of glycans, in which dynamic mixtures dominate.

In this Review, we suggest that, although single-molecule methods are currently used rarely in glycoscience, they enable the discrete structural characterization of glycans and the elucidation of the mechanisms underlying sugar-mediated events. We choose key examples, from within and outside glycobiology, to illustrate the power of single-molecule methods and acknowledge that these methods cannot address all of the issues in the field. Rather than collating all of the methods that can be used to study glycobiology, this Review highlights single-molecule strategies that do, or have the potential to, allow a discrete understanding of the structure, function and dynamics of glycans, and it represents our personal perspective on future directions for advancing the field.

## Major challenges in glycobiology

***Structure determination of glycoconjugates.*** Seemingly minor structural details, such as the anomeric stereochemistry of or type of glycosidic linkage in a glycoconjugate, can influence to varying degrees the biochemical processes that glycoconjugates are involved in. Thus, elucidating the exact structural details of glycoconjugates is an important starting point when studying the mechanisms underlying their biological roles and for

*Department of Chemistry, Chemistry Research Laboratory, University of Oxford, Oxford, UK.*

**e-mail: Ben.Davis@chem.ox.ac.uk*

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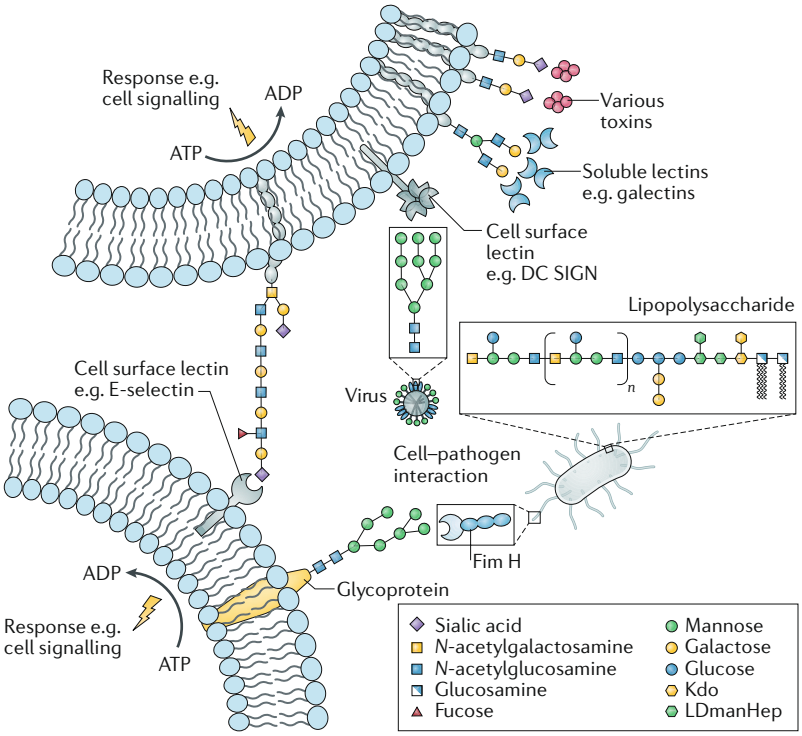


Fig. 1 | **A snapshot of some cell-surface glycobiology.** Carbohydrate-containing structures are central to many biological processes. For example, carbohydrate-binding proteins, such as galectins and C-type lectins, have been implicated in cellular recognition, which may trigger downstream cell signalling pathways that are dependent on ATP. Glycan interactions may also mediate the interaction of cells with carbohydrate-binding toxins, as well as cell–cell interactions and cell–pathogen interactions. Finally, lipopolysaccharides are just one example of a major structural component of bacterial cell walls that may elicit immune responses via glycan-mediated recognition. The key for symbols here and in most other figures in this Review follow the symbol nomenclature for graphical representations of glycans (Symbol Nomenclature for Glycans (SNFG)) convention[100]. Kdo, 3-Deoxy-D-manno-2-octulosonic acid; LDmanHep, L-glycero-D-manno-heptose.

developing system-specific models or analogues that test glycoconjugate-driven hypotheses. Advances in carbohydrate NMR spectroscopy[10,11] and mass spectrometry[12–14] have enabled formidable progress in this direction, yet neither technique is routinely used by all in the biological community. Challenges remain in readily determining the structure and position of glycans in glycoproteins at a sensitivity that allows the proteome-wide survey of glycoproteins (that is, of the glycoproteome) in primary tissue samples[15,16]. Strategies that can distinguish between the isobaric species that are ubiquitous in glycoscience, but more rarely plague proteomics, show promise in enabling us to elucidate the full primary sequence of glycoproteins; such sequence data was once restricted to a report of the ratios of hexose (Hex) to *N*-acetylhexosamine (HexNAc) and to higher sugar types[13,17–20]. Additionally, methods for determining the 'shape' of glycoproteins — that is, for determining secondary and higher order structural motifs[21–25] — are valuable given the relative rarity of glycoconjugates in current structural databases. Thus, 'sugar sequencing' and comprehensive structure–function correlations still remain incompletely solved issues, but promising methods are now touching on useful strategies.

***Understanding glycan dynamics and glycoconjugate modulation.*** Glycoconjugates not only provide a stable matrix for biomolecules, but they are also involved in transferring information during extracellular communication and through signalling networks[6]. Thus, understanding the population dynamics of glycans in such glycoconjugates, and how these dynamics change in response to various stimuli, will enable a deeper understanding of the mechanisms underscoring information transfer and may inform medical diagnostics in the clinic.

Understanding the population dynamics of glycans requires advanced methods for synthesizing and assembling glycans that can be used as key standards and probes, as well as methods for the ready labelling, sample enrichment and rapid and repeatable detection of metabolite glycans in minute quantities within mixtures; many current, widely used methods may not achieve the levels of efficiency or sensitivity needed. Therefore, assays and technologies that can determine, in situ, the functional moiety of the glycoconjugate, if it is present or partitionable, are needed. Recent advances of relevance include the continuing development of automated glycan synthesis[26], the introduction of sensitive labels (for example, fluorophores) into molecules by suitably selective reactions[27], chemoenzymatic strategies[28] for altering cellular glycans to study their function[29] and the increased efficiency of, and alternative strategies in, glycosylation methods[30]. These efforts should help us understand the roles of sugars in disease and misfunction[31–33], perhaps by correlating sugar biomarkers with pathology. Indeed, fluorescent imaging and the use of fluorescence resonance energy transfer (FRET), and even using methods that have not been optimized for the detection of glycoconjugates, have provided valuable insight into processes, including the clustering of glycolipids on the cell surface[34,35], that inform us about glycan function in certain settings. However, despite bioinformatic analyses to identify the most biologically relevant sugars[36], the high number of sugar permutations is a barrier that needs to be acknowledged[36,37] when considering the synthesis and sequencing of such sugar biomarkers.

***Understanding multivalency.*** The role of multivalency is a primary consideration in glycobiology. Carbohydrate–protein interactions are key to many biological processes but often involve weak binding affinities, a seeming contradiction at first inspection. In the limited number of systems that have been studied, nature appears to use differing modes of multivalency. To compensate for their weak binding affinities, multiple copies of ligands and receptors are suggested to cooperate to bind sequentially or simultaneously, providing enhanced binding avidities that convert small affinity interactions into physiologically relevant levels. This phenomenon has been termed the cluster glycoside effect[38,39] and is thought to be integral to the study of many carbohydrate-mediated interactions in glycobiology. Studying this effect requires a continued and improved understanding of models and modes of multivalency[40], as well as the development of appropriate techniques.

