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## The challenges of glycan recognition with natural and artificial receptors

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Glycans – simple or complex carbohydrates – play key roles as recognition determinants and modulators of numerous physiological and pathological processes. Thus, many biotechnological, diagnostic and therapeutic opportunities abound for molecular recognition entities that can bind glycans with high selectivity and affinity. This review begins with an overview of the current biologically and synthetically derived glycan-binding scaffolds that include antibodies, lectins, aptamers and boronic acid-based entities. It is followed by a more detailed discussion on various aspects of their generation, structure and recognition properties. It serves as the basis for highlighting recent key developments and technical challenges that must be overcome in order to fully deal with the specific recognition of a highly diverse and complex range of glycan structures.

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### 1. Introduction

Glycans occur as free polysaccharides or as mono, oligo or polysaccharides conjugated to a wide variety of biological molecules, which include glycoproteins, glycolipids and peptidoglycans (Fig. 1). In contrast to RNA, DNA and protein synthesis, glycan biosynthesis is not a template-driven process. Instead, glycans are assembled by the expression and activity levels of a

series of enzymes present in cells *via* glycosylation.<sup>1</sup> Despite being generated from a limited number of monosaccharides, glycans are characterised by a remarkable structural diversity due to the nature and sequence of the constituent units, the possible branching of the carbohydrate chains and the configuration and position of glycosidic linkages. Monosaccharides have multiple hydroxyl moieties that can serve as linking groups, and the glycosidic bond at the anomeric carbon can have either  $\alpha$  or  $\beta$  stereochemistry, leading to a wide range of potential linkages between two monosaccharide units. Furthermore, glycans can be further diversified by a range of modifications, including sulfation, phosphorylation, and acetylation. It is this diversity that makes glycans perform a vast array of biological

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He then joined the Mendes group (2016), where he investigates glycan recognition with boronic acid derivatives for applications in diagnostics.

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functions and play important roles in various physiological and pathophysiological events, including cell growth, cell signaling, cell-cell interactions, differentiation and tumour growth.<sup>2,3</sup>

Glycans carry information in biological systems that make them an important source of biomarkers for a wide range of diseases, including neurodegenerative diseases, hereditary disorders, immune deficiencies, cardiovascular diseases and many types of cancers.<sup>4–6</sup> During the development and progression of these diseases, glycan expression is altered due to misregulation of enzymes such as glycosyltransferases and glycosidases, leading to changes in glycan structures that can potentially be used to accurately identify the disease at an early stage. In addition to their clinical value in disease diagnosis and management, glycans are an emerging class of therapeutics,<sup>7</sup> ideal markers for identifying and isolating specific cell types, including stem cell lineages for therapeutic transplantation,<sup>8</sup> and among other facets, they are key targets in drug discovery.<sup>9</sup> Regarding the latter, glycans are present on the surface of a variety of pathogens and malignant cells, making them ideal targets for vaccines.

The recognition of glycans by other molecules with high affinity and exquisite specificity is at the heart of current and

further developments in glycan-related basic research and clinically relevant diagnostics and therapeutic applications. However, there are unique challenges associated with such recognition processes since the glycan-binding entities need to be able to discriminate between a large repertoire of carbohydrate structures, including closely related isomers. Subtle differences, such as the stereochemistry of a single hydroxyl group, make selective recognition hard to achieve. Moreover, strong solvation in water poses another major challenge since receptors must be able to compete with an extensive network of hydrogen bonds surrounding the glycans. Unlike nucleic acids or proteins, it is difficult to recognise glycans using complementary sequences or specific antibodies. Glycans are poorly immunogenic, posing major hurdles in the development of highly selective anti-carbohydrate antibodies.<sup>10</sup> While efforts are underway to tackle the low affinities and promiscuous specificity of anti-glycan antibodies, other major strategies involving either lectins,<sup>11</sup> aptamers<sup>12</sup> and boronic acid derivatives<sup>13,14</sup> are concurrently and actively being pursued (Fig. 2).

This critical review focuses on the recent progress on all the different strategies to achieve superior glycan recognition, without considering the substantial efforts concentrated on



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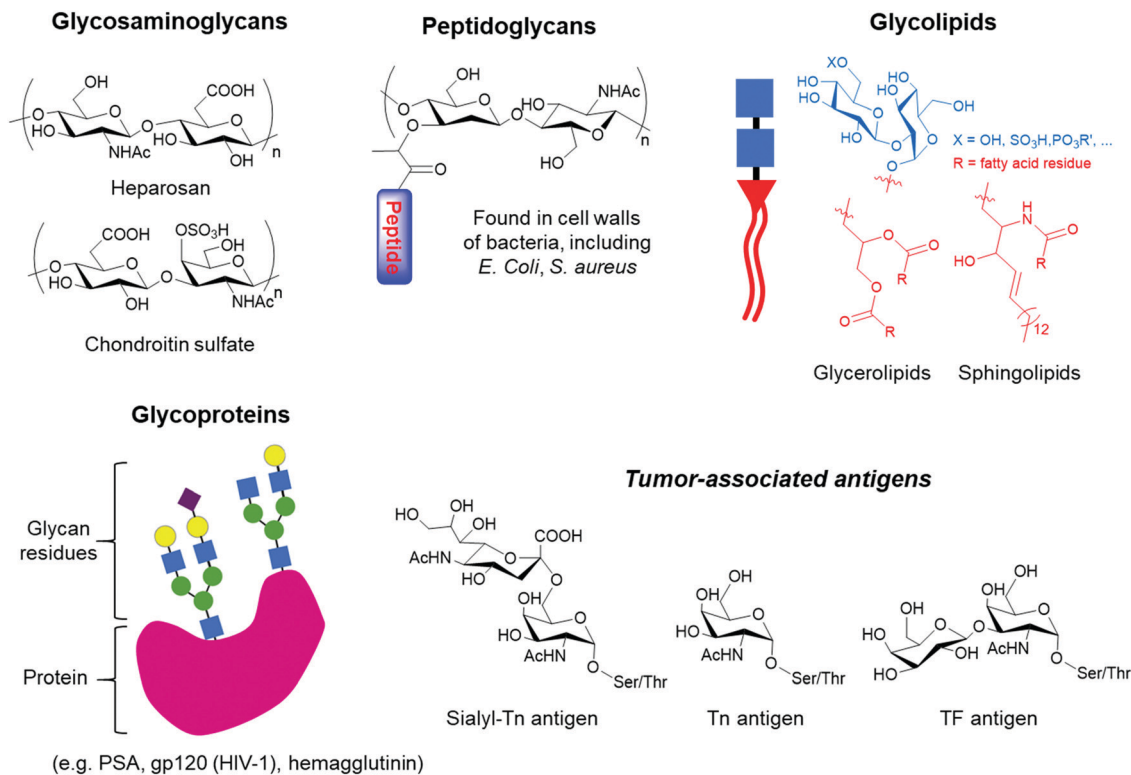


Fig. 1 Examples of glycans that can be found in nature, ranging from glycosaminoglycan polysaccharides to glycoconjugates such as peptidoglycans, glycolipids and glycoproteins, which can bear tumour-associated antigens.

