Analytica Chimica Acta 1155 (2021) 338352

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Tailor-made recombinant prokaryotic lectins for characterisation of glycoproteins

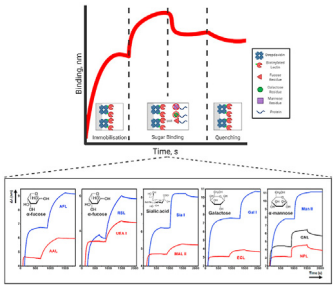
S. Fernandez-Poza [a, b, d, 1], A. Padros [d], R. Thompson [d], Lucy Butler [a, b], Meez Islam [a, b], J.A. Mosely [a, b], James H. Scrivens [a, b], Muhammad F Rehman [c], Muhammad Safwan Akram [a, b, 1, *]

[a] School of Health and Life Sciences, Teesside University, Middlesbrough, TS1 3BA, UK
[b] National Horizons Centre, Teesside University, 38 John Dixon Ln, Darlington, DL1 1HG, UK
[c] Department of Chemistry, University of Sargodha, Sargodha, Punjab, Pakistan
[d] Glycoselect Ltd., South Tees Institute for Learning, Research and Innovation, the James Cook University Hospital, Middlesbrough, TS4 3BW, UK

HIGHLIGHTS

- Novel recombinant prokaryotic lectins with enhanced specificity towards respective glycans.
- Detailed comparison with plant lectins with affinity towards the same glycoforms.
- Example of changing the specificity of one of the lectins through protein engineering.
- Testing performance of microbial & plant lectins against a selection of glycoproteins.
- Demonstrating improved binding kinetics for recombinant prokaryotic lectins.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 26 October 2020
Received in revised form
20 February 2021
Accepted 22 February 2021
Available online 27 February 2021

Keywords:
Lectins
Glycan analysis
Glycan sensors
Glycoprotein purification

ABSTRACT

Development of biosimilars is costly, where glycan analysis is a significant constraint on time and money. This paper provides an in-depth characterisation of several novel recombinant prokaryotic lectins (RPLs), developed through directed evolution, displaying specific binding activities to α-mannose, β-galactose, fucose and sialic acid residues, tested against major biosimilar targets. The binding characterisation of all lectins was performed employing the principles of bio-layer interferometry (BLI), with help of the streptavidin-coated sensor with the biotinylated lectins. The binding activity of the RPLs and the specificity to a broad range of glycoproteins and glycoconjugates were evaluated and compared to those of equivalent plant-derived lectins. While exhibiting better or similar specificity, RPLs displayed significantly better binding in all cases. The binding mechanisms are explained with particular focus on the role hydrogen bonding plays in the change of specificity for a galactose specific lectin. Furthermore, different sets of RPLs and their plant equivalents were assayed against the different glycoprotein targets to evaluate the analytical parameters of the lectin-glycoprotein interaction. The obtained LoDs reached by the RPLs were lower than those of their plant counterparts apart from one, exhibiting RPL:PL LoD ratios of 0.8, 2.5, 14.2 and 380 for the sets of lectins specific to fucose, α-mannose, β-galactose and sialic acid,

* Corresponding author. School of Health and Life Sciences, Teesside University, Middlesbrough, TS1 3BA, UK.
E-mail address: safwan.akram@tees.ac.uk (M.S. Akram).
[1] SFP, MSA contributed equally.

https://doi.org/10.1016/j.aca.2021.338352

respectively. Such enhancement in analytical parameters of RPLs shows their applicability in protein purification and as bioanalytical tools for glycan analysis and biosensor development.

1. Introduction

The patent expiries of several blockbuster biologic drugs have given rise to a rapidly growing (CAGR of 43.85%) %) biosimilar market [1]. The largest growth segment are glycoproteins, particularly monoclonal antibodies (mAbs) with global sales over US$50 billion [2]. This has led to the development of a very strong pipeline of new mAb therapeutics with 50 new investigational drugs [3] and ~180 competitors in development for the biosimilar market [4]. One of the biggest challenges these biosimilar manufacturers face is the control of glycosylation pattern on the biomolecules, which usually changes as the molecule is scaled up. Glycosylation is now part of Critical Quality Attributes (CQA) of a biosimilar approval and the regulatory agencies (FDA/EMEA) demand that heterogeneity should be kept under 5% of the total product volume [5]. The challenge to keep batch-to-batch variations in check contextualizes the application of lectins as an analytical tool [6] because of their high glycan specificity.

Lectins are a group of complex proteins that are defined by their ability to recognise and bind carbohydrate groups specifically in a reversible manner, thus making them excellent candidates for the purification [7] of glycoproteins and for the development of glycan analytical tools [8]. Lectins are known to mediate several cell recognition events, interaction between cells and their adhesion with extracellular matrix [9]. In addition to their immunomodulatory effects including cytokine release, oxidative burst and chemotaxis in immune cells, they also possess antiviral, antimicrobial and anti-tumor properties [10]. This protein-glycan interaction is being exploited in research to precipitate glycoproteins, utilised to agglutinate cells, in the creation of microarrays for cancer diagnosis [11,12], in the exploration of complex biological processes and as potential candidates in medical diagnostics, disease prophylaxis and therapy [13,14]. Since they were first described in 1888 [15], lectins have been isolated and purified mainly from plants [16] and to a lesser extent from animals [17], and from microorganisms for a broad number of purposes [18,19]. Plant-derived lectins in particular have been traditionally employed for all the aforementioned purposes, given their ubiquity and relative ease of isolation. However, these lectins are mixtures of several isoforms owing to their genetic heterogeneity of the plant source and post-translational modifications [20], which limits their use in analytical methods where extreme specificity is required. The second challenge faced by the plant lectins is their scalability, their yield is low and suffer from substantial batch-to-batch and seasonal variations. These challenges limit use of plant lectins as the performance and binding activity per batch varies [21]. RPLs have emerged as a good alternative to overcome all these difficulties. Expression of lectins from a single sequence and lack of post translational modification allows for the expression of only a single specific lectin with an opportunity of scaling it up for industrial production [22]. Recombinant expression also allows for an opportunity to improve upon the binding characteristics of these lectins, and the modification of the carbohydrate-recognition sites becomes feasible through different mutagenic strategies including directed evolution [23,24]. This allows prokaryotic lectins to be tailor-made against various glycan targets with enhanced binding affinity and specificity towards their glycan cognates.