Glycan arrays have been developed to qualitatively and semi-quantitatively probe interactions that involve

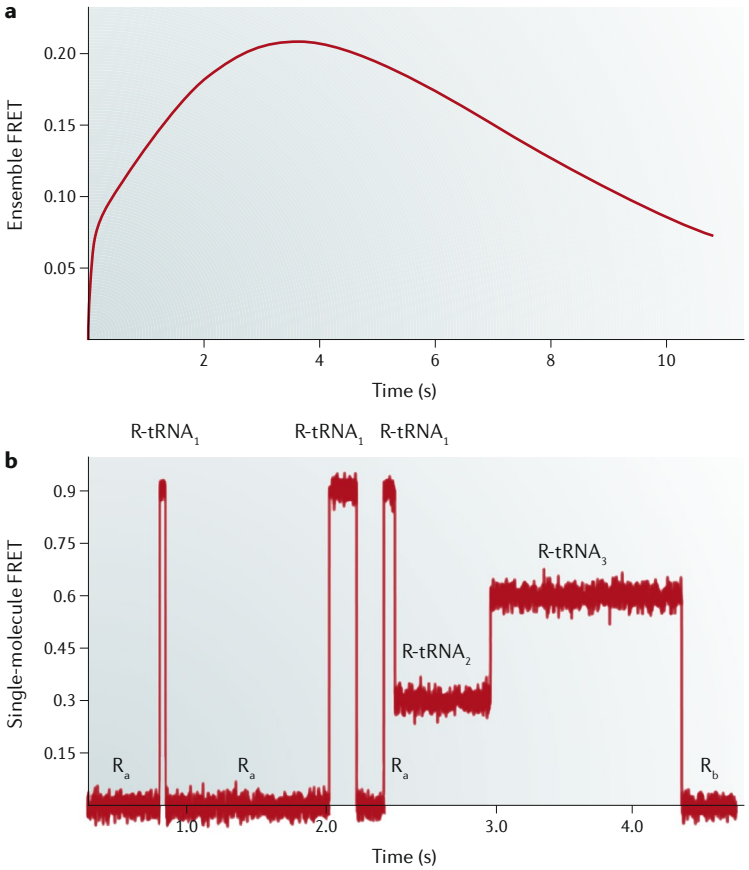


Fig. 2 | **Comparison of ensemble measurements and single-molecule measurements in a simulated non-sugar system. a** | The calculated ensemble fluorescence resonance energy transfer (FRET) versus time for a reaction of ribosome (R)–tRNA interaction that proceeds via three intermediates with different FRET values. The ensemble measurement fails to show such intermediates. **b** | The use of simulated single-molecule FRET to measure the R–tRNA interaction reveals the three different intermediates that are generated in this reaction — namely, $R–tRNA_1$, $R–tRNA_2$ and $R–tRNA_3$ — that have FRET values of 0.9, 0.3 and 0.6, respectively. These data suggest that there is a step-by-step 'movement' from reactant to product[48]. Such observations, within complex systems that are sampling multiple states from within a potential pool of multiple interactions, highlight how incisive such methods could be in glycoscience. Ra, free ribosome before tRNA binding; Rb, free ribosome after release of tRNA. Reproduced with permission from REF.[48], Cold Spring Harbor Laboratory Press.

carbohydrates in various multivalent contexts, including as biomarkers for various diseases[41,42]. Along with surface plasmon resonance (SPR)[43], isothermal titration calorimetry (ITC)[44] and mass spectrometry[12,13], glycan arrays are now semi-routine tools for more detailed but situation-dependent quantification of host–ligand multivalent interactions from which seminal bulk models of multivalency have emerged[39,44–46]. Furthermore, techniques for studying multivalent interactions and delineating the pathways, stochasticity and collective modes of such binding will improve our understanding of many bioprocesses. These bioprocesses include the attachment of pathogenic bacteria to receptors and signalling induced by small molecules and cytokines. We feel that the development of such techniques for studying multivalent interactions could prove invaluable for analysing suggested cluster glycoside effects on a case-by-case basis rather than in a generalized manner. Indeed, it is unclear whether multivalent and/or cooperative and anticooperative effects are routinely or occasionally present in glycoscience, and more regular observation of relevant molecular events will allow us to understand the 'if, how and when' of the multivalency of sugars.

## Studying single-molecule glycobiology

The intricacies of glycobiology have necessitated the development of various bulk techniques that have become indispensable for the study and characterization of the structures and interactions of carbohydrates; some of these techniques are relevant to single-molecule methods for studying glycobiology, and we will highlight these here. Conventional bulk techniques (often also referred to as ensemble techniques) provide the deterministic outcome of a given process, for example, through a comparison between the bound and unbound states of an analyte. However, two potentially confounding aspects of ensembles in glycobiology, in which single-molecule methods could prove particularly useful, are in heterogeneity and collective effects.

Furthermore, kinetics obtained in ensembles can be complex and difficult to analyse[46,47]. Single-molecule studies could reveal information regarding the underlying dynamics of a system and the precise roles of individual components within mixtures to complement ensemble studies, which themselves may simply show the existence of a global interaction. Moreover, spatial and temporal resolution provide unique mechanistic information or different facets of a molecular mechanism that give a common outcome, which cannot be resolved by ensemble analysis (see FIG. 2)[48]. These facets may provide further biological insight to broad effects.

The elucidation of binding mechanisms by new methods could be important for the understanding, exploitation and application of resulting technologies. For example, knowledge of exact carbohydrate–lectin binding modes creates the potential for the design of anti-infectives[49] that block host–pathogen interactions or it may help identify an unexpectedly potent antigen that can be used as a carbohydrate vaccine immunogen[50].

We describe examples that demonstrate strategic and specific potential advances that could be enabled or developed by single-molecule-based technologies in glycobiology. In so doing, we have highlighted both techniques currently used to study carbohydrates in ensembles for which single-molecule counterparts could be valuably developed and techniques that are being used at a single-molecule level in other realms that could be repurposed for sugar-specific interrogation (see TABLE 1). In this way, we hope to chart a possible course for development of the field.