Glycan recognition	
<ul style="list-style-type: none"> <li>Hydrogen bonds, CH-<math>\pi</math>, van der Waals and electrostatic interactions</li> <li>Large binding sites to envelop glycan portions</li> <li>Present in human serum</li> </ul>	<ul style="list-style-type: none"> <li>Mainly hydrogen bonds; CH-<math>\pi</math> and electrostatic interactions possible</li> <li>Multiple interactions with terminal sugars</li> <li>Shallow binding pockets</li> </ul>
<ul style="list-style-type: none"> <li>Single stranded oligonucleotides (typically &lt; 100 bases)</li> <li>Non-covalent interactions (mainly hydrogen bonds)</li> <li>Modifications for the incorporation of sugar binding molecules</li> </ul>	<ul style="list-style-type: none"> <li>Reversible covalent bonds with 1,2- and 1,3-diols</li> <li>pH dependant binding</li> <li>Chemical versatility allows for insertion into different scaffolds</li> </ul>

Fig. 2 Glycan recognition can either be achieved using biologically-derived antibodies and lectins or chemically-derived aptamers or other smaller synthetic receptors such as boronic acids. While they all rely on supramolecular interactions, the methods for their generation differ greatly. These differences provide opportunities but also different types of challenges to create glycan-binding scaffolds with high affinity and exquisite specificity.

the binding of monosaccharides that are extensively reported elsewhere.<sup>15–20</sup> Particular emphasis will be given to the binding characteristics (*i.e.* affinity and selectivity) of the glycan-binding entities and their potential for further structural glycan differentiation. The purpose of this review is not to provide a comprehensive survey of the literature on antibodies, lectins and synthetic receptors such as boronic acid derivatives and

aptamers as entities for the recognition of glycans. Rather examples are provided to highlight main current capabilities and limitations of biologically- and chemically-derived glycan-binding scaffolds. These serve as the framework to bring together as a whole the field of glycan recognition, identify key challenges across the field and potential avenues for further progress on creating glycan-binding scaffolds with high affinity and, more importantly, with great specificity, for a wide, relevant range of glycan structures. The advantages and drawbacks associated with the production and use of glycan-binding scaffolds of biological or synthetic origin are also examined.

## 2. Anti-glycan antibodies (AGA)

Antibodies are produced by the immune system in response to a foreign molecule, namely antigen, entering the body. A given antibody binds specifically only to a small site on its antigen, called an epitope, which usually consists of a few amino acids or monosaccharide units. An ideal antibody should have high specificity and avidity for its antigen. However, glycans pose a serious challenge to antibody development. While selectivity has been observed in some auto-AGAs, other AGAs have been found to interact with the same antigen, even if with different affinities. Development of highly selective anti-glycan antibodies has been hindered both by the inherently poor immunogenicity of carbohydrates, labour intensive antigenic material production and the similarity between various carbohydrate sequences.<sup>21</sup>



There are also difficulties in generating anti-glycan antibodies because a wide variety of complex glycans are intrinsically expressed in standard host organisms used to produce antibodies.

A vast collection of AGAs are present in human sera and play critical functions in many immune processes. AGA expression depends on exposure to both foreign carbohydrate sequences, as seen on pathogen surfaces, and to sequences from the “self” of the same species (*i.e.* blood group antigens A and B). Despite some AGAs exhibiting affinity for naturally occurring glycans, to date they cannot be considered true auto-antibodies due to apparent lack of selectivity for native carbohydrate sequences.<sup>22</sup> Prevalent natural AGAs in human sera include those to foreign glycan epitopes such as *N*-glycolylneuraminic acid (Neu5GC) and galactose- $\alpha$ -1,3-galactose ( $\alpha$ -Gal), antigens responsible for the rejection of xenotransplanted organs in humans from pigs.<sup>23–25</sup> Other extensively studied AGAs are the anti-blood group antibodies against A, B and rare ‘Bombay’ type O antigens, raised *in vivo* in the absence of respective erythrocyte surface antigen. They are highly specific and able to differentiate, for instance, between the glycan structures of the A- and B-antigens, wherein an acetamido moiety in A is replaced with a hydroxyl group in B.<sup>26</sup>

Auto-AGAs found in serum are now useful biomarkers in the diagnosis of inflammatory and autoimmune diseases, including multiple sclerosis and Crohn’s disease.<sup>27</sup> Cancer patients are also able to produce anti-glycan antibodies, which opens up the possibility of their use for early detection of cancer.<sup>28,29</sup> While promising for diagnosis, they are not effective in activating the immune system and eliminating malignant cells, limiting progress in generating new immunotherapies against cancer.<sup>30–32</sup>

Typically, glycan-binding antibodies exhibit lower affinities (with equilibrium dissociation constant ( $K_D$ ) values in the micromolar range for monovalent interactions) than protein-specific antibodies (with  $K_D$  values in the nanomolar range).<sup>33,34</sup> However, affinities can be enhanced by generating antibodies containing two or more glycan binding sites, or through non-covalent assembly into oligomers with multiple binding sites.<sup>35,36</sup> The formation of a multivalent complex can result in higher affinities and enhanced selectivities.<sup>37,38</sup> Remarkably, human antibody 2G12 was found to bind strongly to HIV-1 glycoprotein gp120 ( $K_D$  of 5.6 nM) *via* formation of a dimer that establishes multiple complex interactions with the oligomannose epitope of the glycoprotein.<sup>39,40</sup> Furthermore, phage-display technology has been instrumental in generating AGAs against a broader range of glycans with superior affinity.<sup>41–43</sup> Phage display is an *in vitro* technique developed in the mid-80s that allows the study of protein–ligand interactions between a protein fragment exposed on the surface of a phage particle and a ligand of interest.<sup>44–46</sup> T-nouvelle (Tn) antigen (GalNAc $\alpha$ 1-Ser/Thr), highly expressed in various tumours, is an interesting target for cancer therapy. However, due to its poor immunogenicity, the development of an anti-Tn antibody is extremely challenging. A novel library ( $6 \times 10^5$  variants) of single-chain antibody fragments (scFv) for the Tn antigen was constructed using phage display technology.<sup>41</sup> The best scFv fragments were attached to an antibody constant region (Fc) and converted to scFv-Fc fusion proteins, which showed high specificity against the Tn antigen. Phage display

was also utilised to explore the recognition requirements of 2G12 towards gp120.<sup>47</sup> A set of amino acid residues on a 2G12 scaffold was varied in a restricted manner in order to identify the minimal physicochemical requirements for oligomannose recognition. Investigation of the generated phage display libraries provided insights into glycan-antibody interactions. In addition, the study revealed that there was a high degree of tolerance within the substitution of five to eight residues. Although in this case the binding affinity was negatively affected, the antibodies retained specificity for the target glycan, leaving positive perspectives for future improvements. Phage-display technology, coupled with new advances in protein engineering, are opening up new opportunities to tailor key antibody structural features to endow AGAs with the ability to form high-affinity interactions with glycan moieties.

A growing understanding of the immunological mechanisms by which the immune system refines its antibodies, together with recent technological advances in carbohydrate chemistry and bioconjugation are also enabling the production of high-affinity anti-glycan antibodies.<sup>48,49</sup> By using Q $\beta$  virus-like particles (VLPs) conjugated *via* copper-catalyzed azide–alkyne cycloaddition with short synthetic glycans, anti-glycan IgG antibodies with nanomolar affinity were produced (Fig. 3).<sup>48</sup> The controlled glycan density displayed on the virus-like particles facilitated not only B but also T cell recognition, which subsequently provided T cells with help in promoting affinity maturation in B cells.