In this manuscript, we report on the characterisation of RPLs evolved through employing directed evolution particularly iterative focussed site directed mutagenesis, expressed, and produced in *Escherichia coli*, specific to different sugar moieties: α-mannose, β-galactose, fucose and sialic acid. These lectins are predicated on the natural lectins obtained from the species *Pseudomonas aeruginosa*, *Burkholderia cenocepacia*, *Streptococcus gordonii*, *Aspergillus fumigatus* and *Ralstonia solanacearum*. Literature is replete with instances where lectins are tested with either sugar substrates on their own or linked to BSA with long spacers to make them accessible to lectins. Such arrangements are useful to study properties of lectins however, they are not a good representation of biomolecules that have glycans buried between protein motifs. Therefore, in this manuscript lectin targets have been selected from the glycoproteins which are currently amongst the most active biomolecules for biosimilar development or are more representative of the kind of glycans often encountered in biosimilar development. The binding performance of the presented lectins was compared to that of plant-derived analogues on an octet platform which work on the principles of bio-layer interferometry (BLI) (Fig. 1). Previously binding performances of bacterial lectins have been calculated through Surface Plasmon Resonance (SPR) [25,49] and through FTIR [25], and electronically through field effect transistors (FET) made from chemically converted graphene and single walled carbon nanotubes (SWNT), where SWNT showed better results [26].

For this manuscript, the specificity of the presented RPLs was further assessed against a broad range of glycoproteins displaying different glycosylation patterns. The binding activity was also investigated for both plant and RPLs, providing a thorough analytical performance comparison. Lectins have previously been probed with battery of techniques for example, Frank et al. [27] probed wheat germ agglutinin using Proton magnetic resonance and found the role of tryptophan residues in binding of *N*-Acetyl Glucosamine (GlcNAc) and *N*-Acetyl Neuraminic Acid (AcNeu), where they highlighted the role of hydrogen bonding. In this paper, we present how a change in hydrogen bonding pattern can lead to change in specificity of the lectins.

2. Materials & methods

2.1. Sourcing of lectins

Binding measurements were performed on disposable, high-precision streptavidin-coated biosensors (ForteBio, Portsmouth, UK), dipped in a 96-well, black polypropylene microplate (Greiner Bio-One, Stonehouse, UK) and loaded onto an Octet RED96e platform (ForteBio, Portsmouth, UK). Biotinylated plant lectins employed in this study were GNL from *Galanthus nivalis*, NPL from *Narcissus pseudonarcissus*, ECL from *Erythrina crista-galli*, MAL-II from *Maackia amurensis*, AAL from *Aleuria aurantia* and UEA-I, an agglutinin from *Ulex europaeus*. All of the plant lectins were purchased from Vector Laboratories Ltd (Peterborough, UK). The target glycoproteins, purchased from Sigma Aldrich (Dorset, UK), included porcine thyroglobulin, glucose oxidase from *A. niger*, transferrin from human serum, fetuin from fetal bovine serum and asialofetuin from fetal calf serum. The biotinylated RPLs to these plant-derived

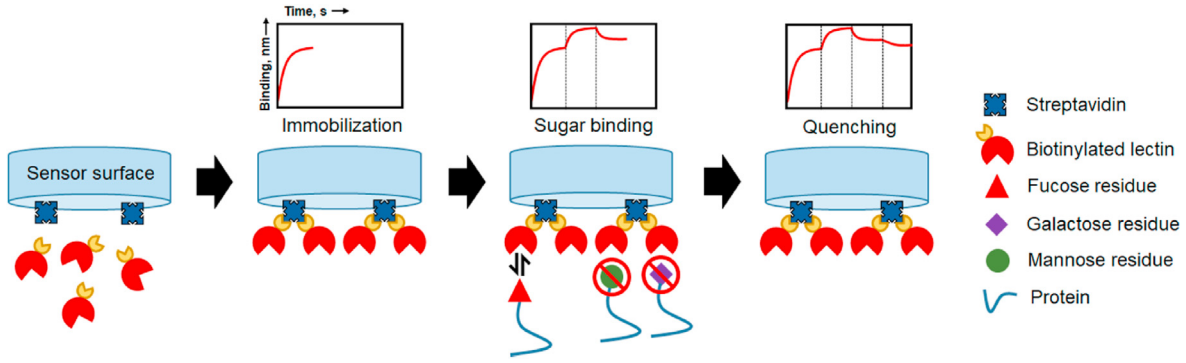


Fig. 1. Scheme of lectin affinity assay performed on an Octet platform and the corresponding sensogram curves involving the most relevant assay steps: (1) Immobilisation of the biotinylated lectins on the surface of streptavidin-coated sensors, (2) binding (including association and dissociation by incubation with glycoprotein and subsequent wash with buffer) and quenching, consisting of the glycoprotein detachment by incubation with a relevant sugar solution in high concentration.

lectins, displayed in Table 1, were prepared through a collaboration with Glycoselect Ltd (Middlesbrough, UK and Dublin, Ireland). They were functionally tuned using directed evolution particularly through iterative focussed site directed mutagenesis, some of the production details including sequences have been excluded due to an ongoing patent submission. All of the prokaryotic lectins were expressed in *E. coli* (DH5α). The RPL against β-galactose is a mutated version of PA-IL against α-galactose from *P. aeruginosa* (described in Ref 22). All measurements were performed in 200 mM TBS buffer (Fischer Scientific, Loughborough, UK), supplemented with 1.5 M NaCl (Merck-Millipore, Watford, UK), 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 0.05% Tween 20, adjusted to pH 7.6. All salts for buffer preparation were of analytical grade and purchased from Sigma Aldrich. Methyl α-D-mannopyranoside, D-(+)-galactose, and D-(+)-fucose were also obtained from Sigma Aldrich. The compositions of the quenching buffers used for binding experiments are 500 mM Methyl-α-D-mannose for GNL, NPL and Man-II; 500 mM D-galactose for ECL and Gal-I; and 500 mM D-fucose for UEA-I and RSL. All of them were dissolved in TBST at pH 7.6. The composition for MAL-II and Sia-I is 1 M $MgCl_2$ in TBST at pH 5.

2.2. Binding measurements

All binding measurements are based on optical detection of biomolecular interactions utilising the principle of BLI. The technique is based on the interference of two white light beams reflected from the external surface of the sensor (on which the capture molecule is immobilised) and an internal reference layer, respectively. Binding events occurring on the sensor surface leading to an overall increase in the optical thickness of the sensor tip, which results in a wavelength shift in the interference pattern, Δλ. This technique allows for the real-time monitoring of the optical thickness of the biosensor tip which directly relates to the concentration of the target molecule present in the sample. The design doesn't allow any shift in wavelength due to unbound molecules or due to the difference in the refractive indices of the medium.