***Force spectroscopy.*** Study of single-molecule force spectroscopy (SMFS) by atomic force microscopy (AFM) is a widely and actively used technique for single-molecule studies in biology. By estimating the strength of certain carbohydrate-based interactions, SMFS has allowed researchers to gain insight into the functional roles of these interactions[51,52]. For example, AFM was used to

Table 1 | **Single-molecule techniques with potential for use in glycobiology**

| Single-molecule technique | Information currently obtained using technique | Chemical tools driving technique | Use of technique in glycobiology | Refs |
|---|---|---|---|---|
| Force spectrometry | Quantitative analysis of binding interactions and binding kinetics | Atomic force microscopy tip coated with lectin and sugar immobilized on, for example, mica | Analysis of the molecular interactions of sugars, including interaction strength | 51–54,60,63–65, 67–69,101 |
| Single-molecule field-effect transistors | Dynamic signals during degradation of glycans | Enzyme-tethered carbon nanotubes | Monitoring dynamic glycan processing | 75 |
| Single-molecule fluorescence microscopy and spectroscopy | Elucidating the mechanisms of biomolecular interactions in biological processes within living cells | Fluorescent labels | Probing single-molecule events in cells | 77,81,84,86 |
| Stochastic optical reconstruction microscopy (STORM) | Distribution and diffusion rate of glycans on the cell surface | Photoswitchable fluorophores | Localization and distribution of glycans and partners on the cell surface | 87,88,90 |
| Single-channel recording using nanopores | Lifetimes of interaction states between glycans, proteins and small molecule inhibitors | A single protein pore (modified or native) that can monitor interactions | Recapitulation of glycan–protein binding processes and screening for molecules that inhibit them | 92–94 |
| Recognition tunnelling | Electrode fluctuations according to identity of sugar | Electrodes functionalized with host molecules | Analysis of carbohydrate epimers | 98 |

assess differences in carbohydrate binding on the surface of cancerous cell lines compared with normal cell lines[53]. Glycoconjugates on the cell surface of HeLa cells (cancerous) and Madin–Darby canine kidney (MDCK) cells (normal) were approached with lectin-coated AFM tips, which were then retracted according to typical SMFS protocols (FIG. 3a) to record lectin–glycoconjugate interactions via force–distance cycles. The lectins wheat germ agglutinin (WGA, which is presumed to be selective for glucose (Glc), and *N*-acetylglucosamine (GlcNAc)) and *Polygonatum multiflorum* agglutinin (PMA, which is presumed to be selective for mannose (Man)) were used to probe cell-surface interaction strengths in a glycan-dependent manner. Although the specificity of these lectins for glycans is likely to be more complex than presumed in this study, the data obtained illustrate what AFM can tell us about carbohydrate-based interactions. Specifically, these studies suggested that the energy of activation for sugar–lectin dissociation is lower in cancer cells than in normal cells, which correlates with some established data showing that cancer cells are less adherent than normal cells[53]. AFM-based molecular recognition has also been used to study the binding kinetics of the lectin domain of the toxin ricin to galactose moieties on HeLa cells. Using approach–withdrawal cycles with a ricin-coated tip gave force–distance measurements that allowed extrapolation to an in situ dissociation constant of $2.2 \times 10^{-3}$ M (REF.[54]). The use of single-molecule techniques to interrogate the binding of glycans to lectins on live cells, as demonstrated by these studies, illustrates the potential contextual advantages of these techniques over bulk studies in obtaining physiologically relevant information.

Certain glycoconjugates on the surface of pathogens can act as immunogens that trigger host responses[55]; thus, the construction of synthetic glycan mimics to develop carbohydrate-based vaccines is an active area of research[56,57]. One prerequisite for their systematic, iterative development is the identification of the antigenic moieties that are bound by antibodies raised in this way, and SFMS (via AFM) might identify correlations between immunity and the molecular properties of glycoconjugate immunogens. For example, data from bulk, enzyme-linked immunosorbent assays (ELISA) suggest that multi-antennary oligosaccharides, such as $Man_9(GlcNAc)_2$, are central to the immune responses associated with the natural, neutralizing anti-HIV-1 antibody 2G12 (REF.[58]). However, synthetic conjugates containing such or related sugars have failed to stimulate the required level of immune response, prompting further investigation[59]. When the carbohydrate specificity of 2G12 was probed by SMFS[60] using a panel of oligosaccharide fragment motifs (FIG. 3b,c), data revealed that 2G12 had lower lifetimes of interaction with segments of $Man_9(GlcNAc)_2$ at the single-molecule level but potentially useful lifetimes with the gp120 glycoprotein displaying multiple glycans. These data highlight the potential value of analysing the kinetics of interactions, an aspect that is less studied in vaccinology[61,62]. They also emphasize the need to determine multiple structural and chemical properties in a system as well as the utility of single-molecule techniques in highlighting which parameters in a system have more functional influence.

Carbohydrate-containing antigens in glycopeptidic fragments may also undergo self-assembly into complex, ordered arrangements, a concept that has also been studied by single-molecule AFM. For example, force–distance measurements of antigens T (Galβ(1–3) GalNAcαThr/Ser) and Tn (GalNAcαThr/Ser) derived from porcine submaxillary mucin revealed stable antigen self-interactions with lifetimes ranging from 0.5–4.5 s; these interactions had not previously been observed in such detail[63]. These antigens are implicated in cancerous tissues, and the authors of the study proposed that the variation of interaction lifetimes observed for these truncated antigens might contribute to aberrant cell-surface signalling.

Protein-mediated or peptide-mediated sugar interactions dominate conventional analyses in glycobiology; direct carbohydrate-to-carbohydrate interactions have

Force–distance cycles
The repeated measurement of force as a function of distance in atomic force microscopy.