Similarly to lectin–carbohydrate interactions, AGA–carbohydrate complexes are held together by hydrogen bonds, CH– $\pi$ , van der Waals and electrostatic interactions.<sup>33,50</sup> However, unlike lectins, antibodies are able to accommodate longer glycan epitopes in their binding sites and can generally interact with all monosaccharide units. Thus, the enveloping capacity of antibody binding sites can allow for very specific recognition.<sup>51,52</sup> A range of experimental techniques, including synthetic chemistry, antibody engineering, microarray technology and NMR, alongside computational approaches have been key to elucidate molecular structural features that modulate glycan–antibody recognition.<sup>53–57</sup> In this regard, antibody engineering and molecular modelling have been applied to demonstrate that in both pyranoside- and furanoside-antibody systems, the affinity and specificity mostly rely on CH– $\pi$  interactions.<sup>54</sup> While hydrophobic stacking interactions and hydrogen bonds are prevalent in the recognition of neutral glycans by AGAs, ionic interactions have been shown to play a key role in the binding of charged glycans.<sup>55</sup> Such interactions result in high glycan-antibody affinities ( $K_D$  values in the nanomolar range), which are generally associated with slow dissociation rates. Antibodies bearing positively charged amino acid residues in their binding domain can similarly recognise charged carbohydrate antigens, such as 3-deoxy-D-manno-oct-2-ulosonic acid (or Kdo, a trisaccharide sequence present in *Chlamydiae*) with  $K_D$  values  $< 1 \mu\text{M}$ .<sup>58</sup> Modulation of the electrostatic interactions can therefore be used to improve the binding properties of antibodies.<sup>59,60</sup> Furthermore, combination of microarray screening and saturation transfer difference NMR provide key information regarding the role of the amino acid linker, such as serine or threonine, in the binding process.<sup>56</sup> A related approach



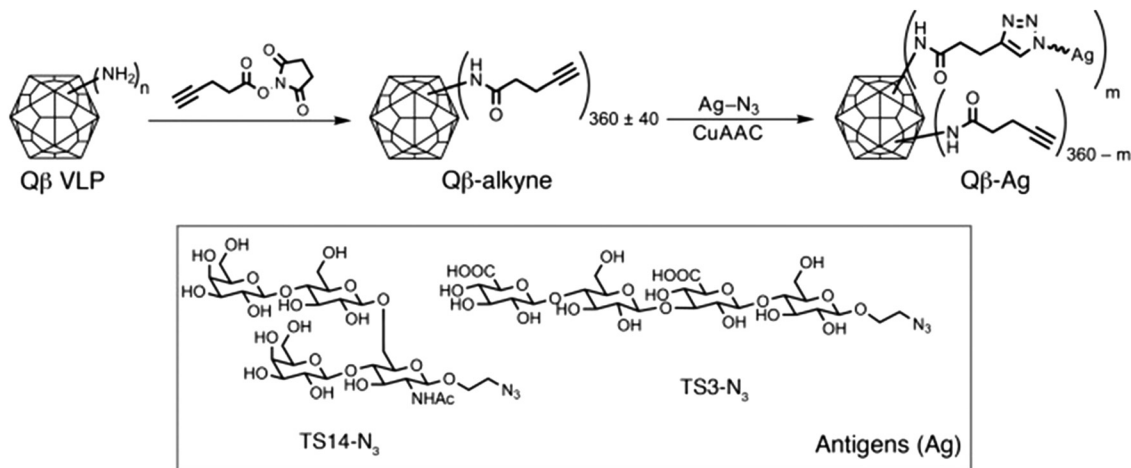


Fig. 3 Synthetic scheme illustrating the functionalization of Q $\beta$  VLPs with two oligosaccharide antigens (Ag), TS14 and TS3;  $m$  represents the number of antigens per VLP. Reproduced from ref. 48 with permission from the American Society for Clinical Investigation, copyright 2017.

has also been used to elucidate the effects of multiple *O*-glycosylation states on antibody recognition.<sup>57</sup> These and future insights on the principles governing glycan-antibody interactions are quite valuable for guiding antibody engineering and creating highly-specific AGAs.

Well-defined validated specificities are now possible due to, recent improvements in glycan microarray-based technology.<sup>61,62</sup> Over 1000 AGAs have been reported, although many of them target the same glycan structure, bringing the current total number of glycans being specifically recognised by AGAs to one-quarter of the AGAs available.<sup>21</sup> Tumour-associated carbohydrates, such as the Tn (GalNAc $\alpha$ 1-Ser/Thr), Sialyl-Tn, (Sia $\alpha$ 2-3GalNAc $\alpha$ 1-Ser/Thr), and the Thomsen–Friedenreich (TF) antigen (Gal $\beta$ 1-3GalNAc $\alpha$ 1-Ser/Thr), have been particularly targeted for AGA development.<sup>63,64</sup> There is also a good number of AGAs for ABH blood group antigens, Lewis antigens and glycolipids.<sup>65–67</sup> Indeed, these developed antibodies are either currently in clinical trials or already employed in disease treatment. They have also been used for diagnostic applications, as is the case for the antibody that targets Sialyl Lewis A for detecting and monitoring several cancers.<sup>68</sup>

Despite considerable efforts, many current AGAs display low affinities for their target glycans and a lack of specificity, recognising families of structurally related glycans, rather than a single distinct structure. Furthermore, available AGAs cover a narrow set of glycan families and epitopes and many important *O*-glycans, *N*-glycans, glycosaminoglycans and modified glycans (*e.g.* sulphated glycans) still lack a corresponding antibody.<sup>21</sup> AGAs that can discern a specific amino acid residue (*e.g.* serine *versus* threonine) involved in the glycopeptide bond are also needed. Therefore, there are still many opportunities for the development of new AGAs for impacting on basic research, as well as for new diagnostic and therapeutic applications. Innovations in and beyond the initial phage display technology and their combination with other emergent technologies, as well as advances in glycan synthesis and glycan arrays for high-throughput AGA analysis, are expected to play a key role in

creating a larger repertoire of AGAs with high binding affinities and selectivities.

### 3. Lectins

Lectins are carbohydrate-binding proteins of non-immune origin that are present in plants, animals and microorganisms. They are involved in many physiological events and currently play a crucial role as tools for glycan probing, purification of glycoproteins, cell labelling, carriers in targeted therapies and detection of cancer and other diseases.<sup>69–71</sup> Lectins display relatively weak affinities for monosaccharides, with dissociation constants ( $K_D$ ) in the millimolar range. Affinities are still generally in the micromolar range for oligosaccharides and glycans, despite the opportunity for multiple contacts with the surface of the lectin. This is due to the shallow binding pockets at the lectin surface, which is exposed to competitive solvent interactions. The absence of deeper binding pockets can also explain their poor selectivities for individual sugars. However, in biological settings, some lectins can assemble into homo-oligomeric structures with multiple binding sites in order to achieve superior affinity and selectivity. Thus, an oligomer can interact in an effective manner with different arms of a branched oligosaccharide or to different glycan sites of the same glycoprotein. In such oligomeric arrangements, a high degree of multivalency can be reached, contributing to affinities in the nanomolar range.<sup>72–76</sup>

Lectins interact with glycans primarily *via* a network of hydrogen bonds between the ring oxygen atom and multiple hydroxyl groups of the carbohydrate residues and oxygen atoms, amide and hydroxyl groups of the protein, as well as van der Waals contacts.<sup>72,77,78</sup> Nevertheless, CH– $\pi$  interactions between the carbohydrate backbone and aromatic amino acids such as phenylalanine, tyrosine, or tryptophan also make an important contribution to the overall binding.<sup>77,79</sup> The *Ralstonia solanacearum* (RSL) lectin can establish CH– $\pi$  contacts with the C3, C4, C5 and C6 carbons of  $\alpha$ -L-Me-fucose *via* the indole ring of a



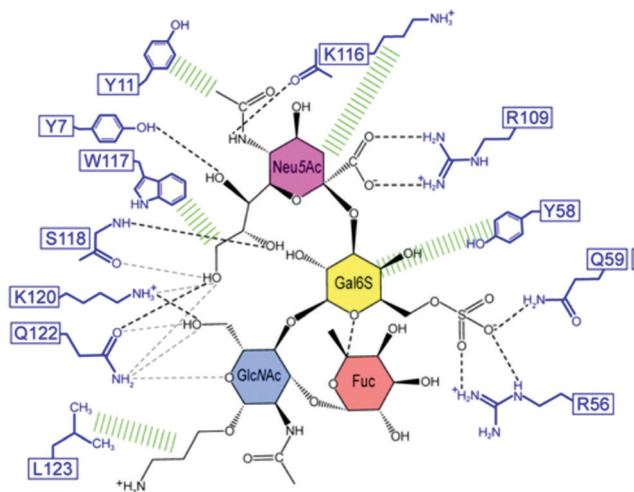


Fig. 4 Schematic illustration of the Siglec-8-6'S sLe<sup>x</sup> interaction network. Black dashed lines indicate hydrogen bonds in the depicted structure; gray dashed lines indicate hydrogen bonds abundantly observed in other structures of the ensemble. Hydrophobic contacts are shown in green. Reproduced from ref. 82 with permission from the National Academy of Sciences, copyright 2016.