All binding assays of this study were performed while keeping constant temperature and stirring conditions at 30 °C and 400 rpm respectively. Output data generated by the Octet's operational software was treated and processed externally, based on the association and dissociation signals extracted from the binding sensograms. The association (k_{on}) and dissociation (k_{off}) rate constants were calculated by non-linear fitting of the experimental sensograms obtained for different concentrations of ligand. The association (K_a) and dissociation (K_d) constants were calculated by linear fitting of the apparent kinetic constant (k_{obs}) as a function of the concentration of ligand.

2.3. Analysis and comparison of PLs and RPLs

SDS-PAGE and mass spectrometry was used to assess purity and to compare the molecular weights of PLs and RPLs. Figure S1-A shows the 12% polyacrylamide gel of nine recombinant lectins: Gal-I to IV, Sia-I & −II, Man-II, RSL and AFL. All the experimental molecular weights matched well with the actual amino acid sequences (Table S2). All the lectins were analysed through mass spectrometry to confirm the molecular weights (Figure S1b). Samples were desalted and mass spectrometry was performed on Waters Xevo G2XS QToF MS, where measurements were obtained in positive ion mode, and mass-to-charge ratio was annotated through MassLynx 4.2. Samples were diluted 1/20 in 0.1% formic acid and 3 μL was injected onto the column. Data from mass spectrometer and its interpretation is presented in Figure S1B and Table S2 respectively.

2.4. Lectin affinity assay procedure

For the preparation of the sensing phase, the biotinylated lectins were first immobilised onto the streptavidin-coated optical

Table 1
Plant and recombinant prokaryotic lectins employed in this study.

Plant lectins		Recombinant Prokaryotic Lectins (RPL)		Sugar affinity
Plant lectin	Molecular weight (kDa)	RPLs	Molecular weight (kDa)	
GNL	50	Man-II	30	α-Mannose
NPL	59			
ECL	54	Gal-I	14	β-galactose
MAL-II	130	Sia-I	27	Sialic acid
AAL	36 (× 2 subunits)	AFL	36	α-fucose
UEA-I	68	RSL	11 (× 3 subunits)	α-fucose

sensors. After being suspended in TBST buffer for 30 min, the sensors were incubated in lectin solutions of typical concentrations of 200 nM for PLs and 600 nM for RPLs. All the sensors have equal number of streptavidin functionalised binding sites and the goal is to saturate the sensor surface. Hence, the concentrations were chosen on the basis that they worked for all the lectins. These experimental conditions were found sufficient to saturate the sensor surface, observed in the sensogram as a plateau-like binding signal profile (Fig. 1). The difference in PLs vs RPLs concentration may appear stark, but size of the proteins should be taken into account e.g. ECL is 54 kDa protein and at 200 nM, its concentration in terms of mass is 10.8 mg while its corresponding prokaryotic lectin, Gal-I is only 14 KDa and at 600 nM is only at 8.4 mg. All lectins employed in the binding experiments are summarized in Table 1. Subsequently, the sensors were washed in TBST buffer and incubated in a 20 μgmL^{-1} solution of glycoproteins, thus enabling the lectin-protein interaction. After another wash in TBST buffer, the sensors were incubated in one of the four quenching buffers mentioned previously (depending on the lectin employed in the assay) and washed again with TBST buffer. These last two steps allowed for sensor reutilization by removing the remaining glycoprotein residues bound to the sensor surface.

2.5. Studying effect of site directed mutagenesis on Gal1

ICM (Internal Coordinate Mechanics) software [28] was used to study the effect of mutations on protein stability and to obtain the ligand dock score with wild-type and mutated PA-IL. The protein structures of PA-IL (PDB ID 2VXJ) and the β-galactose were obtained from PDB (Protein Data Bank). Files were converted to ICM objects. PA-IL monomer (Chain A) was isolated and optimized with an MMFF94 force field [29]. Water molecules and all heteroatoms were removed except calcium ion. β-galactose was docked into the ligand binding pocket of the PA-IL with default parameters implemented in the ICM program DISCO using global energy optimization with fully flexible side chains [30]. ICM PocketFinder was used to find ligand binding pocket surface. The docking method was evaluated by re-docking the already bound ligand into assigned active site of PA-IL. MAESTRO and ICM were used to study the effect of mutations on protein structure. LigPlot [31] was used to plot the interactions and H-bonding between protein and ligand molecules. Molecular Dynamics Simulations (MDS) were performed to obtain the information about amino acid interactions with ligand molecules and protein stability. YASARA Structure ver. 20.7.4 [32] was used to obtain 100 ns long MDS with AMBER14 force field [33]. A density of 0.997 g/mL, 0.9% NaCl, 25 °C temperature, pH 7.4, periodic boundaries and 7.86 cut off for long-range coulomb electrostatics forces were used during the runs of MDS. After completion, simulations were analysed using YASARA Structure for potential energy, RMSF (root mean square fluctuation), and RMSD (root mean square deviation). The molecular mechanics/Poisson–Boltzmann surface area (MM/PBSA) calculations were performed by using YASARA.

3. Results & discussions

3.1. Qualitative comparison of the binding performance

A preliminary interaction study was performed to compare the binding performance of several RPLs and PLs to optimise the assay conditions. As described above, PLs and RPLs were first immobilised on the sensor surface and subsequently assayed with a 20 $\mu g\ mL^{-1}$ solution of glycoprotein. Variation arising due to the loading of the capture molecules (PLs and RPLs in this study) is reflected in the loading baseline and is accounted for subsequent calculations for binding performance. The glycoprotein selection was done individually for each recombinant/plant lectin pair based upon displayed affinity to the glycan groups of the protein. This way, the binding performance of RPLs was evaluated and compared to that of their plant equivalents, employing the same target glycoprotein for each lectin pair. Sensograms shown in Fig. 2 show the comparative binding performance of all different PLs and RPLs presented in Table 1. Note that the assay parameters were customised individually for each lectin, therefore time frame of the different assay steps is not equivalent but the affinity is comparable. AAL exhibits natural binding affinity to proteins displaying α(1 → 6) fucose groups [34]. This lectin and its recombinant analogue AFL were evaluated against porcine thyroglobulin (Fig. 2a), a glycoprotein that contains multiple *N*-linked α(1 → 6) fucose and mannose oligosaccharide groups on its surface [35]. The possible glycan structures present in our target proteins are detailed in the Table S1. UEA-I and its recombinant counterpart RSL displayed specificity to both α(1 → 4) and α(1 → 6) fucose groups. Both lectins were evaluated against Enbrel® (etanercept) (Fig. 2b), a glycoprotein made by fusion of the human tumor necrosis factor receptor (TNFR) with the Fc portion of human IgG1 [36]. This protein contains many glycosylated forms and core α(1 → 6) fucose groups, which makes it a very good target. On one hand, its intrinsic glycosylation facilitates binding to sialic acid specific lectins, such as MAL-II and its recombinant counterpart Sia-I (Fig. 2c). On the other hand, etanercept can be treated with α(2 → 3,6,8) neuraminidase, which cleaves the terminal sialic acid residues exposing β(1 → 4) galactose, which can specifically bind to ECL and its recombinant pair Gal-I (Fig. 2d). Plant lectins GNL, NPL and the recombinant analogue Man-II exhibited specificity to mannose. They were evaluated against glucose oxidase (Gox), which is rich in mannose groups [37] (Fig. 2e). The comparison of PLs and RPLs sensograms evidence an overall higher immobilisation rate for the latter. One obvious cause of this difference is the smaller size of RPLs, which allows for better lectin coverage per unit area of the sensor surface. Smaller size not only allows for better packing, but data also suggest for better access to the binding site and improved specificity and affinity for the substrates (covered in the next section). Moreover, the binding rate, expressed as the difference in wavelength shift at the end of the dissociation stage and the baseline, was also found to be significantly higher for RPLs, as summarized in Table 2.