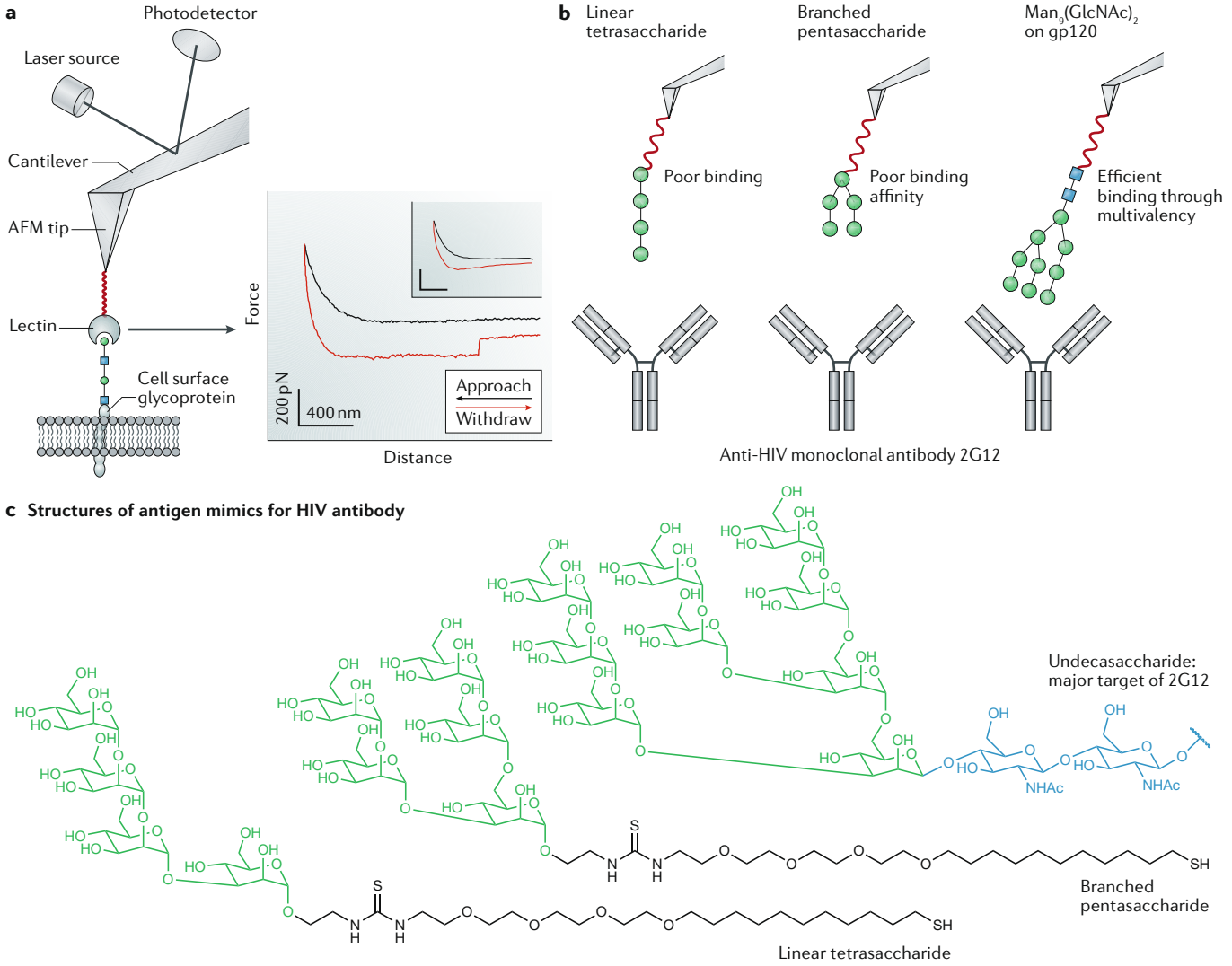


Fig. 3 | **Single-molecule force spectroscopy to study sugar–lectin interactions. a** | Schematic representation of a lectin-coated atomic force microscopy (AFM) tip interacting with cell-surface glycans to study lectin–glycan binding and dissociation. Representative force–distance curves are also presented. **b** | AFM was used to study the antigenicity of branched and linear oligosaccharides from HIV-1 gp120 to the HIV-1 monoclonal antibody 2G12. **c** | Structures of the oligosaccharides from HIV-1 gp120 that were studied for their antigenicity to 2G12 via AFM. GlcNAc, *N*-acetylglucosamine; Man, mannose. The force–distance curves in part **a** are reproduced with permission from REF.[53], RSC. The structures in part **b** are adapted with permission from REF.[60], American Chemical Society.

been less studied. However, the sensitivity of single-molecule methods, and in particular of AFM, has enabled quantitative analyses of carbohydrate–carbohydrate interactions. As an example, the mechanism of carbohydrate-mediated cell adhesion has been studied in marine sponges, providing evolutionary insights into early metazoans that may have broader implications for the development of higher, multicellular organisms[64,65]. Specifically, 'self'-interactions among sulfated glycosaminoglycans (GAGs) that are evolutionarily preserved in metazoans were probed by force cycles using SFMS, revealing that the presence of chelated calcium and sulfation on these GAGs is vital for their self-interaction and, thus, presumably for cell adhesion.

As well as providing useful information on the binding forces of cell-surface glycans in different contexts, AFM has been used to 'map' the localization of cell-surface glycans at the single-molecule level (FIG. 4). For example, AFM tips functionalized with anti-capsular polysaccharide (CPS) antibodies were used to probe both the localization and the density of the outer glycans of CPS and group B carbohydrate (GBC) in wild-type and mutant *Streptococcus agalactiae*[66]. AFM tips functionalized with WGA were then used to probe peptidoglycan underlying these outer glycans, revealing that the adhesive probabilities of wild-type *S. agalactiae* and mutant *S. agalactiae* that do not display GBC were weak (1–3%), whereas those of mutant *S. agalactiae* that do not display CPS were ~30%. These data highlight and quantify the vital role that CPS plays in protecting the peptidoglycan layer of pathogenic bacteria from environmental exposure. A similar study on yeast cells[67] used antibodies conjugated to an AFM tip to map the localization

of α-mannans versus β-mannans. 1,2-β-mannans were markedly detected on pathogenic yeast strains *Candida albicans* and *Candida glabrata* but barely detectable on a non-pathogenic strain, *Saccharomyces cerevisiae*. These data are in line with the fact that 1,2-β-mannans are known to interact with Toll-like receptors to induce an inflammatory immune response against pathogenic yeast.