tryptophan residue, with the dispersion interactions contributing to the binding with an energy between 7 and 8 kcal mol<sup>-1</sup>.<sup>80</sup> Electrostatic interactions become relevant for the recognition of charged carbohydrate residues, as in the case for negatively charged sialic acids. In this regard, positively-charged arginine residues are often involved in sialic acid interaction processes.<sup>81</sup> Examples of intermolecular contacts between lectins and glycans at the binding site can be found in a study reporting the investigation of the complexation of Siglec-8 lectin and its target, 6'-sulfo sialyl Lewis X (6'S sLe<sup>x</sup>) (Fig. 4).<sup>82</sup> Among several other interactions between the lectin and the glycan, the carboxyl group of the sialic acid residue makes a salt bridge with the guanidinium group of Arg109. Moreover, Lys116 and Ser118 engage in a network of hydrogen bonds with the *N*-acetyl amide and the O8 and O9 hydroxyl groups of the glycerol chain. In addition, the binding is promoted by hydrophobic contacts with the surrounding aromatic rings of other amino acid residues such as Tyr11 and Trp117. The binding of the Gal6S is mediated by the side chains of three residues (Arg56, Tyr58, and Gln59), together with a salt bridge with the guanidinium group of Arg56 and/or a hydrogen bond with the amino group of Gln59.

The carbohydrate binding activity of C-type lectins, which are involved in many cell surface recognition events, is mediated by Ca<sup>2+</sup> ions that coordinate carboxylate residues.<sup>83,84</sup> Dectin-2, a C-type lectin specific for high-mannose glycans can for instance, trigger a defence response against pathogens in a Ca<sup>2+</sup>-dependent process.<sup>85,86</sup>

Currently, several hundred lectins have been identified, with about 100 lectins commercially available. A major breakthrough in the use of lectins for glycan recognition came from the development of the lectin microarray. The technique relies on the immobilization of a panel of lectins on a solid support that allows for high-throughput analysis of complex carbohydrates

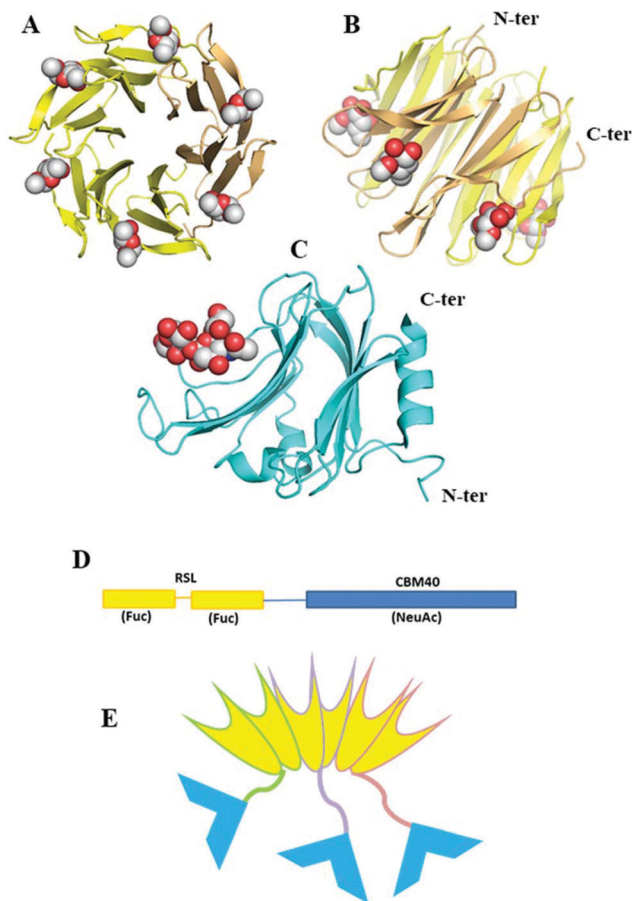
included in serum glycoproteins, bacteria and whole cells.<sup>69,87,88</sup> The advantage of lectin microarrays over other conventional glycan characterization techniques, namely mass spectrometry, NMR spectroscopy and liquid chromatography, is the possibility to rapidly obtain a broad profile or fingerprint of the glycans present in a crude sample, with minute sample consumption and reduced cost. General information about the glycosylation pattern can be determined, such as whether it is *N*-glycosylated, *O*-glycosylated, high mannose, core fucosylated, and fully or partially sialylated, with potential applications in the development of disease-related biomarkers.<sup>11,87,89-91</sup>

Despite the benefits associated with lectin microarrays, the inherent low affinity and specificity of lectins hinder their detection performances. In order to improve their binding properties, researchers have started to focus their efforts on lectin engineering.<sup>92-97</sup> Several types of lectins can be used as scaffolds, such as L-type, F-type, Galectins and R-type, with different engineering procedures available (*e.g.* site-directed mutagenesis, random mutagenesis and DNA shuffle to name a few).<sup>97</sup> Recently, the engineering of a bacterial F-type lectin domain (FLD) from *Streptosporangium roseum*, namely *Sr*FLD, showed significantly improved binding towards multivalent fucosylated glycoconjugates.<sup>98</sup> The engineered lectin, *Sr*DupFLD, contained a partial duplication of the original FLD sequence, affording two possible *L*-fucose binding pockets. Binding studies showed that the engineered lectin had a stronger binding for multivalent fucosylated glycoconjugates, with a dissociation constant 12-fold lower than the wild-type lectin *Sr*FLD. Analogously, lectins in which the *L*-fucose binding residues of the *N*-terminal partial FLD region and the complete FLD region were mutated, respectively *Sr*Dup<sub>TM</sub>FLD and *Sr*DupFLD<sub>TM</sub>, and were expressed and purified. Increased binding avidity was observed for *Sr*Dup<sub>TM</sub>FLD. Against what was its original hypothesis, the study revealed that the increased affinity of the engineered lectins may not be mediated by additional *L*-fucose binding sites in the Dup partial FLD region, instead it could be ascribed to an increased tendency of the lectins for oligomerization.

An interesting example of how artificial lectins can be created has been reported by Ribeiro *et al.*<sup>99</sup> They engineered the first chimeric, bispecific lectin, with two rationally oriented and distinct recognition surfaces, which are able to bind both fucosylated and sialylated glycoconjugates (Fig. 5). The chimeric lectin, named FS-Janus lectin, was obtained by fusing sequences from the lectin of *Ralstonia solanacearum* (RSL), which displays strong affinity for fucose, and a sequence from the NanI sialidase of *Clostridium perfringens* ATCC13124 (CBM40\_NanI), with strong affinity for sialylated oligosaccharides. The binding ability towards fucose, 3'-sialyllactose (3'-SL) and 6'-sialyllactose (6'-SL) functionalised surfaces was evaluated using surface plasmon resonance. The Janus lectin showed nanomolar avidity for fucosylated and sialylated surfaces, with the latter being an important achievement since classic lectins usually display low affinity for sialylated epitopes.

There are many challenges associated with lectin engineering, including the selection of template lectin, construction of a mutagenesis library and high-throughput screening methods.