3.2. Binding specificity and selectivity

Development of biosimilars demands that their glycosylation patterns are kept closer to the originator molecule [4,38]. This demands novel and easier ways of monitoring the glycosylation patterns. The heterogeneity of glycan structures within a molecule poses a difficult analytical challenge as it makes any glycoprotein a target for more than one lectin. For example, microheterogeneity of transferrin (a vital iron transporter) allows it to have 9 different glycoforms containing either no terminal sialic acid residue or up to 8 residues [39]. Therefore, it is useful to compare lectins against a range of glycoproteins (Fig. 3). There is very little activity displayed against transferrin, confirming the glycoform used had low number of terminal α(2 → 6) sialic acid groups. Transferrin has only two sialylated *N*-linked biantennary glycan structures attached to amino-acids Asp413 and Asp611 [39]. Removal of sialic groups known as 'asialo-' form displays terminal β(1 → 4) galactose residues, leading to strong binding to recombinant Gal-I, and to a lesser extent to ECL. On the other hand, only MAL-II and Sia-I displayed affinity to fetuin, as its glycoforms contain multiple *N*- and O-linked α(2 → 3) and α(2 → 6) sialylated ends [40]. Sialylation is an important target for biopharmaceutical development because of its

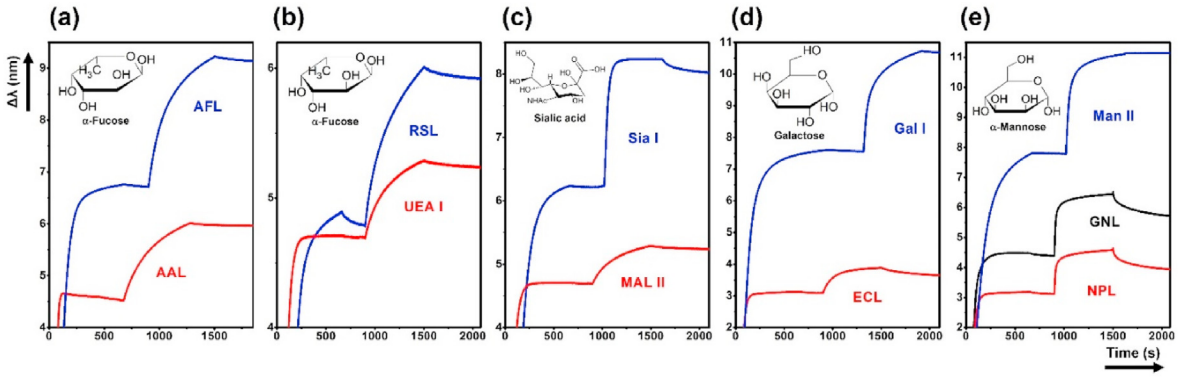


Fig. 2. Binding curves for different plant lectins (red) and the recombinant counterparts (blue) assayed against proteins displaying different glycan groups: (a) AAL and AFL against thyroglobulin (α(1 → 6) fucose), (b) UEA-I and RSL against thyroglobulin (α(1 → 2,6) fucose), (c) MAL-II and Sia-I against enbrel (sialic acid), (d) ECL and Gal-I against asialoenbrel (β(1 → 4) galactose) and (e) NPL, GNL and Man-II against glucose oxidase (α-mannose). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Binding ratios for the different sets of RPLs and PLs.

	α-mannose		β(1 → 4) galactose	α(1 → 6) fucose	α(1 → 2,6) fucose	Sialic acid
	Man-II:GNL	Man-II:NPL	Gal-I:ECL	AFL:AAL	RSL:UEA-I	Sia-I:MAL-II
Binding ratio	2.5	4.1	6.4	1.5	2.1	3.4