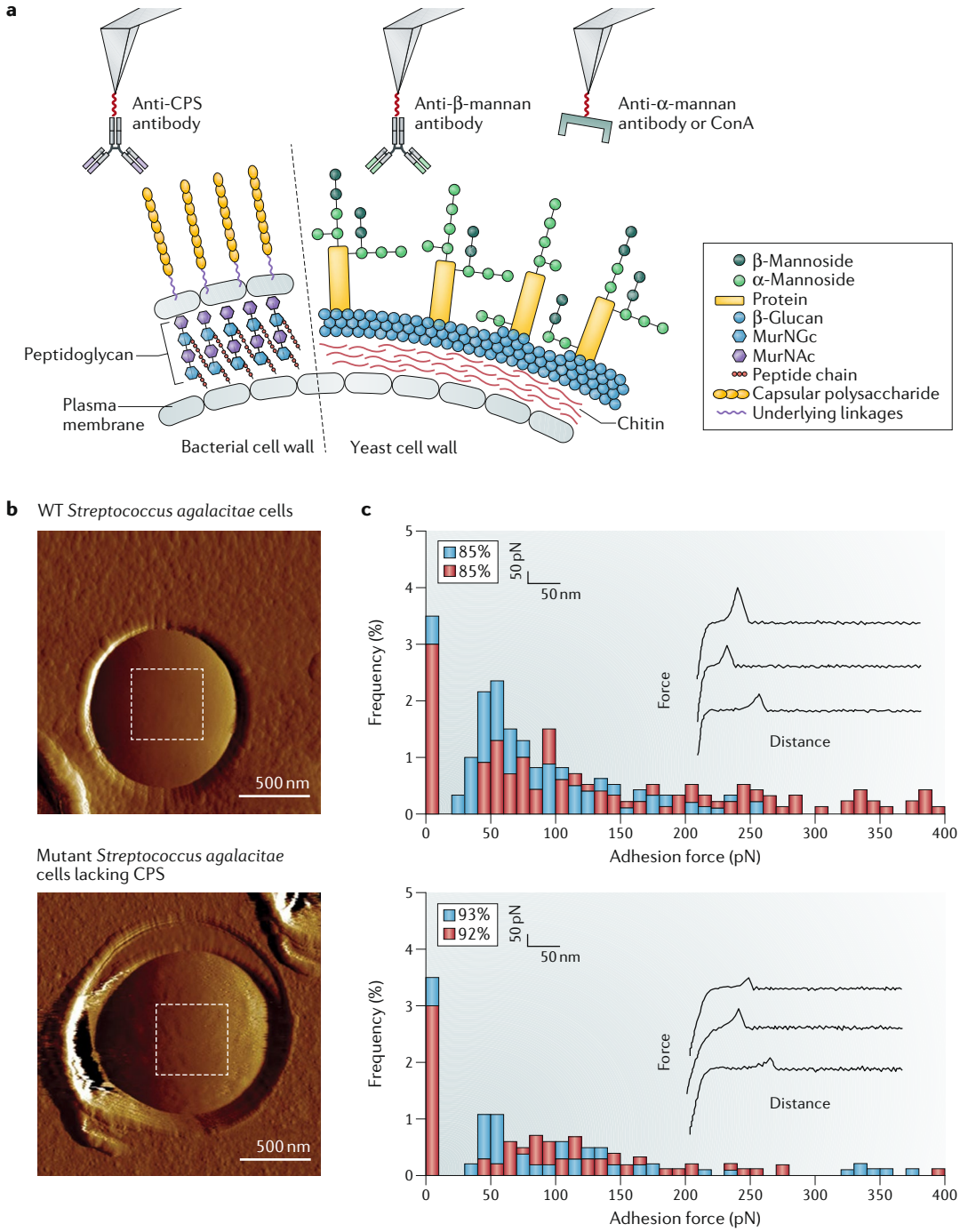


Fig. 4 | **Single-molecule cell-surface glycan 'mapping'. a** | Schematic representation of the use of single-molecule atomic force microscopy (AFM) to determine the composition of the cell wall of pathogenic microorganisms in situ. AFM tips functionalized with anti-capsular polysaccharide (CPS) antibodies were used to probe both the localization and density of the outer glycans of *Streptococcus agalactiae* CPSs. In addition, anti-β-mannan antibodies on AFM tips were used to map the localization of β-mannans on the surface of yeast strains, revealing that their expression was much greater on pathogenic yeast strains than on non-pathogenic yeast strains. **b** | AFM force–volume images of the cell wall of wild-type (WT) *Streptococcus agalactiae* and of *S. agalactiae* lacking CPS probed using anti-CPS antibody. The region in the dashed box was probed to generate the frequency–adhesion and force–distance profiles shown in part **c. c** | The frequency at which the glycan being probed by AFM was present was plotted with respect to adhesive force and the corresponding force distance. Blue and red bars represent two independent experiments. ConA, concanavalin A. Parts **b** and **c** are reproduced with permission from REF.[66], RSC.

Although it is rare for AFM to provide mechanistic insight, one example in which it did so involves family 3 carbohydrate-binding modules (CBM3s) and their 'helper' role in the enzymatic-processing of cellulose. Combined use of AFM imaging of CBM3-functionalized gold nanoparticles and SFMS using a CBM3-functionalized tip allowed the time-dependent monitoring of the alignment of CBM3s along a single cellulose fibril as an assessment of corresponding affinities[68–70]. Using different concentrations of CBM3s and a fixed concentration of cellulose, a minimum initial concentration of $5.1 \times 10^{-7}$ M CBM3 was required for CBM3s to bind to the cellulose after ~5 h (REF.[69]). This elegant study enabled the determination of binding kinetics in this semi-heterogeneous system, which is in contrast to bulk studies in which the heterogeneous distribution of 'free' molecules limited the understanding of real-time processes[71]. This example highlights the unique insights into kinetics that certain single-molecule methods may provide under atypical (for example, interfacial) conditions for substrates that have been difficult to assess, such as insoluble or partially soluble biopolymers (for example, polysaccharides).

Intriguingly, it has been proposed that SMFS can be used as an analytical tool to identify different sugar isomers[72]. A comparison of β-galactan (a polysaccharide of β-1,4-linked D-galactosyl units) and amylose (made up of α-1,4-linked D-glucosyl units) moieties showed that this can be achieved by assessing conformation-associated changes in these sugars. Force–extension curves obtained from measurements using, as a substrate, a single polysaccharide molecule showed a unique fingerprint plateau at 600 pN for β-galactans. A comparison of the force spectrograms of β-galactan and amylose revealed that the energy required to stretch a single β-galactosyl residue in the context of β-galactan was 1.1 kcal per mol higher than the energy required to stretch a single α-glucosyl residue in amylose. This suggests that force spectroscopy could potentially discriminate between sugar isomers when cumulative differences in configuration influence chain conformation; viewed in this way, the differences found between β-galactan and amylose occur through the 'interchange' of axial and equatorial bonds at C1 and C4. It will be interesting to see if, in addition to being successfully applied to this example, which involves homogeneous repeating units, force spectroscopy could be applied to more heterogeneous polysaccharides (for example, those with alternating subunits) or whether the resulting pulling energy spectra would be insufficiently distinct.

In this subsection, we have focused on the use of AFM as a single-molecule method. Given the important influence that trap-based tweezing methods (using optical and magnetic traps, for example) have had on force measurement in other biological fields[73], we foresee a wider use of these methods in glycobiology, including through the use of glycan-coated beads[74].

***Single-molecule field-effect transistors.*** The topology of existing constructs can be altered to provide powerful new systems for use in single-molecule glycobiology. Lysozyme-tethered single-wall carbon nanotubes can be used as intimate field-effect transistors to study the T4 lysozyme-mediated processing of cell wall-derived linear and crosslinked peptidoglycans[75]. By monitoring dynamic electronic signals that correspond to enzyme activity, hydrolytic degradation events were observed. For both linear and crosslinked peptidoglycans, signal switching rates of 20–50 $s^{-1}$ were observed that were correlated with substrate processing. Notably, these observations suggest that the enzyme degrades crosslinked peptidoglycans, albeit at a rate ~15% slower than the rate at which it degrades linear peptidoglycan. Thus, despite the highly heterogeneous structures found in these substrates, single-molecule studies have allowed a more precise understanding of the dynamics and function in what might be considered to be a near-native model of degradation.

***Single-molecule fluorescence.*** Some 20 years ago, fluorescence correlation spectroscopy (FCS) was used to study binding of the lectin concanavalin A (ConA) to a fluorescently labelled β-maltopyranoside. Dilute solutions of this ligand (1 μM) allowed unbound lectins to be distinguished from carbohydrate-bound lectins[76], providing valuable proof of concept that FCS can be used to study glycobiology. Since then, several examples have given a sense of where single-molecule fluorescence is most valuable in glycobiology. For example, CBM–crystalline cellulose interactions have been examined with a method termed defocused orientation and position imaging (DOPI), a technique based on anisotropy of dipole radiation. This was used to study the orientation of CBMs fused to green fluorescent protein (GFP) with respect to cellulose fibrils during binding interactions. The images obtained (under total internal reflection fluorescence (TIRF) illumination with slight defocusing) exploit the clear transition dipole of GFP, allowing a definitive orientation of CBMs along cellulose fibrils to be determined; these proved consistent with current models of CBM molecular recognition processes[77]. Again, in this way, single-molecule methods can explore important glycan–protein interfaces (for example, those in crystalline cellulose) that are less tractable to bulk methods.

In addition to the direct monitoring of sugar–protein ligand interactions, the broader (for example, cellular) regulation and function of sugar-processing proteins may be monitored through the use of fusion fluorescent proteins. For example, SMFS allows real-time monitoring of the expression[78] of the *Escherichia coli lacY* gene that encodes lactose permease (LacY) — a symporter that transports β-galactosides such as lactose into the cell. The *lacY* gene is part of the *lac* operon. Protein production in bacterial cells under non-induced conditions (that is, when a repressor is bound to mRNA) was shown to be a stochastic event that occurs in 'bursts'[79]. The *lac* operon in *E. coli* is repressed by lactose operon repressor (LacI) in the absence of the inducer allolactose (or other galactosides, such as IPTG) (FIG. 5a). The presence of inducer, however, dislodges the repressor and triggers protein production. In this way, using LacY fused to a fluorescent protein, gene regulation can be studied at a single cell level[80]; in the presence of low levels of inducer,

Total internal reflection fluorescence
(TIRF). Microscopy examining a thin region of a specimen (~100 nm) that uses internal reflection at a surface–sample interface for the selective excitation of surface-associated fluorophores, thereby removing contributions from the background.