**Fig. 5** A Janus lectin with two rationally oriented and distinct recognition surfaces, which are able to bind independently to fucosylated and sialylated glycoconjugates. (A and B) Two views of crystal structure of RSL-trimer complexed with six fucose ligands (pdb code 2BT9). (C) Crystal structure of CBM40\_NanI complexed with 30-sialyllactose (pdb code 5FRE). (D) Design of the peptide sequence with two blades of RSL connected to CBM40 through a peptide linker. (E) Schematic representation of the expected FSJanus lectin presenting six fucose binding sites on the upper face and three sialic acid binding sites on the bottom face. Reproduced from ref. 99 with permission from The Royal Society of Chemistry, copyright 2018.

However, as these challenges are overcome, lectins with superior binding properties will broaden the range of applications.

## 4. Artificial receptors

For several years, efforts have focused on the design of artificial receptors that can mimic, or even outperform, the role of antibodies and lectins as carbohydrate binding entities. While many studies have targeted monosaccharides, the list of receptors for more complex systems is considerably shorter. One example is a set of ditopic diaminopyrrolic structures designed for the selective recognition of  $\text{Man}\alpha(1-2)\text{Man}$  dimannosides.<sup>100</sup> Analogous aminopyrrolic derivatives were able to bind the highly mannosylated HIV virial envelope glycoproteins gp120 and gp41, as evidenced by SPR studies, as well as showing antiviral activity against HIV infection of T-lymphocyte CEM cells.<sup>101</sup> Although the aforementioned derivatives showed good binding properties in

polar organic media, solubility in water was very poor, limiting their application in aqueous systems. Receptors with impressive binding abilities in water were obtained by developing polycyclic cages where parallel aromatic surfaces were connected *via* water-solubilising spacers. The contribution of hydrophobic interactions from the aromatic units, together with hydrogen bonding established with the spacers, not only allowed the selective recognition of disaccharides such as cellobiose,<sup>102,103</sup> but also more complex targets like maltodextrin<sup>104</sup> and polysaccharides.<sup>105</sup> Other interesting examples of artificial receptors for disaccharides are cyclic peptides obtained from a dynamic combinatorial approach, which showed selectivity for trehalose.<sup>106</sup> Moreover, polyaromatic nanocapsules were reported to exclusively bind sucrose in water.<sup>107</sup> Interestingly, selectivity arises from the shape complementarity of the capsules, as well as the establishment of CH- $\pi$  interactions with the polyaromatic framework.

The above examples provide interesting insights on some of the key features that give rise to the selective recognition of carbohydrates. However, so far, they may lack a direct application to targets of biological relevance. The rest of this review will focus on the examination of boronic acid derivatives and aptamers for the recognition of glycans, since they have been able to provide a more versatile approach for the development of synthetic receptors for applications in biological settings.

### 4.1 Boronic acids

Boronic acids are known for their ability to covalently bind 1,2- and 1,3-diol groups found in carbohydrates.<sup>108</sup> The binding interaction is reversible and pH dependant. Boronic acids can readily interconvert in aqueous media from  $\text{sp}^2$  to  $\text{sp}^3$  hybridisation in the presence of a Lewis base, with the resulting tetrahedral species in equilibrium with the neutral trigonal form. The reaction with diols in alkaline aqueous solutions leads to the formation of boronate esters. These features make boronic acids interesting molecules for the realization of synthetic receptors for carbohydrate recognition, also named “boronolactins”.<sup>14,19,109–111</sup>

The chemical versatility of boronic acids enables their insertion into various scaffolds, with their integration into polymer backbones useful for a variety of biomedical applications.<sup>112</sup> Vinylphenylboronic acids can be grafted onto suitably modified nanoparticles,<sup>113</sup> while phenylboronic acid–adamantane conjugates have been reported to form self-assembled monolayers on the surface of cyclodextrin vesicles in aqueous solution.<sup>114</sup> These vesicles presented multiple boronic acid receptors on their surface, which were found to bind monosaccharides with binding constants in the range of  $100\text{--}3000\text{ M}^{-1}$ . Moreover, a glucose selective sensor was obtained by the formation of a self-assembled monolayer of bis-boronic acids on gold surfaces.<sup>115</sup> The well-defined intermolecular distance between the boronic acid moieties, together with a suitable spatial orientation, resulted in high selectivity for glucose over other saccharides. These, and other approaches, could enable boronic acids to find real life-applications, such as the production of probes for the sensing of sugars in commercial beverages.<sup>116,117</sup>

Whereas much of the area of sugar binding by boronic acids, in particular monosaccharide recognition and sensing, has



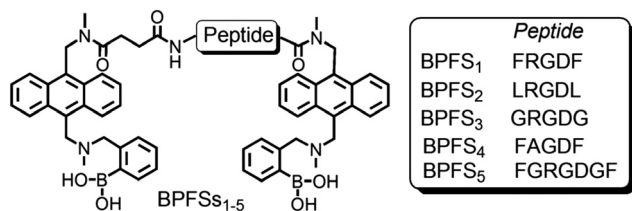


Fig. 6 Molecular structures of BPFSSs: peptide sequences are reported with the single-letter amino acid code. Reproduced from ref. 119 with permission from Springer Nature, copyright 2013.

already been well documented elsewhere,<sup>14,17,19,110,115</sup> glycan and glycoconjugate recognition using such reports is still in its infancy. Promising results have been obtained using boronic acid-functionalized peptidic receptors that act as fluorescent sensors. In particular, diboronic acid compounds of this type could be used to recognise cell surface cancer-associated glycans *in situ*, such as Sialyl Lewis X (sLe<sup>x</sup>), with high specificity.<sup>118,119</sup> The peptide-based linkers connecting two anthracene–phenylboronic acid moieties were employed to improve water solubility and biocompatibility. These receptors show great potential as probes in cancer diagnosis (Fig. 6).

Liposomes bearing boronic acids on their outer surface showed cell-binding ability, providing a tool for drug delivery upon binding with the glycans on the cell surface.<sup>120,121</sup> Further studies could lead to the development of probes with applications in targeted drug delivery, cell labelling and selective imaging.

An effective way to create synthetic carbohydrate receptors is through Molecularly Imprinted Polymers (MIP) using boronic acids as functional monomers.<sup>13,122–127,167</sup> This approach is based on the formation of a polymer network around a template molecule (*i.e.* a glycoprotein), creating artificial binding sites, which upon removal of the template can be occupied by

their target.<sup>126</sup> The use of boronic acids in MIPs can allow the realization of lectin-like surfaces for the specific recognition of glycoproteins, where the controlled orientation of the receptors around the glycoprotein enables the creation of epitopes for specific fragments.<sup>127–129</sup> The approach is versatile and efficient and provides surfaces with strong affinities and high specificities. For instance, a sensor platform with a 30-fold selectivity for Prostate Specific Antigen (PSA) over other glycoproteins was realised.<sup>13</sup> A pre-formed complex of PSA-acrylamido boronic acids was grafted onto a functionalized gold surface, providing the immobilization of the synthetic receptors in spatial arrangements specific for the target glycoprotein. In addition, functionalization of the surface around the template glycoprotein with oligoethylene glycol moieties enabled to create PSA-shaped cavities (Fig. 7). Surface plasmon resonance (SPR) binding studies showed that the nanocavities were specific for PSA, providing high affinity ( $K_d = 1.8 \mu\text{M}$ ) and detection at nanomolar concentrations.

Recently, a strategy using boronic acid-tosyl functionalized glass slides was introduced for the generation of glycoprotein microarray platforms for glycan profiling.<sup>130</sup> The method allows for the immobilization of glycoproteins from complex biological samples, and provides a high throughput alternative for the direct screening of glycan sequences, bypassing the use of multiple techniques and tedious sample preparation. Implementation of this approach could find real-life application in cancer diagnosis.