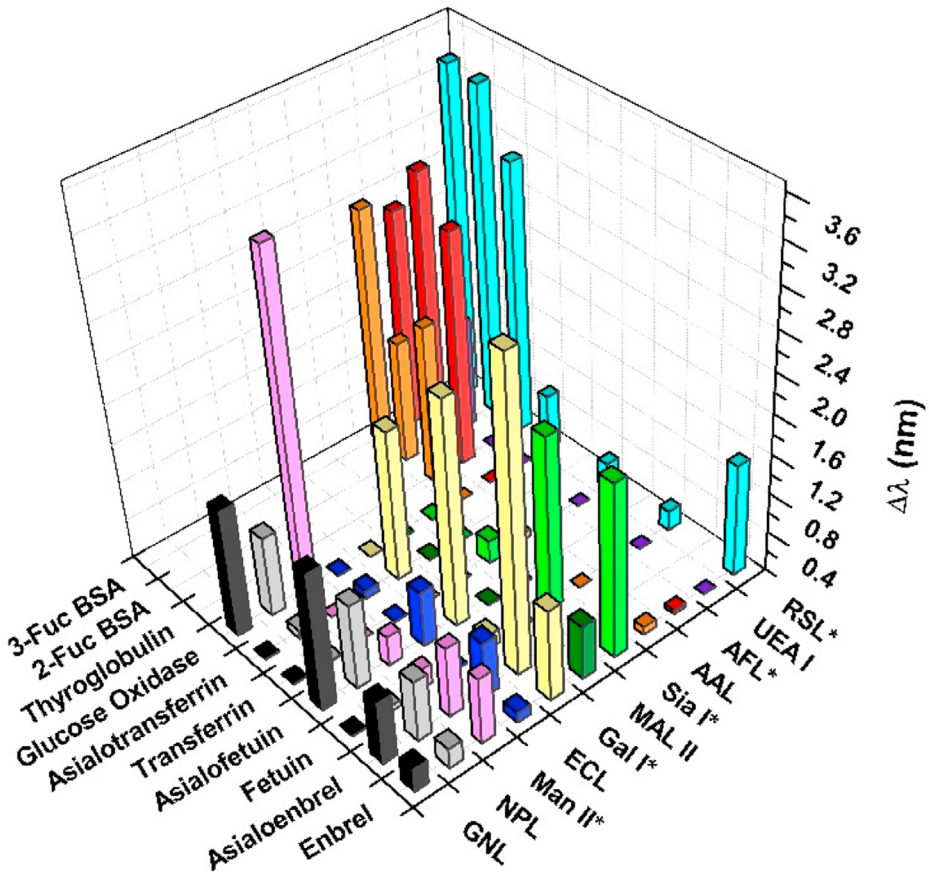


Fig. 3. 3D specificity chart showing the specificity in terms of binding signal of several PLs/RPLs to different glycoproteins and BSA-glycan conjugates (horizontal axes). PLs & RPLs were immobilised on the sensors as capturing molecules and all tested glycoproteins were assayed at 20 μg mL^{-1}. The binding signal (vertical axis) is expressed as a wavelength shift (Δλ, nm).

role in determining half-life of the biologicals. In the body, terminal galactose residues are recognised by hepatic asialoglycoprotein receptor (ASPR) which removes those molecules from blood circulation [41]. Thus, terminally sialylated forms, are cleared slowly from the blood and hence, are more desirable. Chinese Hamster Ovary (CHO) cells, which have become the most dominant cell line for production of glycoproteins, has a limitation that proteins produced in CHO tend not to be fully sialylated. The challenge is due to the fact that there are different ways of linking sialic acid to galactose residues. Most human proteins use α2,6 linkage while CHO utilizes an incomplete α2,3 linkage [41]. This has made sialylation pathways a target for metabolic engineering, particularly for increasing the expression of α2,6−sialyltransferase gene in CHO cell lines. Although, Gal-I shows stronger binding to β(1 → 4) linked galactose, the plant GNL and NPL also display significant binding to asialofetuin, and thus can be used to probe them.

Etanercept, which is a major biosimilar target has several sialylated glycoforms, and in de-sialylated forms it may have exposed mannose, galactose and GlcNAc [36]. The predominance of sialylated species resulted in strong binding of MAL-II and Sia-I. Glycoforms containing galactose and mannose terminal residues also led to significant binding of mannose (GNL, NPL and Man-II) and β-galactose (ECL and Gal-I) specific lectins. It is fascinating to see the difference in binding between fucose specific lectins which are RSL, AAL, AFL and UEA-I; it puts in context the importance of studies like the one presented in this paper. RSL, a recombinant lectin predicated on a small protein (only 90 a.a.) from bacterium *R. solanacearum* [42], outperforms all comparable lectins owing to its structure. The monomers of AAL and AFL are six bladed β sheet propellers, and exist as dimers capable of holding 5 and 6 fucose residues respectively. The RSL monomer has a two-blade propeller structure, that assembles into a trimeric structure which is almost half the size of dimeric structure made by AAL and AFL. RSL has two bindings sites for αFuc1-2Gal and αFuc1-6Gal, one site is within a monomer and one is formed at the interface between monomers. The trimeric assembly can hold 6 fucose residues while taking half the space. The binding mechanism of all these lectins is only dependent on the side chains of some key amino acid residues as opposed to UEA-I that also needs divalent metallic ions (Mn^{2+} and Ca^{2+}). UEA-I is a widely used lectin in agglutination studies of blood due to its binding to the major O antigen H-trisaccharide (Fucα1-2Galβ1-4GlcNAc), but also has broad specificity towards unrelated glycans such as 2' fucosyllactose (Fucα1-2Galβ1-4Glc), GalNAcα1-4(Fucα1-2)Galβ1-4GlcNAc, Galα1-4(Fucα1-2)Galβ1-4GlcNAc, and the Lewis-Y antigen (Fucα1-2Galβ1-4(Fucα1-3)GlcNAc) [43]. Obviously, no binding of MAL-II and Sia-I was observed to the asyliated form of the Enbrel®, increasing the interaction with mannose- and β-galactose-specific lectins.

Biosimilars produced in yeasts such as *S. cerevisiae* face a particular challenge i.e. they end up getting an extra terminal mannose where α1,3-mannose linkage have been found to be immunogenic to humans [44]. Glucose oxidase can act as a good model for terminal mannose. It can be seen from Fig. 3 that recombinant Man-II exhibits stronger binding to glucose oxidase than plant lectins NPL and GNL. Strong binding of mannose is challenging from lectin's structural perspective. The polar-polar interaction of the binding site and hydrophilic nature of carbohydrates poses a threat that water can potentially displace them from the binding sites. Therefore, high enthalpic penalties resulted by desolvation can only be compensated by the quality and number of hydrogen bonds between the active site and hydroxyl groups on the sugars [45]. To ensure stronger binding, evolution has devised strategies from employing divalent cations and making buried pockets. In a similar fashion, Man-II is homo-dimeric, where a monomer has one binding site coordinated through two calcium ions [46]. It is worth noting that all of mannose specific lectins show a bit of cross reactivity with terminal galactose residues (binding to asialofetuin) which is consistent with existing literature [45]. Nevertheless, recombinant Man-II demonstrated least amount of cross reactivity when compared to GNL and NPL. The cross reactivity of all forms with enbrel® and asialoenbrel shows that enbrel® had a higher percentage of glycoform rich in terminal mannose residues (Supplementary Table 1).