Stochastic optical reconstruction microscopy (STORM). Dilute fluorophores are switched on and off through switching or bleaching, and the 'centres' of their location are ascertained using point-spread of photon emission based on a widefield image. After several rounds, a 'super-resolution' image is assembled by plotting the measured positions of these centres of the fluorescent probes.

the cell phenotype can be switched owing to the partial dissociation, slow rebinding or complete dissociation of the repressor from mRNA. Specifically, slow rebinding and partial dissociation of the repressor resulted in only basal levels of LacY production, whereas the complete dissociation of the repressor resulted in a large burst of LacY production and a switch in phenotype. In addition to monitoring its production, the use of other single-molecule fluorescent methods allows the structural dynamics of LacY to be determined; indeed, single-molecule FRET provided a real-time method for monitoring sugar symport in *E. coli*[81]. X-ray crystallography provided structures that form the basis of an 'alternating access' model for LacY-mediated sugar transport[82,83]. This model proposes that sugar binds at the centre of LacY from either cytoplasmic or periplasmic faces, followed by the sequential opening and closing of the faces to allow symport. FRET-based single-molecule fluorescence enabled monitoring of precise distance changes between donor–acceptor fluorophore pairs upon binding of a galactopyranoside ligand and hence validated this model[81].

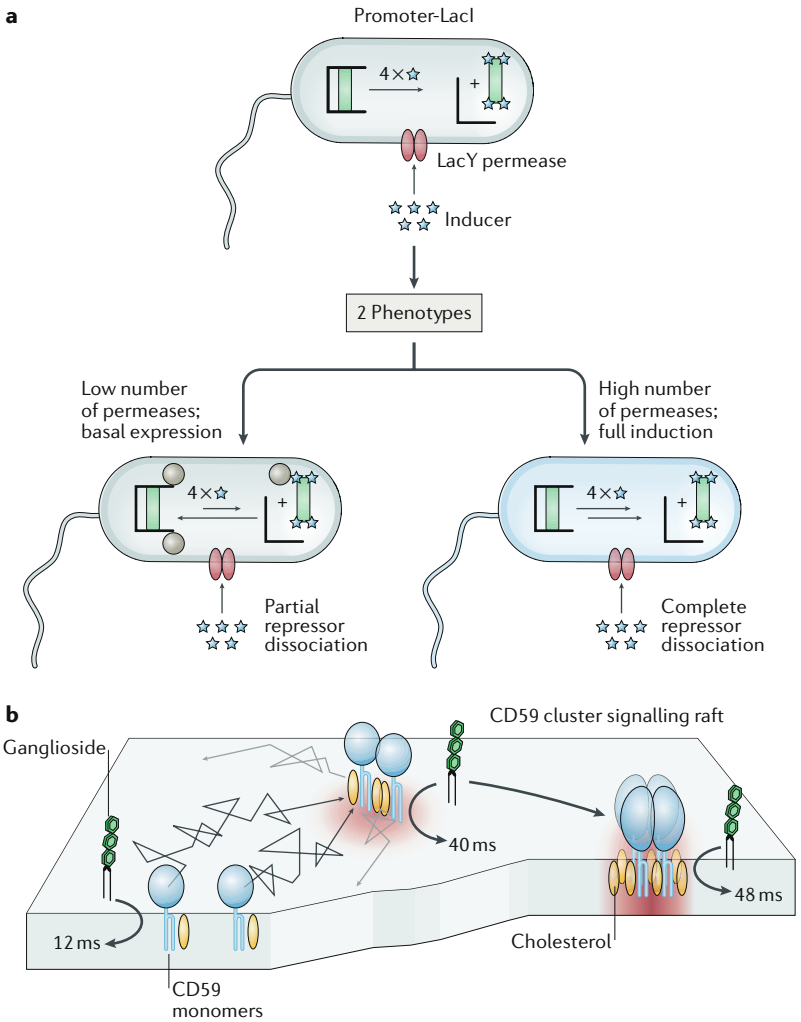


Fig. 5 | **The study of glycan processing and presentation using single-molecule fluorescence. a** | Stochastic phenotype switching in *Escherichia coli* observed at low or intermediate concentrations of intracellular inducer studied using fluorescent lactose permease (using a lactose permease (LacY)–yellow fluorescent protein (YFP) fusion) protein. The observation of precise molecular events allows two distinct states of *E. coli*, based on the expression (and hence function) of the sugar transporter LacY, to be identified. **b** | Clustering and distribution of gangliosides and glycosylphosphatidylinositol on cell surfaces shown by single-molecule fluorescence imaging via total internal reflection fluorescence (TIRF). The observed localization of CD59 and gangliosides in events with varying lifetimes, in both stimulated and unstimulated cells, implicated gangliosides in the formation of longer lasting CD59-cluster signalling rafts as part of stimulation. Part **a** is adapted with permission from REF.[80], AAAS. Part **b** is reproduced from REF.[84], Macmillan Publishers Limited.

In addition to in cellulo examination of their protein partners, fluorescence also allows single-molecule tracking of glycans themselves. For example, the interaction of red-labelled gangliosides with green-labelled CD59 domains (used as analogues of glycosylphosphatidylinositol (GPI) anchors) has been studied. Fluorescent single-molecule tracking using TIRF spectroscopy that allowed precise localization of fluorophores revealed that gangliosides and CD59 domains dynamically colocalize with interaction lifetimes of up to 10 ms. Interestingly, cholesterol supplementation enhanced the formation of CD59–ganglioside raft domains, and, although interactions remained extremely dynamic under these conditions, this nonetheless increased the association lifetime of gangliosides and CD59 domains to 40 ms (REF.[84]) (FIG. 5b).

Cell-surface sugars can also be manipulated to engineer cell-surface morphology. For example, techniques can be used to artificially induce network formation between conjugated glycans at cell surfaces[85,86]. In one example[86], single-molecule tracking via epifluorescence monitored the response of one such network to changes in tuneable parameters, namely, valency of the biotin-binding protein streptavidin (through pre-blocking) and concentrations of streptavidin (FIG. 6), revealing that the mobility of the cell membrane could be tuned from one with physiological mobility to one that was stiff and almost frozen. As might be expected, this enhanced artificial network formation and decreased membrane mobility and permeability in membrane-dependent processes, such as endocytosis. Controlling the mobility of cell-surface glycans, monitored by single-molecule fluorescence, might prove valuable in applications that could exploit the use of such artificial cytoskeletal structures.