According to the examples reported above, the use of boronic acids seems very promising for the construction of synthetic receptors for glycan recognition. There is great potential for the development of sensor devices with applications in several areas, such as biomedical diagnostics and pharmaceutical industry. However, there are some limitations. In fact, boronic acids show good binding ability towards sugars in the furanose form,<sup>14</sup>

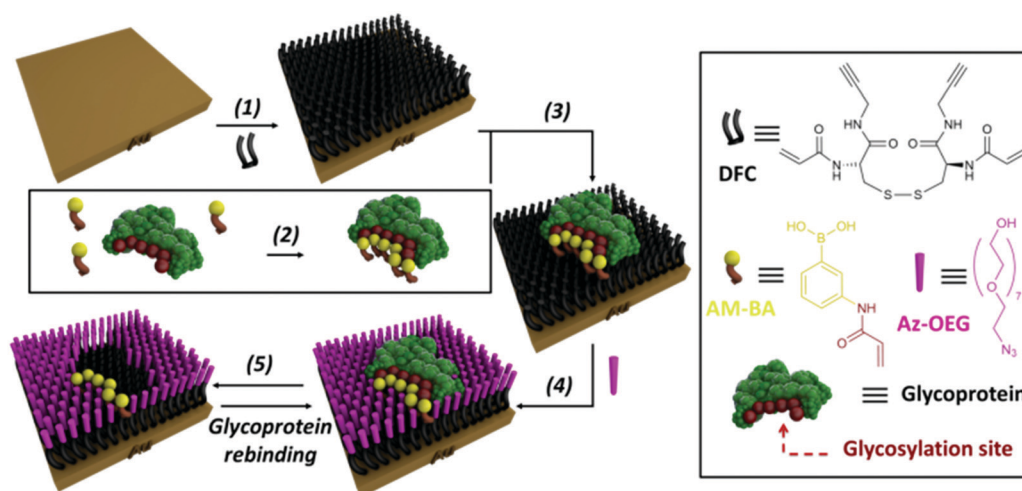


Fig. 7 Strategy for the fabrication of glycoprotein-imprinted surfaces using acrylamido-boronic acids (AM-BAs); (step 1) self-assembled monolayer (SAM) formation on a gold surface with DFC molecule; (step 2) incubation of the AM-BA receptors with the template target glycoprotein; (step 3) grafting of the preformed boronic acid–glycoprotein complex on the SAM *via* acrylamide co-polymerization; (step 4) azide-terminated oligoethylene glycol (Az-OEG) functionalization to provide glycoprotein-shaped cavities; (step 5) removal of the template protein, affording a nanocavity specific for the target glycoprotein. Reproduced from ref. 13 with permission from The Royal Society of Chemistry, copyright 2015.





while most of the carbohydrates in biologically relevant molecules are in the pyranose form. Besides, the best binding constants are observed at alkaline pH values, which might not be compatible with some biological systems. Further studies should be focused on enhancing binding under physiological conditions, as well as improving glycan recognition with higher selectivities and specificities.

## 4.2 Benzoboroxoles

Benzoboroxoles are derivatives of phenylboronic acids with an intramolecular five membered-ring containing the boron atom. Although they were first reported in 1957,<sup>131</sup> these compounds only started gaining greater attention in the late 2000s as their interesting biological activity and hence medicinal relevance, started to become apparent. Several compounds display anti-fungal action with some under clinical trials for psoriasis and human African trypanosomiasis.<sup>132,133</sup> In addition, benzoboroxoles also show inhibitory activity against several enzymes such as carbonic anhydrases and they are potential anti-tuberculosis agents.<sup>134,135</sup> However, this class of compound has recently drawn particular interest from their potential application as carbohydrate receptors, as they can bind sugars under physiological conditions.<sup>136</sup> Compared to their boronic acid counterparts, benzoboroxoles are able to bind sugars in the pyranose form, at pH values that are more suitable for biological systems. This makes them attractive as potential candidates for the development of synthetic lectins for the recognition of glycans on cell surfaces and glycoproteins.

Low molecular weight peptidyl bis(benzoboroxole) receptors were reported to be able to target the cancer-related Thomsen-Friedenreich (TF) antigen.<sup>137</sup> The receptors consisted of two benzoboroxoles for binding the two diol units of the TF antigen, and also a peptide backbone, with a tuneable length and rigidity, to provide additional hydrogen bonding and hydrophobic/CH- $\pi$  interactions (Fig. 8). Even small changes in the stereochemical

configuration of the peptide spacer considerably affected the binding, highlighting the extent to which molecular recognition is sensitive to subtle variations. Screening for the binding of TF antigen using a competitive ELISA assay showed that the most potent receptor exhibited a  $IC_{50}$  value (the concentration of inhibitor required to produce 50% inhibition of an enzymatic reaction at a specific substrate concentration) of 20  $\mu$ M. Although only moderate affinities and selectivities were observed ( $K_d$  in the low millimolar range) the results are promising and encouraging for future studies.

The attractive binding properties of benzoboroxoles have recently led to the development of a carbohydrate-binding ligand for the specific enrichment of glycoproteins.<sup>138</sup> A benzoboroxole-modified ligand with a structure that mimics Trp and Phe (two amino acids frequently present in lectins) was immobilised on a Sepharose<sup>TM</sup> column. This approach allowed the purification of glycoproteins from complex mixtures at neutral pH. When glucose oxidase (GOx) was spiked into an *E. coli* supernatant, purification with the benzoboroxole-modified column afforded GOx with 98% purity, with the protein retaining its enzymatic activity. Thermo-responsive polymers functionalised with benzoboroxole units have also been reported as promising sensing platforms for the detection of diols and polyols (*i.e.* catechols, saccharides and glycopolymers) at physiologically relevant pH values.<sup>139</sup> Moreover, benzoboroxole-containing nanoparticles have turned out to be promising options for immunological applications such as internalisation of dendritic cells and controlled antigen release.<sup>140</sup> These nanoparticles, stable under physiological conditions, were obtained by mixing glycopolymers and a benzoboroxole-containing polymer. Their formation was promoted by the establishment of dynamic covalent bonds between the two complementary polymer chains. The study reported that the nanoparticles can be internalised by dendritic cells which then dissociate when exposed to the acidic environment of the organelles. This feature allowed the controlled release of a preloaded antigen upon internalization, opening up new opportunities for applications in cancer immunotherapy. Modified nanoparticles could also provide a tool for the qualitative screening of glycoproteins, as well as the generation of probes for cancer cell recognition.<sup>141,142</sup> Although reaching high affinity and specificity is still a challenge, benzoboroxoles are certainly a very promising class of compounds for the development of synthetic receptors for glycans and their derivatives, which no doubt will lead to continued interest in these compounds.

In order to disclose the full potential of synthetic glycan receptors, future efforts should focus on the fine control of the spatial arrangements of the ligands. Moreover, the promotion of additional secondary interactions, such as hydrogen bonding and CH- $\pi$  interactions must be considered to achieve selective molecular recognition.