Besides the apparent contrast observed in specificity, it is worth noting the overall stronger binding displayed by RPLs whilst showing similar if not better specificity on all tested glycoproteins. This is partly due to the size, which enables better access to the sugars hidden inside the bulk protein structure, but also due to some clever site direct mutagenesis. From the sensor fabrication or microarray utilisation, it is worth appreciating that the smaller size of RPLs result in higher density of immobilised lectin, meaning more available active sites per unit of sensor surface (see Table 1). Protein sequence particularly the role specific amino acids play in determining lectin-sugar interaction mechanism is extremely important and techniques such as directed evolution and site directed mutagenesis can be applied to improve the binding or to even change the specificity. For example, RPLGal-I, in its wild type form (PA-IL) from *P. aeruginosa*, has specificity towards α-galactose. It binds to its trisaccharide target (Galα1-3Galβ1-3Glc) with a co-ordinated calcium ion through three separate parts of PA-IL monomer. The amino acid residues which co-ordinate calcium binding directly or indirectly are conserved. However, with careful mutations in the amino acids which interact with saccharide residues can be altered to bind β-galactose [22]. To understand this sugar binding environment particularly binding pocket of PA-IL needs careful consideration particularly in terms of the role hydrogen bonding plays in holding on to the sugar molecules. In the native wild type, binding is coordinated through interaction with a calcium ion by the residues Tyr36, Asp100, Thr104, Asn107, and Asn108. The spatial conformation of the binding pocket is maintained by Pro51, Asp52 and Val101, which do not make a direct contact with sugar [22]. Whereas, residues His50 and Gln53 interact with non-reducing sugar (Figure S2), their role has been studied experimentally by site-directed mutagenesis. Interestingly, PA-IL triple mutant (His50-Asn50, Asp52-Asn52 and Gln53-Gly53) showed better protein binding to β-galactose. *In silico* protein stability studies, with ICM, show that compared to wild-type protein, ΔΔG of triple mutant protein increased by 0.873 (0.97 for His50-Asn50, 0.16 for Asp52-Asn52, and 0.18 for Gln53-Gly53). MDS for mutant PA-IL bound with β-galactose show moderately better conformational protein stability in terms of average RMSD and RMSF, (Figure S3 a, c) and ligand binding in terms of MM/PBSA (ΔG for mutant −478.47 kJ/mol vs native −412.21 kJ/mol) as compared to wild-type protein. A decrease in potential energy of the ligand-bound mutant protein also indicates stability of the complex (Figure S4). His50/Asn50 interactions have been found in both native and mutant proteins, while changing Gln53 to Gly53 make this residue more mobile. The mutant PA-IL bound to trisaccharide target (Galα1-3Galβ1-3Glc) shows an increase in RMSD and RMSF while MM/PBSA calculations (ΔG for mutant −549.16 kJ/mol vs native −459.46 kJ/mol) show stable ligand-protein interactions.

While docking β-galactose to wild-type and mutated/recombinant PA-IL it was found that β-galactose coordinates with calcium ion by O3 and O4 (mentioned as O9 and O10 respectively in Fig. 4F). Wild-type PA-IL was found to form hydrogen bonds with His50 (His^{NE2} → β-$galactose^{O5,O6}$, H-bond lengths 3.11 and 2.84), Gln53 (Gln^{OE1} →β-$galactose^{O6}$, H-bond length 2.74), Asp100 (Asp^{OD1} →β-$galactose^{O5}$, H-bond length 2.69), Thr104 (Thr^{OG1} → β-$galactose^{O5}$, H-bond length 2.93), and Asn107 (Asn^{NE2} → β-$galactose^{O2,O3}$, H-bond lengths 3.30, 3.35) Fig. 4C. In case of mutant/recombinant PA-

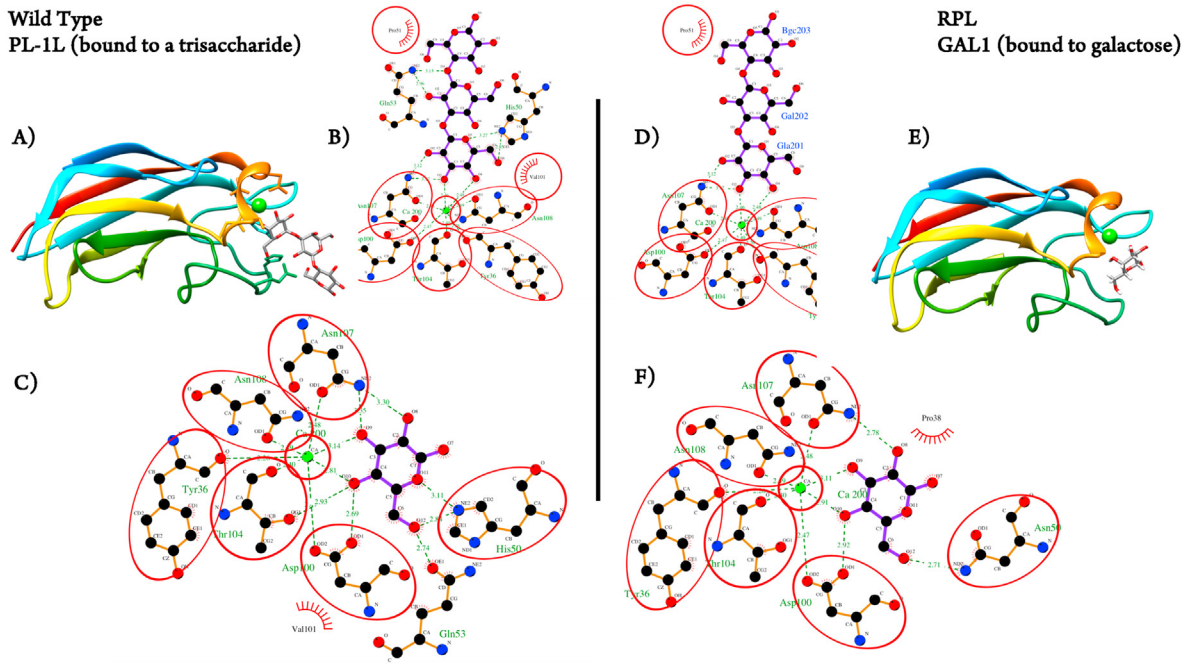


Fig. 4. Comparison of the wild type PL-1L with RPL Gal-I A) Raytraced image of Wild type PL-1L with the trisaccharide substrate B) Interaction of the wild type lectin with the trisaccharide substrate C) Closer look at the hydrogen bonding pattern determining specificity in binding pocket of the wild type lectin D) Interaction of the RPL with the trisaccharide E) Raytraced image of the RPL Gal-I with Galactose F) Closer look at the changes in hydrogen bonding pattern determining change in specificity to α galactose.