***Super-resolution microscopy.*** Several methods in single-molecule fluorescence require some level of cellular 'fixing', generating samples that may not represent those in nature. However, the use of stochastic optical reconstruction microscopy (STORM)[87] can avoid this need by exploiting photoswitchable fluorophores, which, following several cycles of activation, enable the localization of biomolecules on cell surfaces with 'super-resolution'. For example, STORM imaging using Cy5-conjugated lectin enabled the identification of corresponding glycan ligands, which were presumably D-Man-containing based on lectin selectivity, on cell surfaces. Glycan clusters 105–850 nm in diameter were identified, suggesting that these sit on glycoproteins within membranes that form microdomains. Expansion of this application of STORM through indirect visualization allowed the semi-systematic determination of other carbohydrate types found in clustered

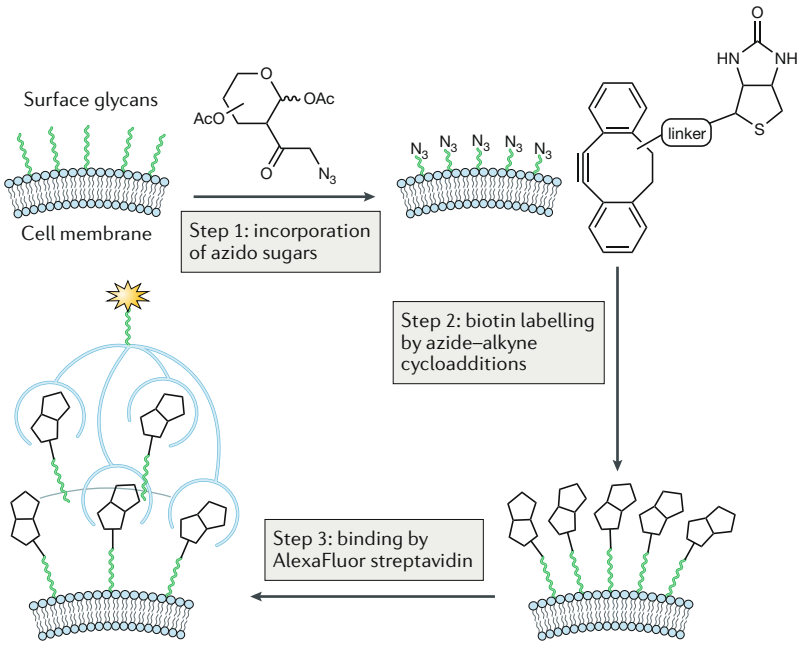


Fig. 6 | **Creation of artificial cytoskeletal networks on cell surfaces via glycans.** Artificial cytoskeletal networks can be generated on cell surfaces by crosslinking. In one mode, this may be achieved by adding azido sugars to cells (step 1), which are then metabolically incorporated into cell-surface glycans. The copper-free azide + alkyne triazole-forming reaction enables the labelling of glycans with biotin (step 2). Finally, the addition of fluorescent streptavidin (that is, streptavidin labelled with an AlexaFluor dye), which can bind to biotin, causes connections to form between glycans (step 3). As the streptavidin is fluorescently labelled, this allows this 'tuneable' artificial process to be potentially monitored at the single-molecule level[86].

states on cell membranes and corresponding membrane components[88]; for example, epidermal growth factor (EGFR) colocalized with GalNAc clusters. The authors propose that these clusters have a more global role in enhancing protein–glycoprotein interactions through the cluster multivalency that is created. It should be noted that the existence of such glycan clusters or microdomains has also been challenged; the direct labelling of unnatural cell-surface glycans with suitable fluorophores (using triazole-forming chemistry) followed by STORM revealed that they have a homogeneous, non-clustered distribution[89]. Such method-dependent differences in findings highlight the need to analyse underlying assumptions and possible attributable artefacts. Among these assumptions, it should be considered that, whereas super-resolution microscopy has a resolution of ~20 nm, which may be suitable for visualizing single protein molecules, this resolution cannot visualize single glycan residues.

The simultaneous fluorescent labelling in live cells of *N*-linked sialic acid and *O*-linked glycans, using alkyne-containing mannose and azido-containing galactosamine, respectively, as metabolic precursors, has allowed their diffusion rates to be estimated by STORM[90]. Glycans on the surface of the mouse mammary cancer cell line PyMT diffused at rates two to three times faster than those on the surface of its highly metastatic variant cell line, Met-1. The diffusion rate was hypothesized to reflect the metastatic potential of these cell lines, suggesting that the imaging of single-molecule glycans is useful for studying the morphology, and even potential phenotypes, of cells.

The related super-resolution method photoactivated localization microscopy (PALM) has been used to determine the influence of surface substrate morphologies upon enzyme activity[91]. Cellulose targeting was used as a model system and PALM was used because AFM-based techniques cannot resolve individual proteins from within a dense, heterogeneous surface. PALM revealed that when crystalline-specific CBMs were added to cellulose, they tended to cluster on cellulose 'ridges'; by contrast, amorphous-specific CBMs were found to be uniformly present across the surface of cellulose[91]. These observations suggest that a combination of CBM types will be more effective when used in aiding cellulose degradation.

***Nanopores.*** A preliminary system based on heptameric α-haemolysin pores showed the potential of nanopores in single-molecule glycobiology (FIG. 7). Specifically, the binding kinetics of the *Bauhinia purpurea* (BP)-lectin were examined in a modified variant of α-haemolysin displaying seven glycans[92]. By monitoring changes in ionic current flow using single-channel recording, pore events with two lifetimes (suggestive of two binding modes) were detected. Although the presence of multiple glycans on the pore prevented the identification of distinct binding modes from many possible permutations, such methods illustrate that strategies in which the 'sensor' is also a biomolecular substrate could prove useful for elucidating the mechanism of glycoprotein–lectin interactions.

In this example, the α-haemolysin pore was used as a sensitive platform on which to mount glycans, but it had no inherent role in glycan binding or processing. By contrast, the putative polysaccharide export protein Wza from *E. coli* is involved in capsular polysaccharide (CPS) export. Its ability to record the passage of sugars through single channels has been explored (FIG. 7). Using synthetic fragments of CPS and single-molecule methods employing droplet bilayers, sugar export was partially recapitulated, and an early sense of the inherent selectivity of this natural sugar pore — even when Wza was not associated with its usual transport helper proteins — was achieved[93]. The results suggest that, even for small oligomers, coordinated aid is needed from underlying proteins for sugar transport in such systems. This Wza system allowed a library of putative Wza blockers to be tested and identified an inhibitor of CPS transport through screening at a single-molecule level; some inhibitors of CPS transport had dissociation constant ($K_d$) values <50 nM (REFS[93,94]). As inhibiting CPS transport prevents the formation of the bacterial capsule, these inhibitors may synergize with antibiotic and antibacterial vaccination strategies[94,95]. Chitoporins, which are involved in sugar transport in marine bacteria, have also been tested in single-channel recordings. The studies suggest chitoporin monomers have multiple traps, or binding sites, for sugars; stochastic binding of one sugar may cooperatively assist the sequestration of multiple sugar units, perhaps enabling the marine bacteria to achieve high sugar translocation even in extreme

Photoactivated localization microscopy
(PALM). Similar to STORM but has typically used photoactivation and then bleaching to switch fluorophores on and off. It is more often associated with the use of protein fluorophores such as photoactivatable fluorescent proteins.