## 4.3 Aptamers

Aptamers are synthetically derived, single stranded oligonucleotides that bind to non-oligonucleotide based targets. They are typically constructed from less than 100 bases, with sequences determined from the Systematic Evolution of Ligands

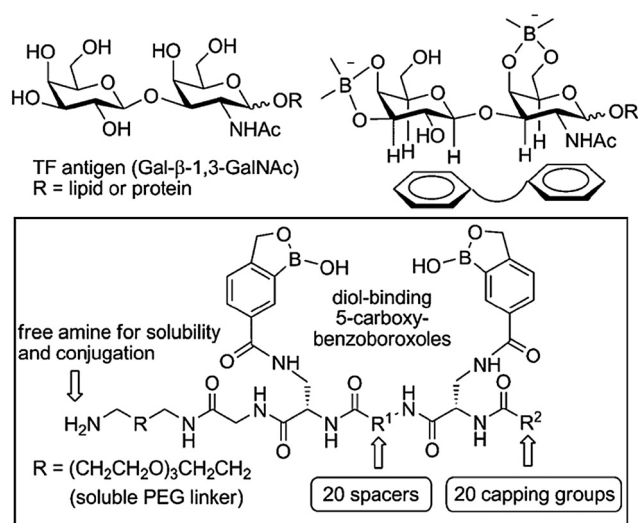


Fig. 8 Design of peptidyl bis(benzoboroxoles) for the recognition of TF antigen; the library was generated by combining different spacers R<sup>1</sup>, natural and non-natural amino acids, and carboxylic acids as capping groups R<sup>2</sup>. Reproduced from ref. 137 with permission from Wiley-VCH, copyright 2010.



by EXponential enrichment (SELEX) method, first adopted by Tuerk and Gold.<sup>143</sup> Aptamers interact with a variety of targets, ranging from small molecules to whole cells, with similar, if not improved, affinities than antibodies.<sup>144</sup> There is limited recent literature on aptamers targeting glycans, with most sources identifying saccharide-binding sequences from over a decade ago.<sup>145</sup> The relative lack of new developments in this field may be due to limited options regarding non-covalent binding interactions between sugars and oligonucleotides. For example, the absence of charged groups and aromatic ring structures in simple sugars limits interactions to hydrophobic sites and hydrogen bonding.<sup>146</sup> There have been attempts to overcome this particular problem by the use of modified aptamers. Oligonucleotides bearing boronic acid groups have been employed to promote affinities of aptamers to glycan regions of glycoproteins.<sup>147</sup> Although there are now a few recent reports on this approach,<sup>148–150</sup> there are no other examples of the use of any other modification designed specifically to enhance glycan–aptamer interactions.

Current advances in this area have focused mainly on aptamers that target sugars with charged moieties, such as the sialic acids. These sugars are known to be overexpressed in cancer cells and are thus ideal candidates to probe in cancer diagnostics and therapeutics.<sup>151</sup> Kim and co-workers used the SELEX process to identify an RNA aptamer for the sialic acid, *N*-acetylneuraminic acid (Neu5Ac).<sup>152</sup> They reported a high affinity ( $K_D$  of 1.35 nM) and high selectivity, with very little interaction observed between the aptamer and non-Neu5Ac containing oligosaccharides. They attribute these properties to the directional immobilisation approach undertaken for the selection of the aptamer (Fig. 9). Typically, in SELEX rounds utilising solid supports, target molecules with multiple functional groups are immobilised in random orientations, leading to oligonucleotide pools recognising various epitopes of the same target but with reduced specificity towards a particular epitope. By directing the immobilisation of Neu5Ac to adopt a more natural conformation by exposing the carboxylic acid group of the sugar, the authors were able to identify an aptamer highly selective for both Neu5Ac alone and Neu5Ac modified glycans. The binding of the aptamer to Neu5Ac was also shown to prevent enzymatic hydrolysis of the sugar. In another study, six DNA aptamer sequences which specifically targeted another member of the sialic acid family, *N*-glycolylneuraminic acid (Neu5Gc), were identified. One of the aptamers produced a high affinity constant ( $K_A$ ) of  $6.68 \times 10^9 \text{ M}^{-1}$ .<sup>153</sup> The technology was later developed into aptamer-nanoparticle biosensor strips for visual detection of Neu5Gc.<sup>154</sup> Both of the examples above highlight the latest use of aptamers as novel probes targeting sialic acids, for rapid and effective sensing of this particular type of glycan.

Specific DNA aptamers have been also generated to recognise cell-surface based glycoconjugates. One such target is the cancer antigen, glycosphingolipid Globo H,<sup>155</sup> the binding of which was enhanced by extending the strands of the aptamers, resulting in dissociation constants in the low micromolar range. The specificity of the aptamers towards Globo H was also investigated. However, the probes showed comparable affinities to oligosaccharides similar in structure to Globo H. Another target is

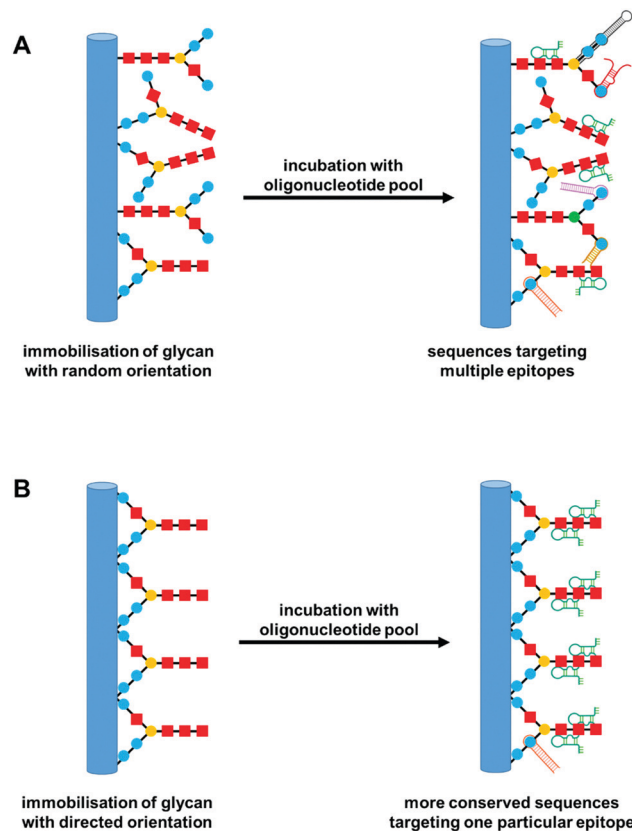


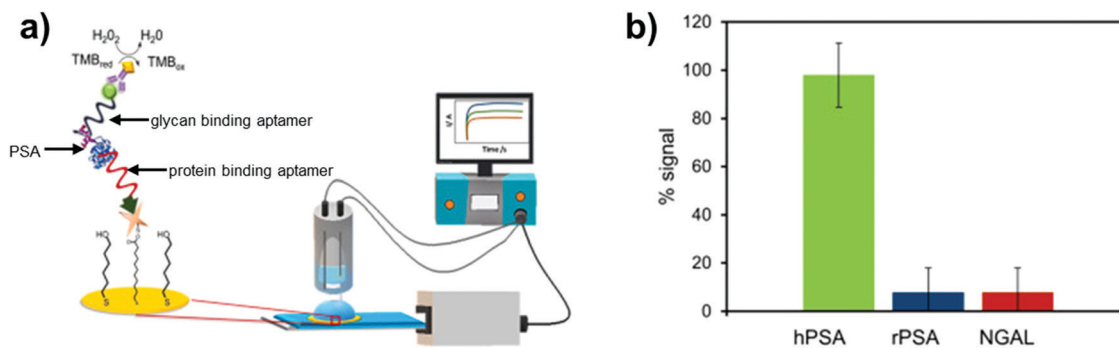
Fig. 9 SELEX process used to enhance selectivity of sequences towards a particular epitope. Target glycan is immobilised to a solid support in either a random orientation (A) resulting in reduced selectivity, or a directed orientation (B) resulting in high selectivity.

membrane-bound *N*-acetylgalactosamine (GalNAc). A fluorescently tagged aptamer selected for GalNAc was used to image HeLa and Cos-7 cells with precise localization.<sup>156</sup>

Other recent developments have reported aptamers which target peptidoglycans and glycan regions of glycoproteins. For instance, RNA aptamers have been selected against two classes of glycosaminoglycans, heparosan and chondroitin.<sup>157</sup> The non-immunogenic nature of these glycosaminoglycans had previously made their detection in biological samples using popular antibody based technologies difficult. High affinities were observed, with  $K_D$  values in the range of 0.71–1.0  $\mu\text{M}$ . An RNA aptamer that binds specifically to the glycosylated receptor-binding domain of a hemagglutinin protein on the surface of the influenza virus (HA1, subtype H5) has also been isolated.<sup>158</sup> The binding of the aptamer to the glycoprotein blocked attachment of the virus to host cells, indicating a potential use of the aptamer as an antiviral reagent against influenza.