IL, Asn50 (Asn^{ND2} → β-galactose^{O6}, H-bond length 2.71), Asp100 (Asp^{OD1} → β-galactose^{O5}, H-bond length 2.92), Asp107 (Asp^{ND2} → β-galactose^{O2}, H-bond length 2.78). While docking trisaccharide to mutant/recombinant PA-IL, it was observed that O^6 of α-galactose was not involved in H-bonding with any of mutated amino acid but in case of β-galactose O^6 is involved in H-bonding with Asn50 (Fig. 4F). β-galactose $C3^{OH}$ interaction with Asn107, observed for wild-type protein, is missing in mutant protein, but shows relatively strong coordination with calcium ion. MDS shows strong interactions of His50/Asn50, Asp100, Thr104, Asn107, Asn108 with the ligand molecules and confirms the docking results (Figure S3 and S4). Previously, PA- 1 L/αGal12GalOMe docking showed that O^3 and O^4 of the second monomer and the $Gln53^{OE1, NE2}$ were involved in H-bonding [47]. For β-galactose/wild-type PA-1L system, $Gln53^{OE1}$ interact with O^6 of β-galactose and may interfere with its coordination with calcium. Mutating Gln53-Gly53, His50-Asn50 may change the overall H-bonding patterns leading to better calcium coordination as well as deep binding into the cleft. Same was observed with MDS where changes in amino acid fluctuations and deviations show an overall change in binding pattern of both β-galactose and trisaccharide. MDS also showed some amino acids considerably effecting the stability of the protein (Figure S3). In future, mutating these amino acids *in vivo* and performing binding analyses can provide valuable information to even further enhance the efficacy and efficiency of these RPLs towards glycoconjugates.

3.3. Kinetic evaluation & the dose-response binding curves

The higher affinity of RPLs can be further justified by the binding kinetics. The kinetic characterisation of lectins, like any other biomolecule, provides reliable and conclusive information about the affinity of the protein-target (carbohydrates in this case) interaction. One of the advantages of bio-layer interferometry is the possibility of measuring binding kinetics in real time, very much as is done with other techniques like SPR [48,49]. The kinetic study of all PLs and RPLs employed in this study was carried out in two subsequent steps: (1) the values of the apparent kinetic constant (k_{obs}) for different concentrations of ligand were obtained through the non-linear fitting of the association step of each experimental sensogram, (2) followed by the linear fitting of this constant plotted as a function of the ligand concentration (Fig. 5).

The values of the equilibrium dissociation ($K_D = k_{off}/k_{on}$) and association ($K_A = 1/K_D$) constants obtained for each lectin are shown in Table 3. Both constants relate directly to the actual affinity of the lectin towards its complementary ligand. In order to make a comparative study of the different RPL/PL pairs, both lectin types were assayed with the same glycoprotein ligand. As it can be concluded from Table 3, all the studied RPLs turned out to exhibit lower K_D values than their PL peers. This result is a clear indicative of the stronger binding interaction and thus, higher binding affinity of the tested RPLs, supporting the behaviour observed in Section 1.

The binding activity of the studied RPLs was quantitatively characterised and compared to that of their plant analogues by registering the corresponding dose-response curves against the same target glycoprotein. The selection of the target glycoprotein for each RPL/PL pair was based on the lectin specificity, and against which both lectins displayed the highest affinity. Lectin immobilisation on the sensor phase was performed as described previously, followed by incubation with increasing concentrations of glycoprotein in all cases, typically ranging from 0.01 to 1000 μg mL^{-1}. Fig. 5 depicts the registered dose-response curves for four PL/RPL sets specific to different sugar residues.

The analytical parameters obtained for the selected PL/RPL sets are summarized in Table 3. On the whole, RPLs showed comparatively lower limits of detection than PLs. Among the four pairs, it is worth highlighting the dramatic difference in lowest binding concentration displayed by MAL-II and Sia-I, showing the latter a 380-fold reduction against fetuin. This difference was followed by ECL and Gal-I (~14-fold) and GNL and Man-II (~2.5 fold), assayed against glucose oxidase and asialofetuin, respectively. AFL and AAL, however, exhibited the opposite tendency, showing AAL a slightly lower limit of detection than its recombinant peer. However, in