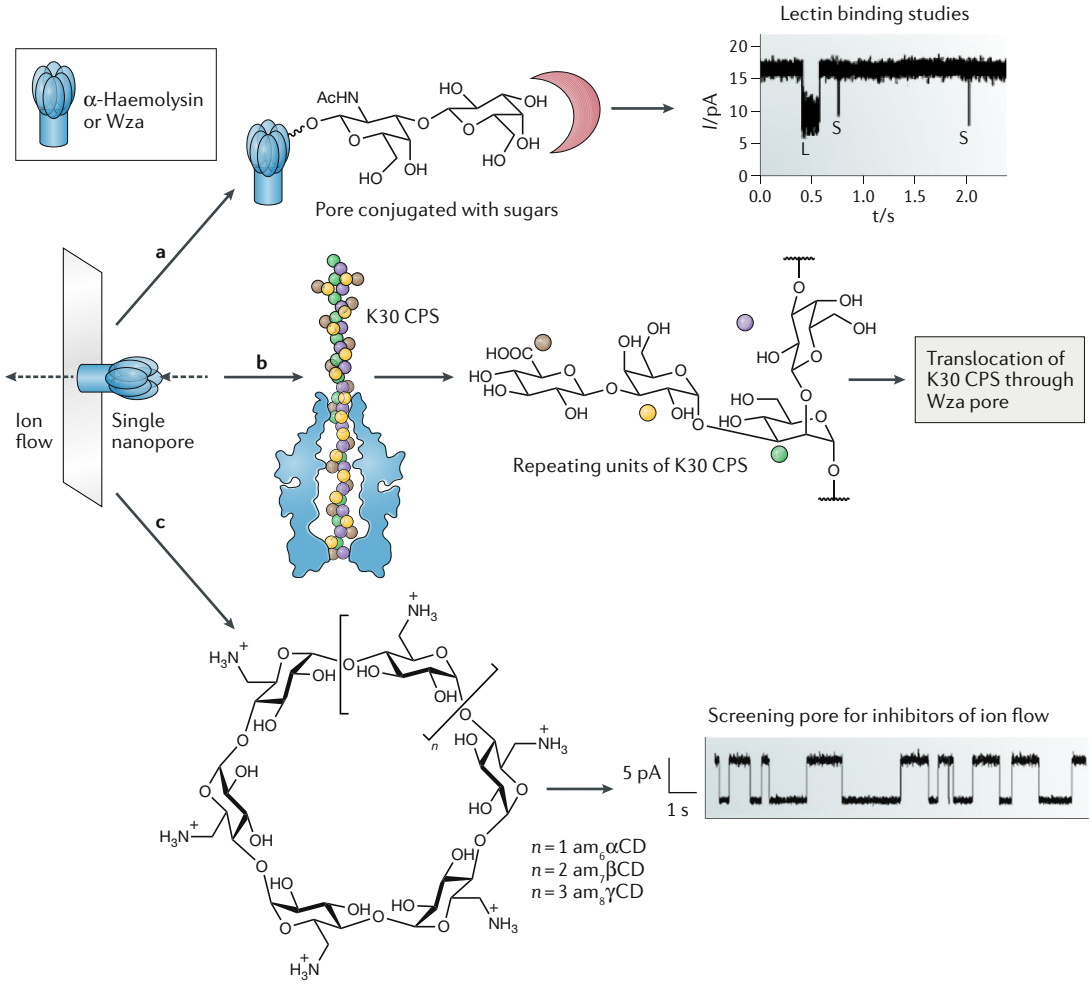


Fig. 7 | **Nanopore single-channel recording in glycobiology.** Monitoring changes in current transmitted by ion flow through nanopores has allowed the study of various carbohydrate–protein interactions. **a** | Multivalent carbohydrate–lectin interactions can be monitored by functionalization of the pore with glycans and seeing the modulation of current upon binding[92]. **b** | Parts of polysaccharide export in bacteria can be recapitulated by using the relevant export pore itself as the nanopore[93]. **c** | Screening for inhibitors[94] of polysaccharide export in bacteria also allows the discovery of compounds that may represent a new class of antibacterial agent. Note that in this figure, the colouring of the spheres is arbitrary and does not follow the symbol nomenclature for graphical representations of glycans[100]. CPS, capsular polysaccharide. The trace in part **a** is reproduced with permission from REF.[92], Wiley. Parts of part **b** and **c** are reproduced from REF.[94], Macmillan Publishers Limited.

environments[96]. It is notable that such nanopore-based traps have not yet, to our knowledge, been complemented in glycobiology by other trap types[73,97].

***Recognition tunnelling.*** An approach enabling the identification of sugar anomers and isomers at the single-molecule level by 'recognition tunnelling' has been identified[98]. In this process, fluctuations in current observed when a single molecule is trapped in the space between two electrodes that have been functionalized with appropriate recognition molecules can distinguish between anomers or epimers. 4(5)-(2-mercaptoethyl)-1*H*-imidazole-2-carboxamide (ICA), which can form multiple hydrogen bonds, has been proposed to be a suitable recognition molecule for analysing carbohydrates by recognition tunnelling. By analysing changes in the spikes in current produced by different epimers, the authors proposed that α-D-glucopyranoside could be discriminated from β-D-glucopyranoside (these are C1 epimers and/or anomers). They also showed that various C4 epimers could be distinguished — Glc, $GlcNH_2$ and GlcNAc from Gal, $GalNH_2$ and GalNAc, respectively — and that the C5 epimers D-glucuronic acid and L-iduronic acid could be discriminated, all with an accuracy >98%. Like SMFS for polysaccharides (see above), the applicability of recognition tunnelling to more complex sugar units remains to be explored.

## Challenges and future directions

Although the widespread application of some single-molecule techniques in studying glycobiology has been partly impeded by the need for bespoke methods, for example, nanopore technologies or certain fluorescence techniques, the development of commercially available equipment and reagents is increasingly reducing this barrier. That said, for techniques in which methods have

been more accessible, such as AFM, somewhat time consuming data recording and result analyses still necessitate high levels of technical expertise (and/or dedication) and prescient experimental design. Moreover, artefacts arising from defective samples, nonspecific interactions or the use of indirect probes (for example, labelled lectins) can lead to observations for which caveats need to be carefully considered and declared. Above all, the current lack of studies in more complex (for example, in cellulo or even in vivo) settings suggest such experiments as an interesting mid-term challenge[99].

Despite such ongoing challenges (from observing sugar transport though a single pore to testing the mode in which glycans are displayed on cell surfaces or assessing their multivalency), single-molecule interrogation is leading us towards an improved understanding of the more complex aspects of glycobiology. When bulk studies provided useful possible models but insufficient detail, single-molecule interrogation has, in certain cases, already allowed the validation of proposed mechanistic models. This progress may open new avenues in the development of inhibitors of sugar–protein interactions or more potent antigens for use in vaccination, as well as in mapping and correlating distribution patterns of glycoconjugates as potentially important functional biomarkers. They also, in turn, raise questions and highlight the current limitations of using single-molecule techniques to study glycobiology. For example, can (or should) all of glycobiology be studied at the single-molecule level? And which single-molecule methods can be developed and utilized to study a given problem without losing contextual or functional relevance? The use of an accessible technique will need to be balanced by its functional relevance.

Although single-molecule analysis of sugar biology is still in its relative infancy, a wealth of opportunities exist. Perhaps most importantly (and perhaps more so than in other areas of biology), single-molecule methods could prove essential in enabling the proper mechanistic understanding of glycobiology. For example, these techniques might be essential for our full understanding of the functional roles of heterogeneity and multivalency in glycans. Thus, with careful experimental design, some molecular causes and drivers of sugar function in the biology of complex organisms may emerge. This, in turn, could enable precise and coherent kinetic analyses of carbohydrate-processing in context and hence allow sufficient selectivity for more effective medical diagnostics and therapies. By providing specific information and unearthing hitherto unknown details in areas in which bulk analyses of mixtures have limits, single-molecule technology is a valuable tool in glycoscience.

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**Competing interests**
B.G.D. is named as an inventor on patents that describe new antibiotics based on some of the Wza science described in this Review. If licensed, these would afford B.G.D. royalties in line with normal university practice.

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