Apart from RNA aptamers, DNA aptamers that selectively bind glycan domains in glycoproteins have also been developed. For instance, DNA aptamers have been shown to target only the glycan moiety of the prostate cancer biomarker, prostate-specific antigen (PSA).<sup>159</sup> Counter SELEX rounds were introduced to remove sequences showing affinity for non-glycosylated PSA. The selected sequences were able to discriminate human PSA





**Fig. 10** Prostate specific antigen (PSA) biosensor utilising both glycan and protein binding aptamers in a sandwich based sensing approach: (a) biosensor set up; (b) data showing selectivity of the sensor towards glycosylated PSA (hPSA) over the non-glycosylated version (rPSA) and a similarly sized glycoprotein (NGAL). Reproduced from ref. 159 with permission from Elsevier, copyright 2019.

from the non-glycosylated protein but exhibited comparable affinities to a similarly glycosylated protein (lipocalin-2). To overcome this problem, the authors utilised their glycan targeting PSA aptamer along with the anti-PSA aptamer (shown to bind only to protein regions of PSA) in a sandwich assay for quantification of human PSA (Fig. 10). The assay displayed enhanced selectivity for human PSA over both non-glycosylated PSA and a similarly sized glycoprotein (NGAL), with a limit of detection of  $0.66 \text{ ng mL}^{-1}$ . While the assay was able to detect human PSA at low amounts, it was difficult to ascertain whether it could discriminate between different glycan forms of PSA, some of which are indicative of more aggressive forms of prostate cancer. Lower readings were obtained for patients with benign prostate hyperplasia compared to the antibody based ELISA, with the authors suggesting that the sensor could detect a fraction of PSA with distinct glycan structures.

Two peptidoglycan-targeting DNA aptamers have also been identified that displayed high affinities towards *Staphylococcus aureus* cells.<sup>160</sup> The dissociation constants of the two aptamers towards peptidoglycan were  $0.415 \mu\text{M}$  and  $1.261 \mu\text{M}$ . It was observed that the aptamers had similar binding to both Gram negative and Gram positive bacterial cells, despite the latter typically consisting of a higher content of and more exposed peptidoglycan. The probes were selective towards bacterial cells over human fibroblasts, making them promising tools in the diagnosis of bacterial infections. DNA aptamers have also been produced against advanced glycation end (AGE) products to target macromolecules associated with age related disorders.<sup>161</sup> Although results were promising, it was stated that further testing of the aptamers needed to be performed for safety and efficacy, before consideration for clinical use.

Although the challenges faced by aptamers mainly stem from the lack of interactions that these oligonucleotides can establish with glycans, the easy adaptation of aptamers to incorporate sugar-binding moieties such as boronic acids, to enhance interactions, is one way to overcome this limitation.<sup>147</sup> The biggest challenge for sensing glycoconjugates using aptamers appears to be enhancing specificity towards a particular glycan target. Nevertheless, progress can be achieved by optimising the aptamer selection process (e.g. by utilising directional

immobilisation and counter SELEX methods), and/or by employing multiple probes in a sandwich based sensing approach.

## 5. Advantages and drawbacks of natural and synthetic receptors

Approaches towards the targeting of glycans based on antibodies, lectins, boronic acid derivatives and aptamers have their own advantages and drawbacks. Various factors have to be considered when choosing the appropriate receptor system, according to the glycan being targeted and the application for which it is intended. The extended binding sites of antibodies provide opportunities for enhanced affinities and specificities for glycan targets. However, these improved binding properties are currently underexploited due to major technical challenges associated with the poor immunogenicity of glycans and the lack of high-throughput methods to evaluate the glycan binding properties of newly generated antibodies. Nevertheless, high-throughput analysis methods are available by means of lectin microarrays, yielding information about the glycosylation state of glycoconjugates, with potential applications in the development of biomarkers. Moreover, despite usually showing low binding affinities and poor selectivity for glycans that share the same extremity, lectins can display higher affinities when assembled into oligomeric structures.

Thanks to their chemical versatility that allows for the introduction of various functional groups, boronic acid derivatives can be incorporated into different scaffolds, namely surfaces, nanoparticles or vesicles, unlocking new opportunities for the creation of sensing devices with potential applications in diagnostics. A major drawback arises from the strong pH dependence of the binding, which is favoured at alkaline pH. In this regard, benzoboroxoles can overcome the issue since they are able to bind sugars under physiological conditions, therefore being more compatible with biological targets. Although selectivity can be achieved, binding constants are usually in the low millimolar/micromolar range. Small changes in the structure of the receptor can affect the binding significantly, hence the accurate tuning of the spatial orientation of the binding units is essential.



Aptamers, like boronic acid derivatives, possess heightened stability and amenability to chemical modification compared to antibodies and lectins, whilst displaying similar or even higher affinities for glycan targets. Current research appears to be focused on the detection of specific glycan-containing biomarkers, including the sialic acid family and various glycoproteins such as PSA, for disease diagnostics and therapeutics. Future efforts should be dedicated to tackling the selectivity challenge concerning aptamer technology, improving the so far limited techniques that aim at tweaking the aptamer selection process to focus on particular epitopes and exclude sequences that bind molecules similar in structure to the target glycan.

All the glycan-binding entities described in this review show great potential, although there are still important challenges that must be tackled in order to successfully achieve specific glycan recognition. Research on antibodies is moving towards the rational design of glycan derivatives to improve immunogenicity, and glycan arrays to speed up antibody screening and advances in carbohydrate chemistry to create more complex glycans. However, antibody production still remains a complicated and costly procedure and, thus, in many settings, lectins, boronic acid-based entities and aptamers represent highly suitable and attractive alternatives. Many efforts are currently focussed on lectin engineering and despite the several difficulties associated with the creation of artificial lectins, recent studies have shown that superior affinities for glycans can be achieved. Moreover, for both boronic acid derivatives and aptamers, the identification and introduction of functionalities able to establish additional interactions with the substrate of interest will be crucial for the development of more effective receptors. Due to the vast amount of hydroxyl groups, hydrogen bonding is probably the most sought-after type of interaction,<sup>15,20,100,162</sup> followed by CH- $\pi$  interactions between glycan backbones and aromatic molecules.<sup>107,163</sup> Structural features (*i.e.* the size of the aromatic substituents or the presence of electron-rich groups) can have a significant impact on the stability of the stacking complexes.<sup>164</sup> Finally, the establishment of electrostatic contacts in the appropriate positions can afford enhanced binding affinities, particularly with charged molecules such as glycosaminoglycans.<sup>165,166</sup> As technology continues to advance, progress is expected in all these areas of research.

## 6. Summary and outlook

Glycans are of great importance in signifying disease states and in fundamental biological processes. The diversity of possible monosaccharide combinations, linkages and modifications, require a variety of strategies to achieve the selective recognition of biomedically relevant glycans and their derivatives. In this regard, various approaches, based on antibodies, lectins, aptamers and boronic acid derivatives, have been pursued. While these approaches already contribute to cancer biomarker research, diagnostics and therapeutics, the development of new and better glycan binding entities would be invaluable, coupled with better tools to investigate these targets in various biological settings, including *in vitro* and *in vivo* conditions. Furthermore, there is the

need for more powerful high-throughput screening methods (*i.e.* fluorescence methods, UV and SPR) and efficient synthetic methodologies for the synthesis of complex glycans in high yield. Finally, a common major challenge across natural and synthetic receptors is enhancing the selectivity of the receptor towards a specific glycan target. Thus, the development of new glycan binding entities will continue to be a high priority for the foreseeable future.

## Conflicts of interest

There are no conflicts to declare.

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