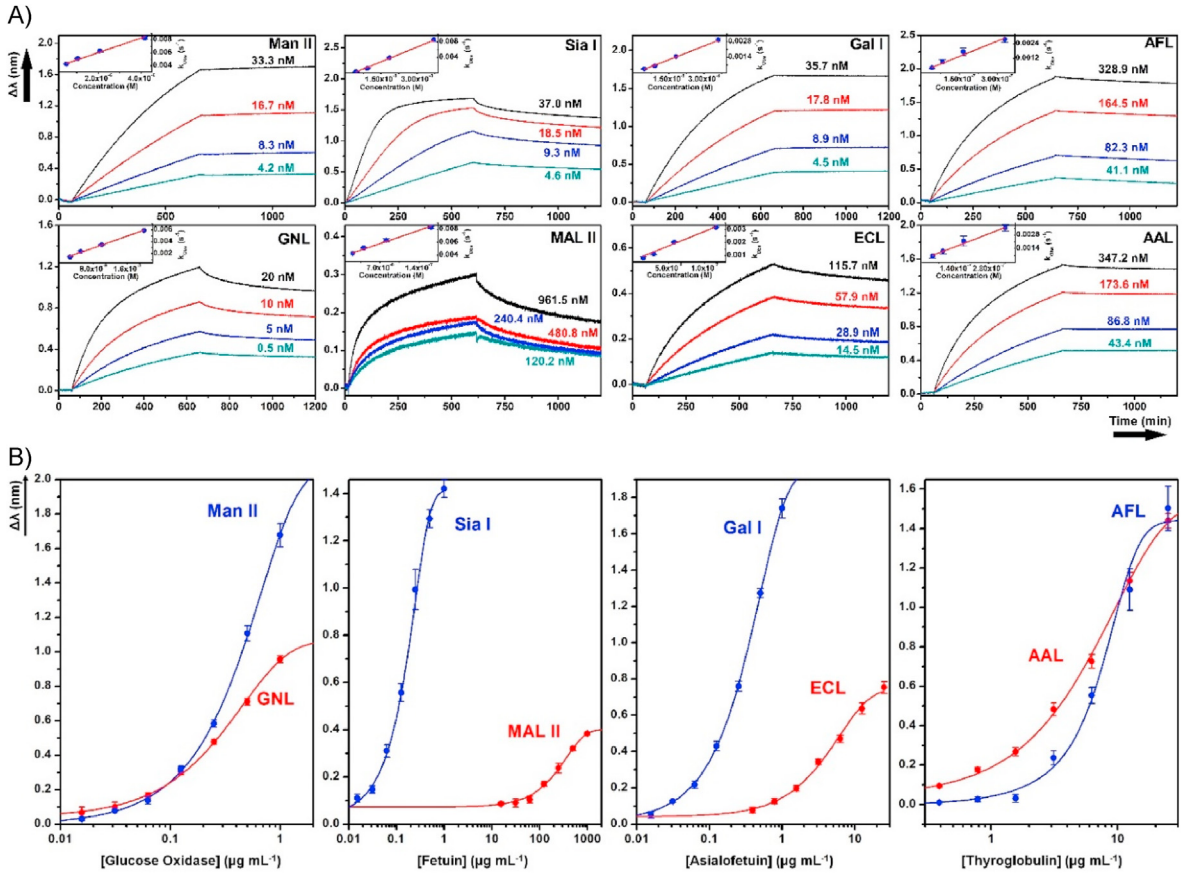


Fig. 5. A) Kinetic study of the four pairs of RPLs (top row) and their plant counterparts (bottom row). The apparent kinetic constant (k_{obs}, s^{-1}) is obtained in each case by the non-linear fitting of the sensogram association step for different ligand concentrations: (1) The formal association (k_{on}, nM s^{-1}) and dissociation (k_{off}, s^{-1}) binding rate constants are subsequently obtained by the linear fitting of the observed apparent kinetic constant plotted as a function of the ligand concentration. (2) This last plot is inserted as an inset in each sensogram. GNL and Man-II (first column, assayed against glucose oxidase), MAL-II and Sia-I (second column, assayed against bovine fetuin), ECL and Gal-I (third column, assayed against bovine asialofetuin), AAL and AFL (fourth column, assayed against porcine thyroglobulin).
B) Dose-response curves (n = 3) of several PL/RPL sets (colored in red and blue, respectively): (a) GNL and Man-II (α-mannose, assayed against glucose oxidase), (b) MAL-II and Sia-I (sialic acid, assayed against bovine fetuin), (c) ECL and Gal-I (β-galactose, assayed against bovine asialofetuin) and (d) AAL and AFL (fucose, assayed against porcine thyroglobulin). Error bars are standard deviation. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Analytical parameters of the dose-response curves of PLs and RPLs sets assayed against different glycoproteins.

Lectin	Targeted glycoprotein	LoD (ng mL^{-1})	IC_{50} (μg mL^{-1})	K_D (nM)	K_A (nM^{-1})
GNL	Glucose Oxidase	29	0.58	5.3 ± 0.9	0.19 ± 0.04
Man-II*		12	0.71	1.6 ± 0.7	0.7 ± 0.2
ECL	Asialofetuin	170	6.23	7.0 ± 0.6	0.14 ± 0.01
Gal-I*		12	0.501	2.3 ± 0.2	0.44 ± 0.04
MAL-II	Fetuin	950	286.6	998 ± 4	0.0010 ± 0.0004
Sia-I*		2.5	0.18	4.1 ± 0.2	0.25 ± 0.01
AAL	Thyroglobulin	290	10.7	27.7 ± 0.2	0.036 ± 0.002
AFL*		380	9.73	12.9 ± 0.3	0.077 ± 0.002

comparative terms, the LoD ratio for these two lectins was not as stark as observed for the others, measured as less than 1.5-fold. In fact, the obtained LoD values can be used as a direct indicator for the activity of the lectins, defining this parameter as the minimum concentration of glycan (glycoprotein in this case) necessary to trigger binding. This way, the RPLs presented in this manuscript can be compared to other reported examples in literature. Tronchin et al. purified and indirectly evaluated the activity of lectins produced by *A. fumigatus* against several sugars and glycoconjugates, reporting high binding exhibited against sialic acid groups [50]. When tested with fetuin, the minimum concentration of this glycoprotein that caused interaction with the lectin was 666 μg mL^{-1}, much higher than 2.5 × 10^{-2} μg mL^{-1} obtained with Sia-I (Table 3). Another example was reported by Chen et al. who isolated a plant lectin from *Phaseolus coccineus*, specific to sialic acid groups [51]. Similar to the previous example, the lectin was observed to bind to fetuin at a minimum concentration of 100 μgmL^{-1}. Regarding mannose-binding lectins, Zhang et al. isolated a new lectin from salt-stressed rice (*Oryza sativa*) which indirectly evaluated with mannose, resulting in an inhibition IC_{50} of 12 μgmL^{-1} [16]. As for galactose, Pajic et al. characterised a new lectin from the sponge *Haliclona crater*, which showed an inhibition IC_{50} of 4.5 mgmL^{-1} [52]. Lastly, Kuboi et al. characterised AfuFleA, a fucose-specific lectin from *A. fumigatus* [53]. The authors reported

an indirect minimum concentration of fucose (70 μg mL^{-1}) for total interaction with the lectin, which yet remains far from the dynamic range obtained for AFL.

4. Conclusions

In the last few decades, lectins have become key elements in the recognition and analysis of glycan structures, contributing massively to the development of modern glycobiology. In this study, we report the development of novel RPLs which display high affinity to different sugar residues, including α-mannose, β-galactose, α-fucose and sialic acid. The binding performance and specificity of the lectins were thoroughly evaluated and compared to analogous plant lectins (PLs) with similar binding targets. Besides exhibiting similar specificity to different glycoproteins and BSA glycan conjugates with different glycosylation patterns, the presented RPLs displayed substantially higher binding activities to the selected targets. The analytical parameters of the lectin-glycoprotein interaction for the RPLs were also assessed by registering the corresponding dose-response curves against certain glycoproteins, and ultimately compared to those of their plant equivalents. All the studied RPLs except AFL showed an exceptional analytical performance, this one being similar to its plant analogue AAL. This exceptional analytical performance could be of great benefit in the characterisation of glycoproteins, evaluations of monoclonal antibodies and development of biosensors.

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. ‡SFP, MSA contributed equally.

CRediT authorship contribution statement

S. Fernandez-Poza: Methodology, Formal analysis, Investigation, Writing – original draft. **A. Padros:** Investigation, particularly collection of data on Octet®. **R. Thompson:** Resources, procurement of lectins. **Lucy Butler:** Investigation, SDS-PAGE and sample prep for mass spectrometry, Visualization. **Meez Islam:** Funding acquisition, helped writing the grant and discussion on analytical aspects. **J.A. Mosely:** Investigation, Data acquisition, analysis on mass spec. **James H. Scrivens:** Data analysis on mass spec. **Muhammad F Rehman:** Formal analysis, support om bioinformatics aspects. **Muhammad Safwan Akram:** Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Supervisor to SFP and LB, Funding acquisition, PI on the grant.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Dr. Muhammad Safwan Akram has joined Scientific Advisory Board of GlycoSelect Ltd. Nevertheless, he has no shares or financial interest attached to the company.

Acknowledgement

Authors will like to acknowledge the KTP funding from Innovate UK titled ‘Development of simple, rapid & high-throughput glycan-analytics for biopharmaceuticals’ (Project no. 010802), and THYME (Teesside, Hull & York - Mobilising Bioeconomy Knowledge Exchange - ccf14-7167) from Research England. MSA is thankful to John Clayton appreciates help from Robert Dunne, R. Roy and rest of the team at GlycoSelect Pvt Ltd. MSA would also like to thank Prof. Vikki Rand for reading the manuscript..

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2021.338352.

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